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Comparative Binding Analysis of N- Acetylneuraminic Acid in Bovine Serum Albumin and Human α-1 Acid Glycoprotein

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Abstract

In this present study focus that determination of biologically significant *N*-Acetylneuraminic acid (NANA) drug binding interaction mechanism between bovine serum albumin (BSA) and human α-1 acid glycoprotein (HAG) using various optical spectroscopy and computational methods. The steady state fluorescence spectroscopy result suggests that the fluorescence intensity of BSA, HAG was quenched by NANA drug in a static mode of quenching. Further time- resolved emission spectroscopy measurements confirm that mode of quenching mechanism of NANA drug in BSA and HAG system. The FT-IR, excitation - emission matrix and Circular Dichroism (CD) analysis confirms the presence of NANA drug in HAG, BSA system and fluorescence resonance energy transfer analysis shows that the NANA drug energy transfer between HAG, BSA system. The molecular docking result shows good binding affinity in both protein complex and further molecular dynamics simulations and charge distribution analysis was performed to understand more insight binding interaction mechanism of NANA drug in HAG, BSA complex.

1. Introduction

N-Acetylneuraminic acid (NANA or Neu5Ac) are widely involved in the biological activity and which is found in glycoprotein or complex of glycans on mucins at the cell membrane. NANA drug is essential for pathogenic bacteria also it can use as a nutrient by giving carbon and nitrogen atoms to the bacteria. In some cases, we found activated pathogens in the cell surface. N-acetylmannosamine or 6-phosphate mannose contraction caused synthesis of Neu5Ac with activated pyruvate and followed up by dephosphorylation within the cell. In several parts of glycoconjugates chains, most sialic acids are naturally originated at the terminus of N-glycans, O-glycans, and glycosphingolipids [1-3].

Being at the consequence of essential cell-surface glycoconjugates, sialic acids are admirably located to arbitrate carbohydrate–protein communications in cell–cell understating phenomena. For example, sialic acids are required in the Sialyl Lewisx-selection binding that appears in the enrollment of leukocytes in the course of inflammation mechanism [4]. Also, sialic acids are performing as receptors for a few toxins, bacteria, and viruses. For example, the contact between Neu5 Ac and heamaglutinin add up to the primary step of infection by the influenza virus. Also, sialic acids play significant role in masking to counter the biological recognition. Alternations in sialic acids can prevent with the mechanism of cell interaction.

In this regard, it is essential to understand the binding interaction mechanism of NANA drug in both HAG and BSA molecule to explore the biochemical mechanism in a biological environment. Human α-1 acid glycoprotein consists of 10 – 15 % of sialic acid residues in the total weight of the protein molecule, also it is a major plasma glycoprotein. Which contain 181 amino acid residues with single polypeptide chain and the molecular weight are about 41 kDa, and it is heavily glycosylated made up of 40% of carbohydrate, 10 – 15 % of sialic acids by weight of the protein. HAG is from lipocalin protein family, and it has common structural motifs comparing its super family member. Eight anti-parallel beta sheets circularly closed by forming a barrel type structure provide a ligand binding region [5-8].

In other hand serum albumin also one of the primary class of transport protein, which can balance osmotic pressure and transport both exogenous, endogenous compounds. In many cases, bovine serum albumin (BSA) is considered as a model protein to perform binding analysis, because it can distribute many compounds into the particular target. BSA is a one of globular protein which consists of 583 amino residues and contains three domains (I, II, III), each as divided by subdomains A and B respectively. Also, the molecule structure contains 17 disulfide links and in both BSA, HAG protein molecule, tryptophan residue plays a major intrinsic fluorophore [9].

In this context, the present study focus on the binding mechanism of NANA drug in both BSA and HAG is explained and compared by various spectroscopy techniques such as fluorescence stead -state analysis, time -resolved life time decay studies, FT-IR. The molecular docking and molecular dynamics analysis clearly explain the interaction mechanism and stability of NANA drug in both BSA, HAG protein environment. Further charge distribution analysis explores the intermolecular interaction of NANA drug in both protein molecule environments [10 - 13].

2. Materials and Methods

2.1 Materials

The bovine serum albumin (A2058), human α-1 acid glycoprotein (G 9885) products were purchased from Sigma Aldrich, India. *N*-Acetylneuraminic acid (16091) purchased from Cayman Chemicals, USA.

2.2 Preparations of BSA, HAG and NANA solutions

All the experiments were done using a phosphate buffer solution (pH 7.2). Both protein molecules were used without any further purification. BSA and HAG protein molecules were also broadly dialyzed using in the same buffer and for both protein molecules, 1 mg/ml stock solutions prepared in the same buffer, the purity of both molecules were determined UV–visible absorption spectrophotometer (Perkin–Elmer Lamda35, Waltham, MA) at 280 nm wavelength regions. *N*-Acetylneuraminic acid (NANA) drug was dissolved using DMSO (Dimethyl sulfoxide) and stock solution was prepared (3.5 mM) using the same solvent.

2.3 Steady-state fluorescence quenching measurements

Fluorolog-3, ISA; Jobin-Yuvon-Spex, Edison, NJ was used to perform the emission spectral analysis of both BSA and HAG –NANA drug complex, the spectra were recorded between 280 nm – 500 nm wavelength range with 5 nm excitation, emission slit width. The emission spectrum of BSA, HAG – NANA drug complex was recorded at 298K with 280 nm excitation and the maximum emission wavelength observed at 350 nm, 347 nm respectively. The titration of BSA (10 μM), HAG (10 μM) and the NANA drug concentration vary from 0 to 2 μM with 0.4 μM for BSA complex and 0.2 μM for HAG complex. The decreasing fluorescence intensity of BSA, HAG – NANA drug complex quenching mechanism was determined using Stern – Volmer Equation 1[14]. Also, due to the inner filter effect the following steady state spectrum must be corrected using the following equation [2]

$$F_0/F = 1+k_q\tau_0 [NANA] = 1+K_{SV} [NANA] \quad [1]$$

$$F_{corr} = F_{obs} . e^{(A1 +A2)/2} \quad [2]$$

Where, K_{SV} is the quenching constant, k_q rate constant of the biomolecule quenching reaction τ_0 is the average lifetime value of fluorophore (tryptophan, tyrosine) which is present in biomolecule complex and, Fcorr and Fobs denote the fluorescence intensities after and before correction at the emission wavelength A1 and A2 are the total absorbance of all components at the λ_{ex} and λ_{em}, respectively[50,51]. In many reported literature mentioned that the average lifetime of protein molecules is in the range of 3 to 6 ns and it varies depending upon many parameters. The binding constant and number of binding sites for BSA, HAG –NANA complex were determined using double logarithmic plot equation 3 [15].

$$\log [(F_0\text{-}F)/F] = \log K_b + n \log [NANA] \quad [3]$$

Where, F_0 and F are the initial and final fluorescence intensities of BSA, HAG –NANA drug complex, K_b is the binding constant and n is the binding sites of protein – drug complex. Also, the free energy changes ΔG° for protein – drug complex was calculated using equation 4.

$$log\ \text{Kb} = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} \quad , \Delta G^\circ = \Delta H^\circ - T\,\Delta S^\circ = -RT \ln K_b \quad [4]$$

Where T is the temperature (298K, 300K and 304 K), R is the gas constant (1.987 cal mol^{-1} K^{-1}), enthalpy (ΔH°), entropy (ΔS°) and free energy (ΔG°) and K_b is the binding constant of BSA, HAG – NANA complex [16 -20].

