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# **A combined computational/experimental study on HSA binding of two water soluble Schiff base ligands derived from pyridine derivative and ethylenediamine**

Hajar Molaee, Mehdi Sahihi\*, Majid Moghadam\*, Valiollah Mirkhani, Shahram Tangestaninejad, Iraj Mohammadpoor-Baltork, Hadi Amiri Rudbari

*Department of Chemistry, University of Isfahan, Isfahan 81746-73441, Iran*

## **\*Corresponding authors:**

a) Mehdi Sahihi

Assistant professor of biophysical chemistry

Department of chemistry

University of Isfahan

Isfahan 81746-73441

Iran

Tel.: +98-31-37934929

Fax: +98-31-36689732

E-mail: [m.sahihi@chem.ui.ac.ir](mailto:m.sahihi@chem.ui.ac.ir)

b) Majid Moghadam

Professor of inorganic chemistry

Department of chemistry

University of Isfahan

Isfahan 81746-73441

Iran

+98-31-3137934920

Fax: +98-31-36689732

E-mail: [moghadamm@sci.ui.ac.ir](mailto:moghadamm@sci.ui.ac.ir)

**Keywords:** HSA binding, Schiff base ligand, Stabilizing agent, Fluorescence quenching, Molecular docking, Molecular dynamics simulation.

## **Introduction**

Due to the interesting chemical and physical properties, Schiff bases have received significant attention in recent years (Amiri et al., 2017; Shahraki, Shiri, & Saeidifar, 2017). In these compounds, imine bond is formed easily by condensation of primary or secondary amines with carbonyl groups. The Schiff base ligands have high coordination ability and very strong biological properties (Shahraki, et al., 2017). Although in the past decade these compounds have been extensively studied, still they are highly considered. These compounds exhibited many biological activity such as antitumor, antibiotics, antifungal, antimicrobial, anticancer and antibacterial properties and finally, they have appropriate for binding to DNA and HSA macromolecules (Dehkhodaei, Sahihi, & Amiri Rudbari, 2017). In the field of bioinorganic chemistry, the Schiff base ligands that derived from heterocyclic compounds such as pyridine are of high importance because of the presence of one or more ring containing nitrogen atom with a localized pair of electrons (Shokohi-Pour, Chiniforoshan, Sabzalian, Esmaeili, & Momtazi-borojeni, 2017).

Human serum albumin (HSA) is the most abundant plasma protein. Also, it is known as the dominant transporter plasma protein for endogenous and exogenous ligands (*e.g.* fatty acids and hormones) and is one of the most important targets in binding of various drugs (*e.g.* warfarin, diazepam and Ibuprofen) and metal ions. The HSA-binding of a drug increases its solubility in plasma, decreases its toxicity, protects it from oxidation, prolongs its *in vivo* half-life and increases its pharmaceutical effect. Understanding of these interaction mechanisms help us to know the pharmacodynamics and pharmacokinetics effects of drugs. Hence, investigation of the

interaction of small drug molecules with HSA is very important for the discovery of the mystery of life and the interaction mechanisms of drugs (Shahraki and Heydari, 2017).

In this paper, two water soluble Schiff bases were synthesized by the reaction of 4-pyridinecarbaldehyde or 4-pyridylmethyl ketone with ethylenediamine (Scheme S1). Then the bidentate Schiff base compounds were characterized by  $^1\text{H}$  NMR spectroscopies and elemental analysis. In addition, the molecular structure of one of these compounds was determined by single crystal X-ray diffraction technique. Then, the binding ability of these two Schiff bases with HSA was investigated using UV-Vis and fluorescence spectroscopy, molecular docking and molecular dynamics simulation methods.

## Experimental

### *Materials and instruments*

4-Pyridinecarboxaldehyde, 4-pyridylmethyl ketone and ethylenediamine were obtained from Merck chemical company and used without further purification. Human serum albumin (HSA), was purchased from Sigma–Aldrich. All salts used for buffer preparation were analytical grade and dissolved in double distilled water. All of the solutions were used freshly after preparation.

$^1\text{H}$  NMR spectra were recorded on a Bruker Avance 400 spectrometer using  $\text{CDCl}_3$  as solvent (**Figure S1**). The UV–Vis spectra were obtained on a JASCO V-570 spectrophotometer and the UV-Vis spectra for PE and MePE are shown in the **Figures S2** and **S3**, respectively. Also their details were stated in the **Synthesis** part. The fluorescence spectra were carried out on a Shimadzu RF-5000 (Kyoto, Japan) spectrofluorimeter equipped with thermostated cell compartment, was used at  $\pm 0.1$  °C.

### *Synthesis of the Schiff base ligands*

Two Schiff base ligands PE and MePE have been synthesized in various articles for different applications, before (Yang, Tao, Huang, & Zheng, 2010). Here, these compounds were resynthesized in a simple method in order to investigate their interaction with HSA. To a stirring solution of 4-pyridinecarboxaldehyde (2 mmol) or 4-pyridylmethyl ketone (2 mmol) in methanol (10 mL), a solution of ethylenediamine (1 mmol) in methanol (10 mL) was added dropwise. The reaction mixture was stirred for 3 h at the 50 °C and then filtered. Suitable single crystals of PE for X-ray crystallography were obtained by leaving the filtrate undisturbed at room temperature in stable state for 2 days. The crystals were filtered off, were washed with cold methanol, and were dried in vacuum. Crystal data, together with other relevant information on structure determination for PE, are listed in **Table S1**. The molecular structure and atom-numbering scheme of **PE** is presented in **Figure S4**. Selected bond distances, and bond angles are also given in **Table S2**. Also inter and intra molecular hydrogen bond geometries (Å) and their informations were shown in **Figure S5** and **Table S3**.

**PE:** Yield 93%. Anal. calculated for  $\text{C}_{14}\text{H}_{14}\text{N}_4$  (M.W. = 214.02 g/mol): C, 70.57; H, 5.92; N, 23.51. Found: C, 71.43; H, 5.92; N, 23.48.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz, 298 K)  $\delta$ /ppm: 8.4 (d, 4H,  $\text{H}_{\text{Ar}}$ ), 8.2 (s, 2H,  $\text{H}_{\text{imine}}$ ), 7.5 (d, 4H,  $\text{H}_{\text{Ar}}$ ), 4 (s, 4H,  $\text{H}_{\text{en}}$ ). UV–Vis:  $\lambda_{\text{max}}$  (nm): 286 ( $\pi \rightarrow \pi