2.4 Time- resolved emission spectroscopy analysis (TRES)

The fluorescence lifetime measurements were carried using Time- Correlated Single Photon Counting system (TCSPC), Fluorolog – 3, HORIBA Jobin Yvon, INC, Edison, NJ. The protein molecule sample was exited using 280 nm Nano LED source (Pulse Width: < 1 ns) with rapid response red- sensitive photomultiplier (PMT; R928P, Hamamatsu Photonics, Shizuoka-Ken, Japan) detector. The fluorescence decay profile of both protein molecules was compiled at 90° from the exciting path of the light source. The obtained signal was amplified using a pulsed amplifier (TB-02, Horiba) model and delivered to the single channel fraction timing discriminator using Model No. 6915, Philips Scientific, and Mahwah, NJ. Initially detected photon was used by time to time amplitude (α) converter (TAC) until the signal peak reached 1000 counts for 350 nm emission. Using the Ludox - 40 reagents, the instrument response was obtained at 280 nm excitation wavelength region before performing the protein –drug interaction decay experiment. The obtained final output was analyzed by using Decay Analysis Software (DAS6 v6.0, Horiba) and the excellent linearity of the fitted decay profile for protein – drug complex is verified by chi – square values. The average lifetime value is calculated from the equation 5[21].

$$\tau_0 = \frac{\sum_{i=1}^{i=3} \alpha_i \tau_i^2}{\sum_{i=1}^{i=3} \alpha_i \tau_i} \quad [5]$$

2.5 Fourier transforms infrared spectra (FTIR) analysis

The FTIR spectroscopy analysis was carried out using a JASCO instrument model no FT/IR-6600 type A series, for both protein and drug complexes. The experiment was conducted at ATR (Attenuated Total Reflectance) mode for both protein and drug complexes at room temperature 298K. The background of the sample was subtracted before performing the experimenting BSA, HAG – NANA complex. Using the instrument software the spectral noise, CO_2 was reduced, and base lines are corrected for all samples. The wave number ranges starts from 399.193 cm^{-1} to 4000.6 cm^{-1} with 0.964233 cm^{-1} data interval at 45° incident angle for all samples, outputs were collected by Triglycine sulfate (TGS) detector system for further sample analysis [17].

2.6 Circular Dichroism (CD) analysis

The CD analysis was carried out using JASCO instrument J-715 series for both protein and drug complexes. The concentration of both BSA and HAG is 20 µM and the NANA concentration also 20 µM range (1:1 ratio). The spectrum was recorded between 180 nm to 300 nm range for all the samples at room temperature. Further, obtained spectrum was analyzed using http://bestsel.elte.hu/index.php server.

2.7 Molecular docking studies

The crystal structure of HAG (PDB ID: 3KQ0), BSA (PDB ID: 4F5S) were obtained from the protein data bank (PDB). The compound structure of NANA drug was obtained from PubChem and further, the structure was drawn using ACD/chemsketch software. To perform molecular docking the before the compound structure should be energetically minimized, here the NANA compound structure was minimized using conjugate gradient algorithm 5000 steps which are inbuilt in Schrödinger software package 2018-3. Further, both protein molecules were optimized, and energy minimized using OPLS 2005 force field in protein preparation wizard panel. The co-crystal sites were noted before optimization of the protein molecule, and during the docking simulation the binding site grid box was set into that native structure region. Induced

fit docking panel is used to perform the molecular docking simulation for both proteins – NANA drug complex. Grid -based ligand docking with energetic (GLIDE) was used to find favorable interactions between ligand and the receptors with flexible conformations of ligand. Glide scoring function is given by the equation [6] below:

$$GScore = 0.05 \times vdW + 0.15 \times Coul + Lipo + Hbond + Reward + RorB + Site + hydrophobicity \quad [6]$$

Glide scoring with XP descriptor with Induced Fit Docking (IFD) rewards hydrophobic interactions in-between ligand and the receptors. IFD also reduces false positive of true ligand binder where by IFD induces side chain flexibility in the receptors.Maestro panel and pymol software is used to analysis output files [22-28].

2.8 Molecular dynamics analysis

Once the receptor -ligand complex was generated, it was subject to molecular simulation protocol using Desmond Schrödinger software package 2018-3 [29, 30]. Protein system was build involving periodic boundary condition with 10 $Å^3$ orthorhombic boxes from the center of mass with protein. TIP3P water solvation system was used as a buffer system with charged ions placed isotopically, to neutralize the Ewald charge summation of the solvated protein entity. The system was minimized with maximum iterations of 5000 steps with a gradient convergence threshold of 1.0 kcal mol^{-1} $Å^{-1}$.Once the system is minimized, the system is subjected to Newtonian dynamics of the model system to evaluate energy of the system. 2ps steps were integrated to record the simulation. Six stage NPT ensemble default relaxation process was carried out before performing molecular dynamics simulation. Initially at first state solute restrained Brownian dynamics of the ensemble was carried by keeping the energy constant using NVT condition. In the second stage using Berendsen thermostat the NVT (*canonical)* ensemble was allowed to relax with respect to temperature with velocity resembling of every 1ps applied to the non-hydrogen solute sample. Subsequently, NVT ensemble was changed to NPT ensemble with Berendsenbarostat with the system kept at 1 atm pressure followed by system equilibration of 1ns. Then the ensemble was subjected to 50 ns Molecular dynamics run.

2.9 Density functional theory (DFT) calculation analysis

The best docked pose for both protein – NANA drug complexes were taken for further understand charge distribution analysis. The obtained free NANA and NANA in complex structures were converted and created as a Gaussian input file with needful geometrical parameters and basis set. After getting the docked complex, the NANA molecule is taken from the active site of both protein molecules for further DFT single point energy calculation using B3LYP/6-311G** level in Gaussian 03 software package. The wave function of NANA drug in both gas phase and active regions (quantum chemical calculations) were obtained and used it for charge distribution analysis [31, 32].

3 Results and Discussion

3.1 Steady- state fluorescence spectroscopy analysis

Fluorescence spectroscopy is most effective and sensitive method to understand the effect of drug binding nature in a protein molecule. There is three major fluorophore natured amino acids such as tryptophan, phenylalanine and tyrosine residues, plays a vital role in understanding protein molecule binding interaction mechanism. Among these three amino residues, tryptophan contributes more, and this can change the micro environmental surrounding of tryptophan in protein molecule. So that changes in the intrinsic fluorescence of the protein molecule can get more meaningful data and, that can be utilized to understand the conformational changes in the protein molecule. In general, the emission maxima of protein molecules obtained in 350 nm due to tryptophan residues

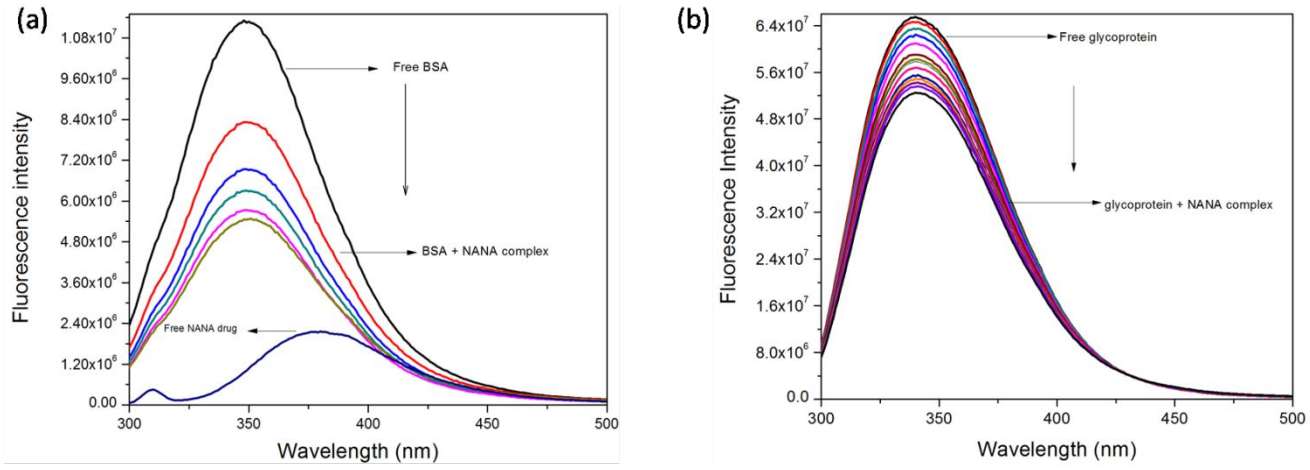

Figure 1 Steady state emission spectrum of (a) free BSA with BSA-NANA complex (b) free glycoprotein with glycoprotein – NANA complex

. Figure 1 shows the steady state emission spectrum of (a) BSA – NANA complex (b) glycoprotein – NANA complex. Here both protein BSA (10 µM), HAG (10 µM) molecule kept as a control and NANA drug varies from 0 to 2 µM with 0.2 µM interval in HAG, 0.4 µM interval in BSA system respectively. From the figure 1 (a) and (b) the result shows that when increasing the concentration of NANA, the fluorescence intensity of both protein molecules is decreased without any further shift, this may be due to the influence of NANA in both protein complex. This result suggests that NANA molecule may develop hydrophobicity in both BSA and HAG complex, also possibilities of deterioration in polarity around tryptophan residue due to the presence of NANA in both protein complexes. So that from this result we may conclude that NANA molecule acts the same in both the BSA and HAG protein molecule environment [33].

3.2 Determination of quenching mechanism and binding constant

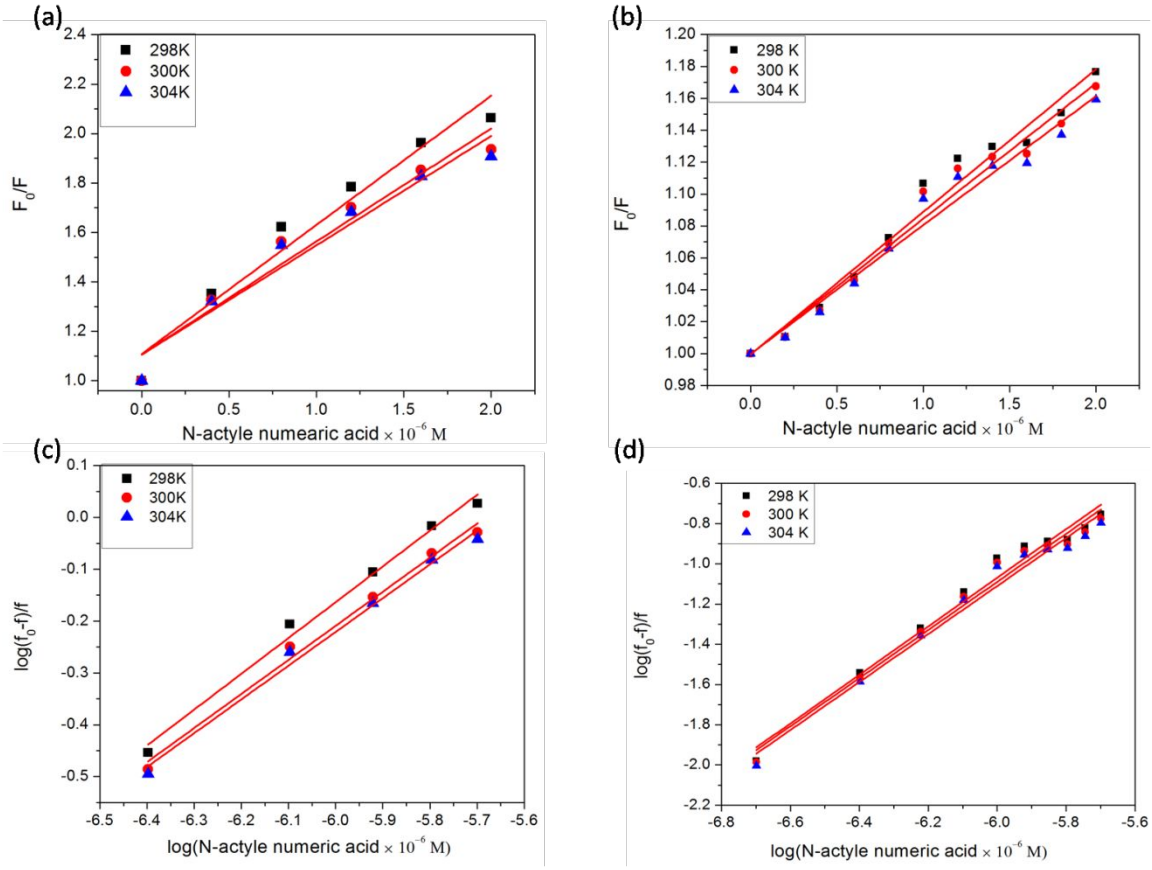

Figure 2 Stern-Volmer plot and log plot of (a) & (c) free BSA with BSA-NANA complex, (b) & (d) free glycoprotein with glycoprotein – NANA complex

The quenching fluorescence data explore the mode of binding information for NANA drug in both BSA and HAG system. In general, the fluorescence quenching is classified by two modes, one is static quenching and another one is dynamics quenching. Static quenching mode explains about the ground state complex formation between protein – drug molecule, for example before excitation occurs, but in dynamics quenching refers to after the excitation of quencher (excited state complex). Figure 2 (a) and (b) shows the Stern-Volmer plot of BSA-NANA, HAG-NANA complex system. Based on equation 1 quenching constants were calculated for both complexes (Table 1).

NANA in complex with	T(K)	K_{SV} ($\times 10^8$L/ mol/ s)	Kq ($\times 10^{17}$ L/ mol/ s)	K_b ($\times 10^6$L/mol)	n	ΔH^o (KJ/mol)	ΔG^o (KJ/mol)	ΔS^o (J/mol/K)
BSA	298	0.68	0.119	3.97	0.96	-77.90	-34.69	-149.47
	300	0.62	0.107	3.73	0.93		-34.93	
	304	0.60	0.105	3.68	0.95		-35.39	
Glycoprotein	298	8.50	2.83	6.17	1.21	-97.02	-36.72	-127.02
	300	8.10	2.70	6.08	1.19		-36.97	
	304	7.96	2.57	6.04	1.09		-37.46	

Table.1 Fluorescence binding Parameters for BSA, glycoprotein –NANA complex (pH-7.4)

The maximum scattering collision quenching constant Kq, of various quenchers with macromolecules is about 2.0×10^{10} L mol^{-1} s^{-1}.The calculated Kq values show that NANA binding mode is higher than the maximum scattering collision quenching constant of Kq and mainly due to static in nature for BSA, HAG protein molecules and also the plot shows good linearity in both complexes. Further to understand the relationship between BSA, HAG and NANA drug complex, the double logarithmic plot was plotted and shown in figure 2 (c) and (d). The binding constant for BSA – NANA complex K_b is 3.97×10^6 L/mol but in the case of HAG –NANA complex which showing, K_b value is about 6.17×10^6 L/mol at 298 K. Also, the binding stoichiometry for NANA in both protein molecules is almost same in the order of 1; this result reveals that the NANA can be a one class of binding in both protein molecule environments [34, 35]. Also, according to Ross and Subramanian theory the binding mode

association of small molecule into the protein molecule microenvironment can be divided into three sub class which is (i) $\Delta H° > 0$, $\Delta S° > 0$ correspond to hydrophobic forces,(ii) $\Delta H° < 0$, $\Delta S° < 0$ correspond to van der Waals interaction, hydrogen bond formation,(iii) $\Delta H° < 0$, $\Delta S° > 0$ correspond to electrostatic interaction and the thermodynamic binding parameters of NANA in complex with BSA and HAG calculated through the van't Hoff curve (Fig S1) and $\Delta H°$, $\Delta S°$ and ΔG^o values are given in Table 1, from that result the binding forec of NANA in both HSG and BSA complex mainly due to van der Waals interaction/ hydrogen bond formation [51-53].

3.3 Time- Resolved Emission Spectroscopy analysis (TRES)

Time- resolved emission measurement is a most effective method to confirm the mode of quenching in a protein molecule. The alteration in BSA, HAG protein microenvironment and conformational changes were analyzed using lifetime and its amplitude. In general, the protein molecules decay profiles were measured at 350 nm emission using 280 nm excitation LED source. But, here time resolved emission spectroscopy (TRES) method is carried between 280 nm to 400 nm wavelength region with 10 nm excitation interval to understand the NANA behavior in both BSA, HAG system. Figure3 (a) and (c) shows average photon counts of TRES data of BSA – NANA, HAG – NANA complex system respectively. The average photon count plot shows that NANA drug made little impact on both protein molecule fluorophore regions without any further shift only quenching is happen between 280 nm to 400 nm wavelength region; this result concluded that presence of NANA drug in both BSA, HAG system. Further, the obtained decay amplitudes for BSA, HAG-NANA complex systems were analyzed using DAS 6.0 software and triple exponential global fitting parameter was used to extract data sets for this biomolecule. The average lifetime value τ_0 is calculated for both free protein and protein - ligand complex system and plotted wavelength viruses τ_0 (Figure 3 (b) & (d)). Figure 3 b clearly shows the average photon count difference between free BSA and BSA – NANA complex, initially for 280nm wavelength there is no much difference between free BSA and BSA – NANA complex but the real difference start from 320 nm and these counts intense increases up to 360 nm and saturated after that until 400 nm end wavelength region.

The similar thing happens for free HAG and HAG – NANA (Figure 3 c) complex and these results suggest that NANA presence in both protein molecule. Figure 3 b & d shows the calculated τ_0 average life time value for free BSA, BSA – NANA complex and free HAG,

HAG-NANA complex respectively. Figure 3b clearly shows that free BSA lifetime value starts from 1.05 ns at 280 nm and it increases towards 5.54 ns for 330 nm, after that saturated until 400 nm, but in the case of BSA- NANA complex this values is decreases to 0.118 ns for 280 nm and for 330 nm it reaches 2.54 ns and sutured after that until reaching 400 nm. In the case of free HAG the lifetime value is between 2.75 ns (350 nm) to 2.82 ns (360 nm -400 nm) and it decreases 2.66 ns (350 nm) to 2.77 ns (360 nm to 400 nm) for HAG – NANA complex. These results suggest that the NANA drug can form ground state complex in both protein molecule during the binding interaction period [21, 35-36].

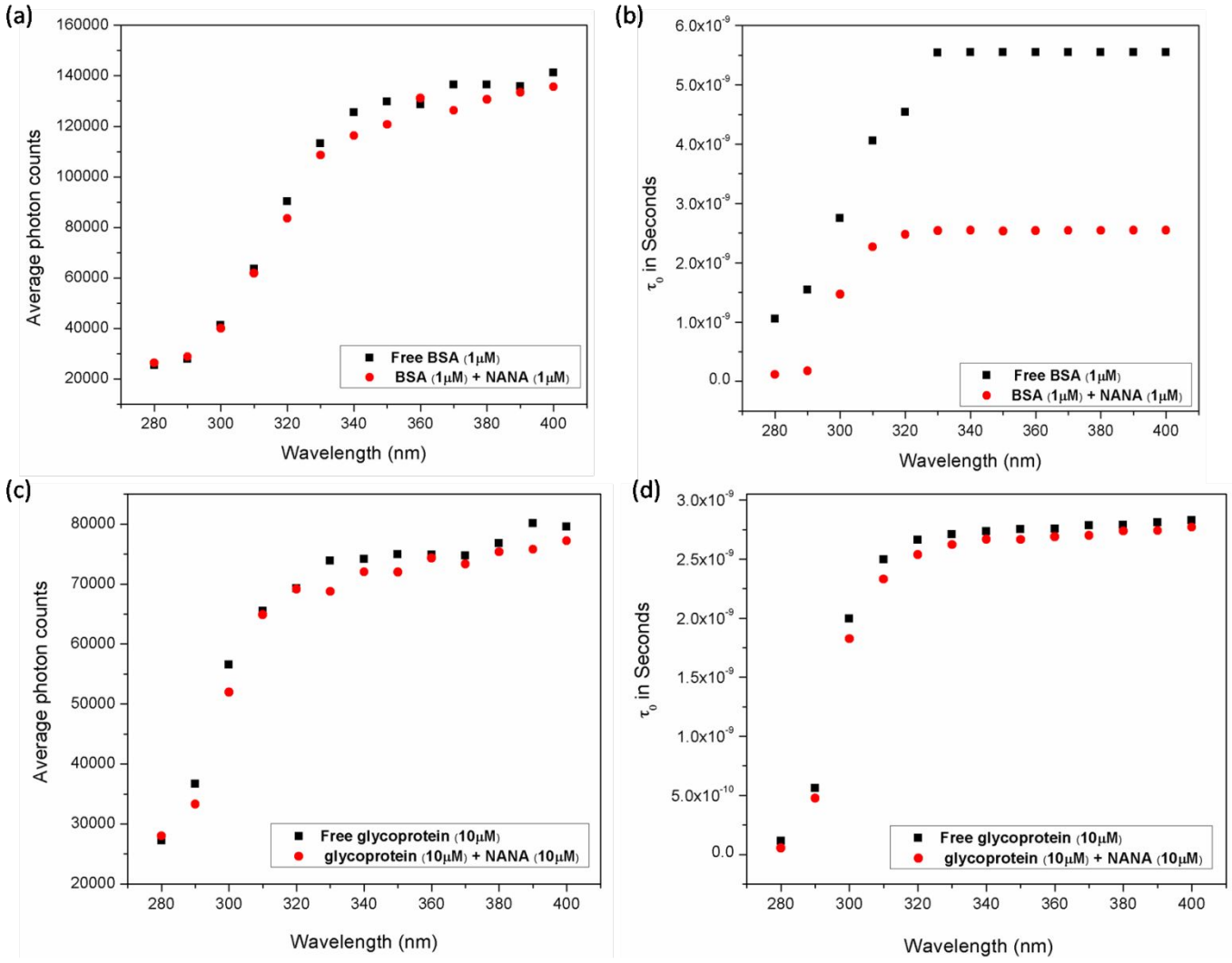

Figure 3 Time resolved emission spectroscopy analysis of (a) free BSA (b) BSA-NANA complex (c) free glycoprotein (d) glycoprotein – NANA complex

3.4 Forster Resonance Energy Transfer analysis (FRET)

The resonance transfer energy analysis explains the quantitative determination of transfer efficiency between donor and acceptor. The efficiency of energy transfer for NANA in both BSA and HAG (1: 1 μM) calculated according to Forster theory using the following equation 7

$$E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + r^6} \quad [7]$$

Where E is energy transfer efficiency, R_0 is critical energy distance, r is distance and F_0, F is initial and final intensities of free BSA,HAG and BSA,HAG- NANA complex.

R_0 critical energy is calculated equation 8.

$$R_0^6 = 8.79 \times 10^{-25}\left[k^2 N^{-4} \varphi_D J\right] \quad [8]$$

Where k^2 =2/3 is dipole spatial orientation factor, N =1.336 is a refractive index, φ_D = 0.198 is quantum yield, J is overlapping integral of donor (BSA,HAG) and acceptor of NANA molecule

$$J(\lambda) = \int_0^\infty F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda \quad [9]$$

The overlapping integral is calculated using equation 9, F_D (λ) is donor (BSA,HAG) fluorescence intensity; $\varepsilon_A(\lambda)$ is acceptor (NANA) molar extension co-efficient.

Figure S2 (a) & (b) shows the overlap emission of BSA, HAG and absorbance of NANA spectrum, from this data integral overlapping value is calculated for both molecule and calculated J is 1.902×10^{-21} cm^3 L/ mol for BSA -NANA and 6.809×10^{-21} cm^3 L/ mol for HAG-NANA. The R_0 also calculated from above equation 6 which 1.78 nm for BSA- NANA complex and 2.31 nm for HAG-NANA complex. The efficiency E for BSA – NANA is 0.0601 and for HAG – NANA is 0.0234. The final distance r is 3.1 nm for BSA-NANA and 4.3 nm for HAG – NANA. Since, the average reported distance r value is < 7nm. Hence, form the above calculated result suggests that NANA drug is within the binding region and possibilities of energy transfer for both BSA and HAG molecule [16-18,55].

3.5 Excitation emission matrix analysis

The excitation emission matrix analysis clearly explains about the presence and absence of NANA drug and conformational changes in both BSA, HAG microenvironment. The three dimensional fluorescence spectral data of free BSA (Fig S3a), BSA – NANA (Fig S3b) and free HAG (Fig S3c), HAG – NANA (Fig S3d) complex is shown in figure S3. The concentration of both protein and NANA molecule here kept as 1: 1 µM. In three dimensional panels, the excitation range was set into 230 nm to 700 nm with 10 nm intervals and emission was 2 nm integral with 5 nm bandpass filter for both excitation emission. From the figure S3a and S3b there is no shift and the new peak is observed, but the emission maxima are decreased for BSA – NANA complex comparing with free BSA, this result suggests that presence of NANA in BSA molecule and this result similar to the free HAG, HAG –NANA complex also. The overall result concludes that NANA drug forms a complex and rearrange the native structure of both protein molecule hydrophobic binding cavity [17,56,58].

3.6 FT-IR analysis

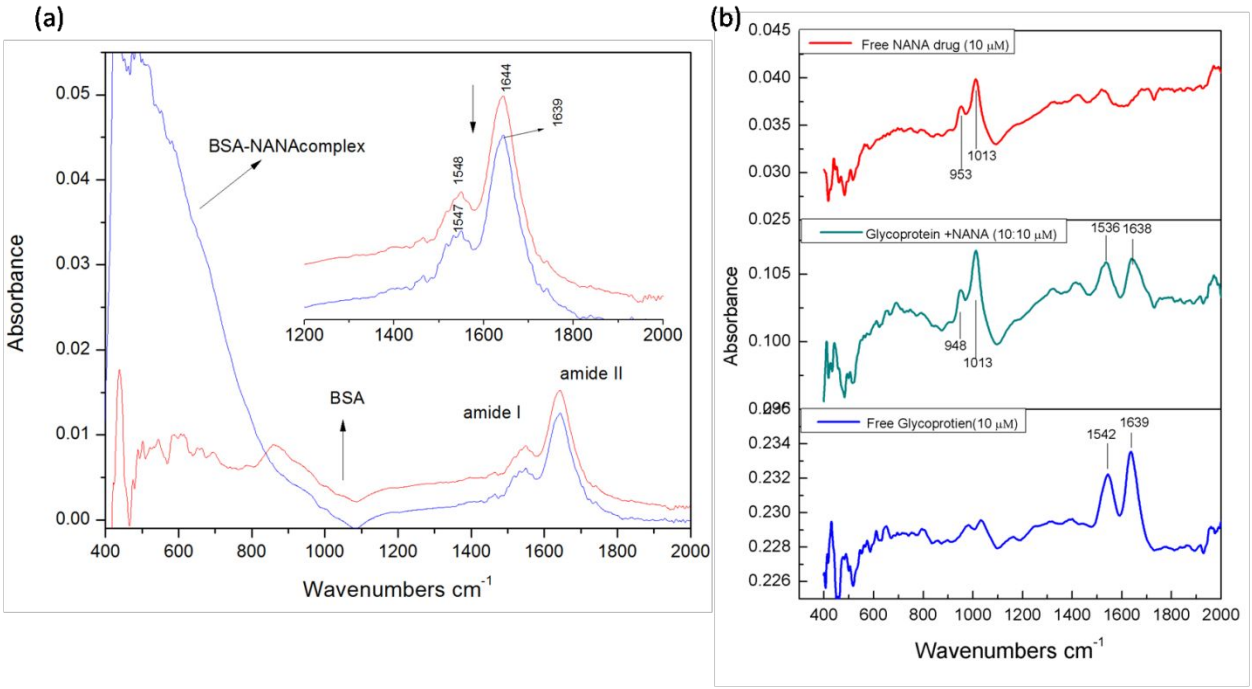

Figure 4 FT-IR spectrum of (a) free BSA with BSA-NANA complex (b) free glycoprotein with glycoprotein – NANA complex

FT-IR spectroscopy technique is used to understand the conformational changes in BSA and HAG, and also to explore the effect of NANA drug in both protein molecule environments. Figure 4 (a) & (b) shows free BSA and BSA –NANA complex, free HAG and HAG – NANA complex respectively. In general for the protein molecules amide peaks will be in the region of 1500 – 1700 cm^{-1} due to C=O stretch and N-H bend in the protein peptides. Also the amide I band (C- N stretch) is more sensitive while comparing with amide II band (C-N stretch, N-H bend). Here both protein and drug molecule are kept 10:10 µM ratio at 298K. Figure 4a shows the absorbance spectrum of free BSA molecule and the amide I and II bands are observed at 1548 cm^{-1} and 1648 cm^{-1} respectively.

While adding the NANA drug, the absorbance intensity is decreased and slight lower wavelength region shift is occurs in the amide II (1547 cm^{-1}) and amide I (1639 cm^{-1}) which confirms the presence of NANA in BSA microenvironment. On the other hand figure 4 b shows a similar effect of NANA in HAG microenvironment. From this overall result suggest that NANA could able to bind in both protein molecule and there is a possibility of structural rearrangements in NANA binding region [17-18, 37,57].

3.7 CD analysis

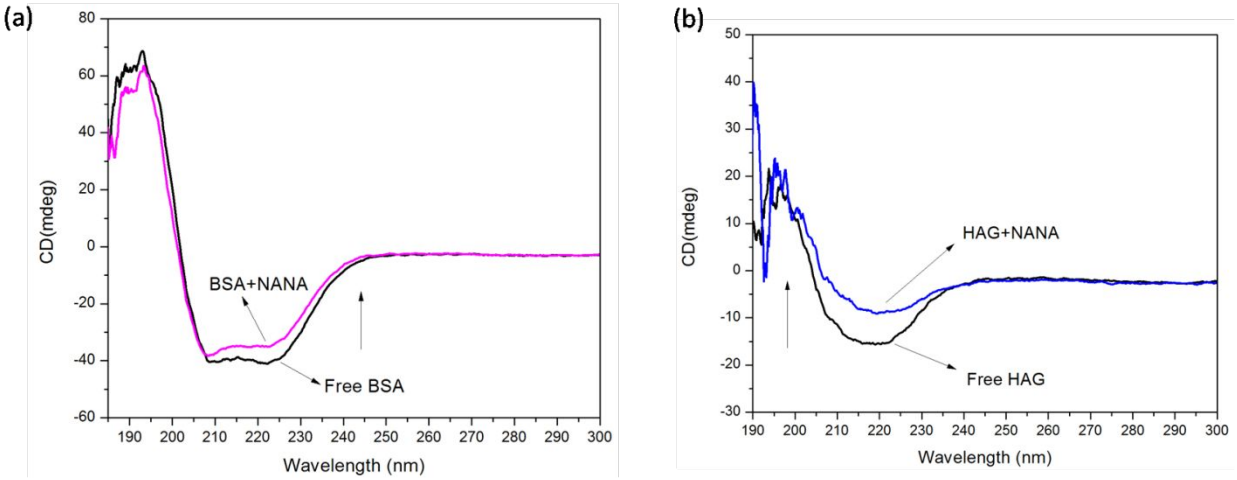

Figure 5 CD spectrum of (a) BSA-NANA system (b) HAG-NANA system

` The CD analysis was performed to understand the secondary structural conformation in both BSA, HAG in NANA complex. Fig 5 (a) and (b) clearly shows the difference between free BSA, HAG and BSA, HAG with NANA complex. From that Fig 5a shows two negative bands between 208 nm and 222 nm wavelength region due to the alpha helix but in the case of Fig 5b shows one negative band mainly due to the presence of beta strands. Also, to NANA in both BSA and HAG system shows that notable increase and slight shift. It is mainly due to the presence of NANA in both protein molecular secondary structural elements (SSE) (alpha helix (BSA), beta strands (HAG)) loss in their stability or it may re-arranged in total the orientation of total secondary structural elements. Also, the calculated SSE is shown in table S2 and the experimentally obtained result also compared with MD SSE analysis shown in Fig S7 (BSA-NANA) S8 (HAG –NANA) and the overall result suggest that the presence of NANA in BSA and HAG SSE of both protein molecule may partially unfold or re-oriented[53,54,59].

3.7 Molecular docking analysis

The molecular docking studies were performed to understand more about the NANA binding insight mechanism in both BSA and HAG molecule. The above experiment section may confirm the NANA drug presence in both BSA and HAG, but in molecular docking results reveals the specific interaction mechanism of NANA in two essential protein molecules. Figure S4 (a), (b) & (c) shows the best binding pose of NANA in BSA and its inset shows binding position, also interacting active site residues. Similarly, Figure S4 (d), (e) & (f) shows the best binding complex of NANA in HAG, inset is respective binding position and finally NANA interacting active site residues in HAG environment. The NANA molecule forms five hydrogen bonding interaction with (ASN 404, TYR 400, LYS 524, MET 547) BSA microenvironment with four amino acid residues (Figure S4c) , but in the case of HAG complex NANA drug forming seven hydrogen bonds with six amino acid (TYR 127, ARG 90, THR 47, GLU 64, TYR 27, SER 125) residues (Figure S4f). Interestingly the tyrosine residues were in active site region in both NANA – protein complexes, this residue is one of the significant fluorophore in the fluorescence study and this results may value added to the fluorescence spectroscopy results for confirmation of the presence of NANA in both protein complex. Also, based on the binding energy, the best docked poses were taken for BSA- NANA and HAG-NANA complex.

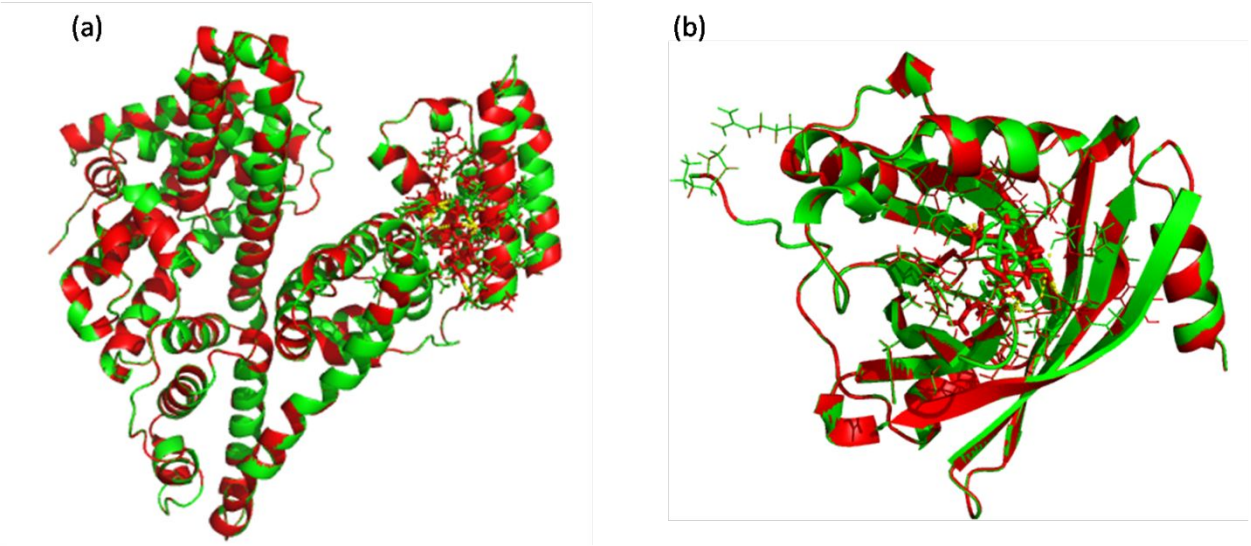

Figure 6 shows the superimposed structure of initial (green) and final (red) MD outputs of (a) BSA – NANA complex (b) HAG –NANA complex

The calculated binding energy for BSA –NANA is -48.93 KJ/mol and -44.38 KJ/mol for HAG –NANA complex among 20 different binding scores. Based on equation 3 the obtained docking scores were compared with experimental and theoretical data. From that the for BSA – NANA complex theoretically obtained docking score is -48.93 KJ/mol and -44.38 KJ/mol is almost in the same order and this results again giving concrete evidence for fluorescence experimental studies [38, 39].

3.8 Molecular dynamics analysis

The molecular dynamics study was performed after the molecular docking to understand more about the NANA stability in both BSA and HAG system (Figure 6). In general, the dynamics studies were performed at aqueous medium at drug binding in the region of a protein molecule to understand the effect of the drug in structural level. Root mean square deviation (RMSD) data is mainly used to obtain conformational changes in the protein – ligand complex. Figure 7 (a) shows the RMSD plot of BSA – NANA complex system and the simulations were performed up to 50 ns. The figures represent the stability of NANA in a protein molecule environment and there is no much fluctuation in the secondary structure of the BSA during the

interaction session of NANA. Figure 7 (b) & (c) shows the BSA and NANA root mean square fluctuation plot, from that the backbone of protein molecule Cα is observed and ligand RMSF gives the information about insights of NANA fragments in BSA binding environment, and this result suggests that the due to binding impact of NANA, the BSA secondary structural region may be rearranged during the molecular dynamics simulation process. In Figure 8 (a) shows the NANA –HAG system of RMSD and these result shows that NANA drug balanced after reaching 10 ns and the HAG secondary structure Cα also explore the same result.

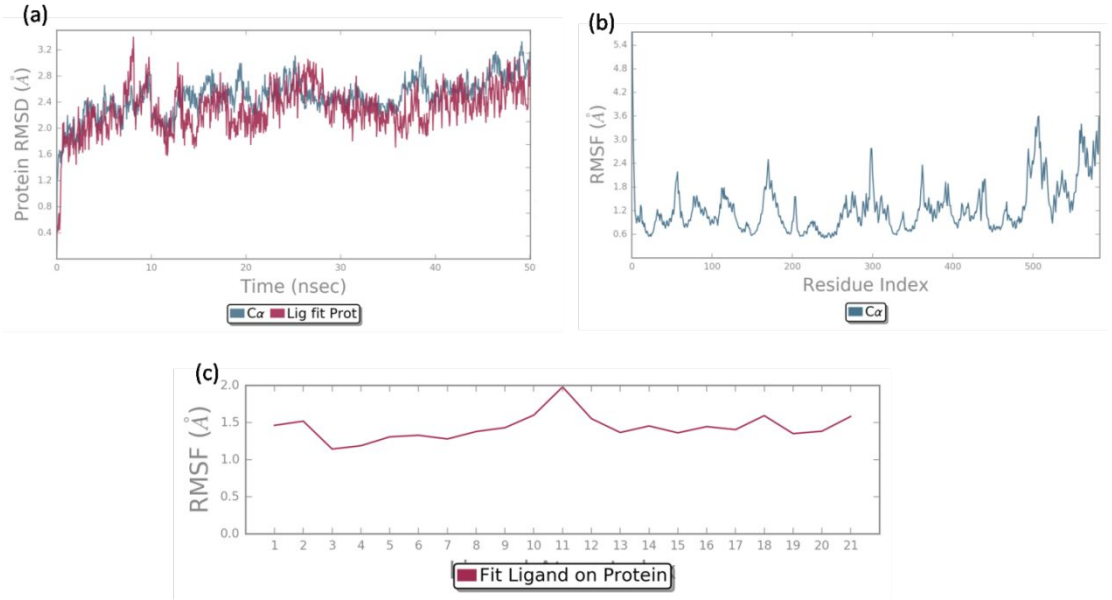

Figure 7 Molecular dynamics simulation of BSA – NANA complex (a)RMSD (b) RMSF of BSA (c) RMSF of NANA in fitting BSA

The RMSF of HAG – NANA system (Figure7 (b) & (c)) result shows that HAG does not affect the backbone of HAG during the MD simulation and this effect similar to BSA system also. The figure 7 and 8 results conclude that the binding of NANA did not affect C -alpha of both protein molecules. Figure S5 (a) and (b) shows the protein –ligand contacts of BSA – NANA, HAG-NANA respectively.

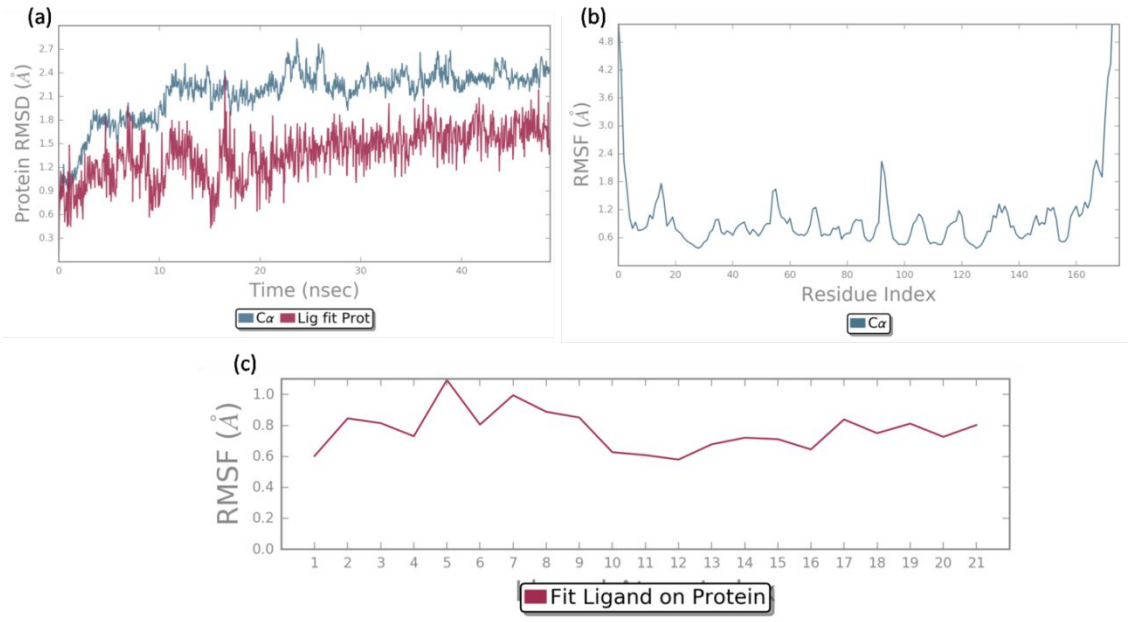

Figure 8 Molecular dynamics simulation of glycoprotein – NANA complex (a)RMSD (b) RMSF of glycoprotein (c) RMSF of NANA in fitting glycoprotein

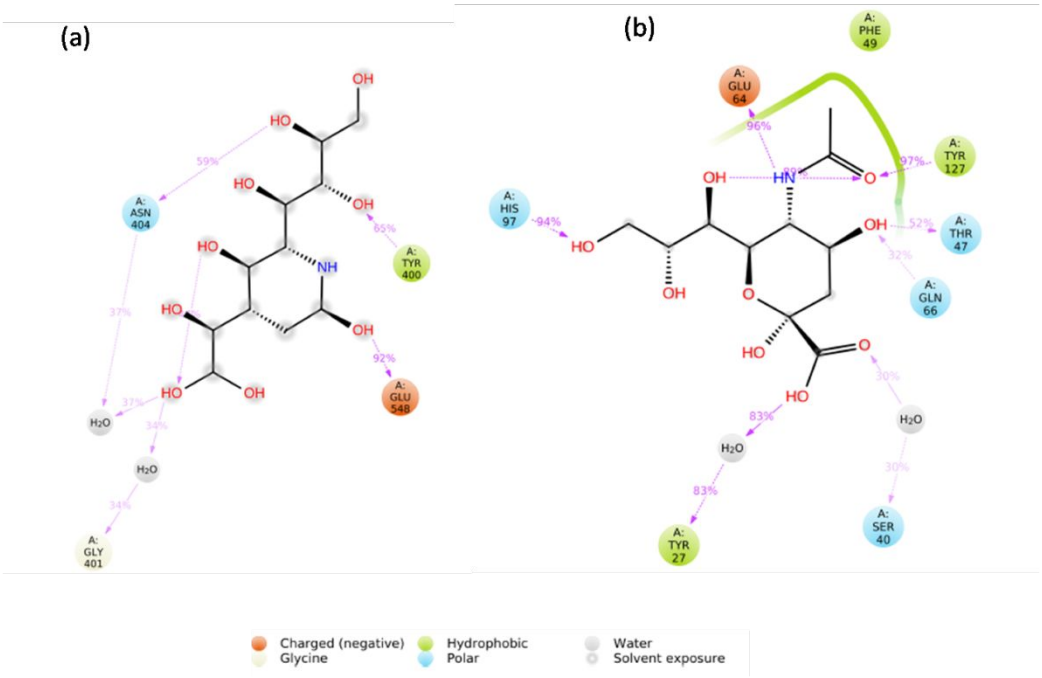

Figure 9 shows the after the simulation binding interaction plot of NANA in complex with (a) BSA (b) HAG

The active site residues were monitored throughout the simulation process from those hydrogen bonds, hydrophobic, ionic and water bridges contact were observed for NANA in both protein molecule environments. Comparing with all other active site residues tyrosine have more contacts with NANA in both protein molecules (Figure 9), around 70% of H-bond contact and 20% of water bridge in BSA microenvironment (TYR 400) and in HAG TYR 27 give 90% of water bridge, TYR 127 contributes 97% of H-bond with NANA molecule.

Further figure S6 (a) and (b) shows the H-bond stability of BSA- NANA and HAG – NANA active site region. This result also value added for TYR residues stability for both protein molecule active site NANA microenvironment and hence this may be comparable with fluorescence results, but the molecular dynamics result concludes that NANA can be bind into both protein molecule environment. In addition to this to know the secondary structural elements (SSE) of both BSA, HAG in NANA complex is also monitored and shown in figures S7 &S8. Also, looking at the histogram and torsion profile (figure S9 & S10) may give more insights into the conformational strain of NANA in BSA, HAG complex[40 -42].

3.9 Atomic charges and dipole moment analysis

After the docking simulation of BSA- NAN and HAG- NANA complex, there is one more possibility to understand about charge distribution analysis of NANA in both protein molecules binding site environment. Mullikan charge distribution analysis (table S1) can reveal the information of NANA molecule charge distribution environment in gas phase (free NANA) and in both BSA, HAG active site (single point) position. Form the calculated Mullikan values it is observed that most of the carbon and hydrogen atoms are positive value due to high electro negativity which is mostly bonded with oxygen and nitrogen atom. Similarly, the NANA molecule in both protein molecule active site environment reflects the same result with slight integer changes because of inter molecule interaction in both protein molecule active site region. Further, the dipole moment of NANA was also calculated to understand about polarity in protein environment and shown in figure S11. For free NANA the dipole moment was 4.51D and which is increased 4.71D for BSA active site region and 4.80D for HAG active region and this result concludes that NANA molecule could redistribute structurally as well as geometrically in both protein molecule environment [43,44].

3.10 Electrostatic potential and frontier molecular orbital analysis

To understand the reactive site of NANA in both protein molecules the electrostatic potential analysis was done. In general from the potential map a positive atom contains electrophilic sites and negative atoms are nucleophile selectivity. Figure 10 shows the electrostatic potential map for (a) NANA, (b) BSA –NANA active site and (c) HAG – NANA active site region. Form the figure10 (a) it is observed that carbon –oxygen bounded region are more nucleophile and other hydrogen atoms regions were surrounded by electrophilicity. Figure 10 (b) and (c) explains about that positive potential regions attract more amino acid residues instead of negative potential and this result suggests that NANA could form the intermolecular interaction between both protein molecular environments.

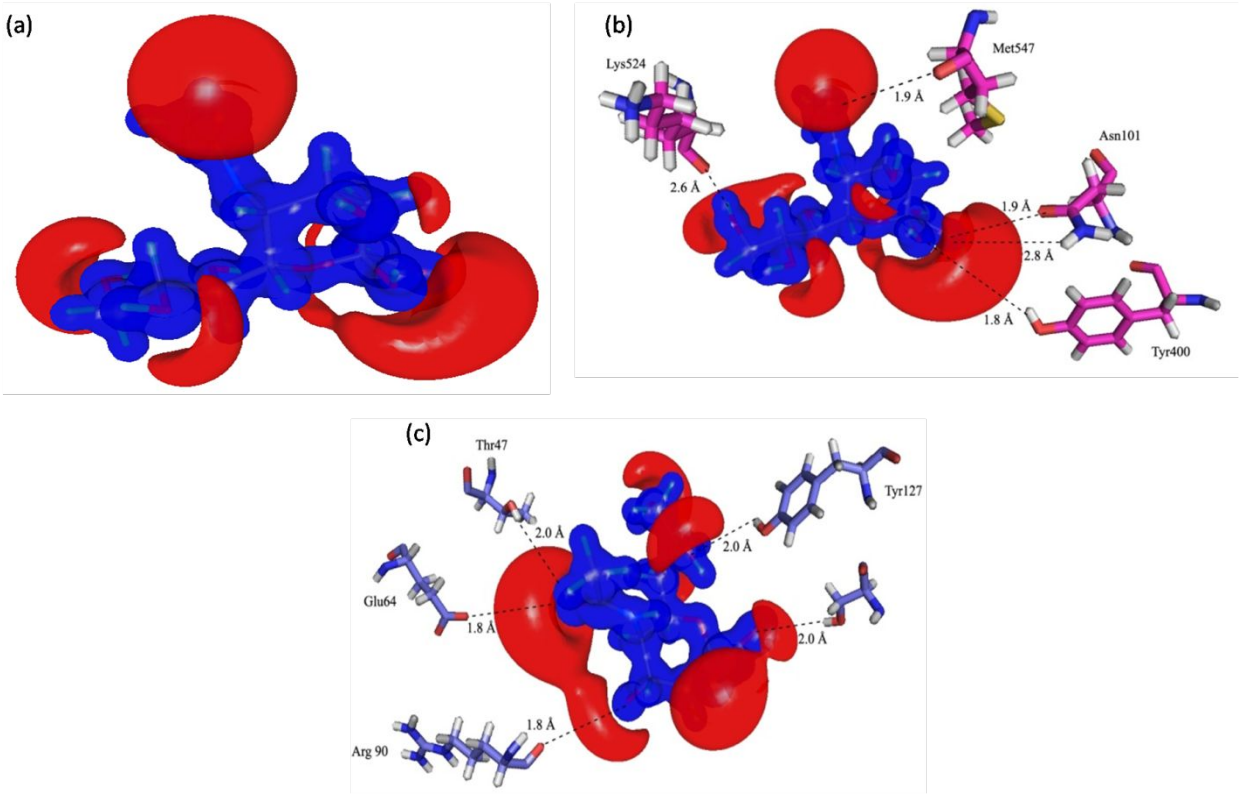

Figure 10 The molecular electrostatic potential of both molecules in isosurface representation with amino acid interactions are shown in pictorially [Blue: positive potential and Red: negative potential]. The surface values are +0.5 and -0.05 eÅ^{-1}

Figure S12 shows the HOMO – LUMO molecular orbital maps of (a) NANA (b) NANA in BSA and (c) NANA in HAG complex system. This result can suggest that electron donor acceptor of highest and lowest molecular orbital during the chemical reaction. The global reactive parameters of NANA molecule calculate and shown in Table 2. The overall result concludes that NANA could form strong intermolecular interaction during the binding of both protein molecules [45 – 49].

Global Reactivity Descriptors	**DFT-I** Energy (eV)
Band Gap	-5.45
HOMO Energy	-5.84
LUMO Energy	-0.39
Ionization Potential $I= -E_{HOMO}$	5.84
Electron Affinity $A= -E_{LUMO}$	0.39
Global Hardness $\eta= (I-A)/2$	2.73
Electronegativity $\chi= (I+A)/2$	3.12
Electrophilicity $\omega=\mu^2/2\eta$, $\mu= -\chi$	1.78

Table 2 The calculated global reactivity properties of NANA

4. Conclusion

In summary, the overall results obtained by different techniques suggest that NANA molecule binds well in both BSA and HAG molecules. In fluorescence technique confirms the nature of quenching of NANA is a static mode in both molecule and time resolved analysis confirm that the presence of NANA in both protein molecule was stable in full wavelength region. Energy transfer analysis suggests that there is a possibility to occur between NANA and BSA, HAG and calculate r values are 3.1nm, 4.3nm respectively. The excitation emission matrix analysis, FT-IR and CD report suggest that NANA could bind BSA and HAG without disturbing

both protein native structures. Molecular docking analysis clearly shows the evidence of NANA binding in both BSA and HAG molecule also, the calculated docking is almost in the same order of experimental value again this result makes a bridge between experimental results. Further molecular dynamics simulation concludes that NANA molecule could bind without altering the native structures of the both protein molecule. The charge distribution analysis provides detailed insights and intermolecular interaction of NANA in both protein molecule environments. These types of results will improve more in future drug design and these feedbacks will help to design some new effective drugs.

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Conflict of Interest:

There are no conflicts to declare

Supporting information:

Supplementary materials are provided that describe more detailed information on Plots of $\ln K_b$ versus 1/T for NANA binding to (a) BSA (b) HAG, FRET analysis of (a) free BSA with BSA-NANA complex (b) free glycoprotein with glycoprotein – NANA complex, Excitation Emission matrix analysis of (a) free BSA (b) BSA-NANA complex (c) free glycoprotein (d) glycoprotein – NANA complex, Pymol view of (a) BSA- NANA complex (b) inset of BSA-NANA complex (c) ligplot of BSA –NANA complex (d) glycoprotein – NANA complex (e) inset of glycoprotein – NANA complex (f) ligplot of glycoprotein – NANA complex, Interaction fraction of (a)BSA – NANA complex (b) glycoprotein – NANA complex, protein – ligand contacts during simulation

(a)BSA –NANA complex (b) glycoprotein – NANA complex, Table of Mullikan population analysis of NANA compound complex with BSA and HAG, The BSA and HAG secondary structural elements (SSE) reports, NANA torsion profile during simulation with BSA and HAG, dipole moment vectors of NANA in both molecules in gas phase and in the binding site of BSA and glycoprotein, HOMO and LUMO of NANA and NANA in molecules were plotted at the isosurface value at 0.02 au

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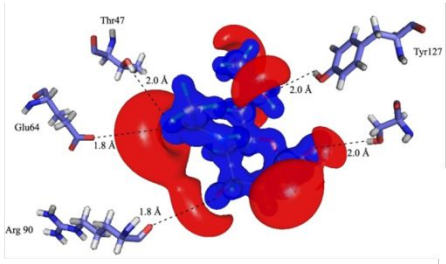
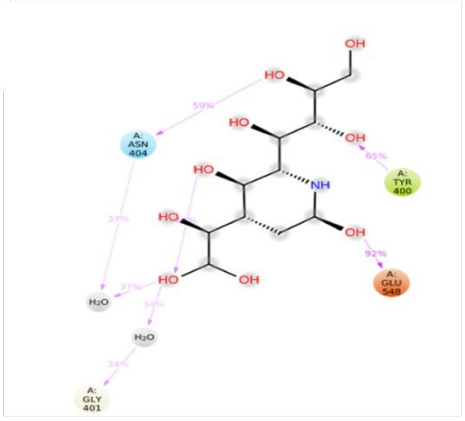
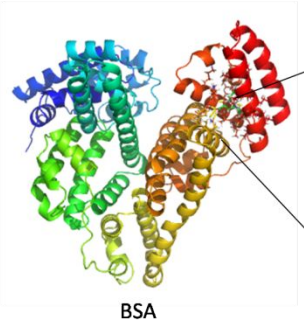
Graphical TOC Entry



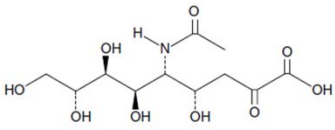
Inset of BSA-NANA complex



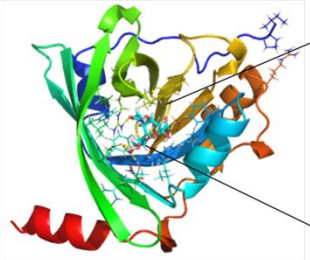
Interaction plot of NANA after MD simulation



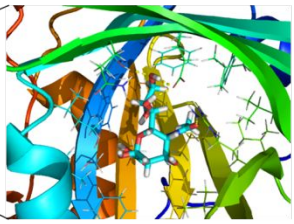
BSA



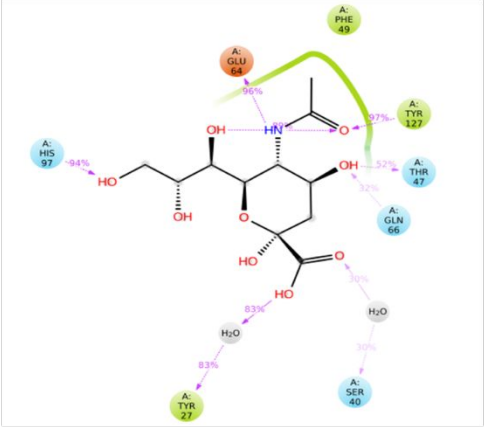
N- acetylneuraminic acid

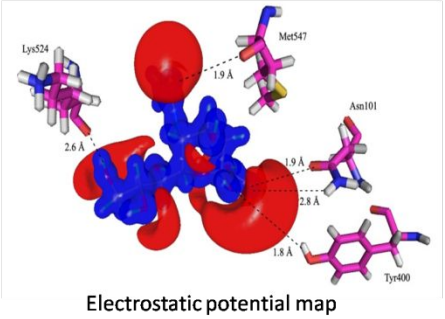


HAG



Inset of HAG-NANA complex





Electrostatic potential map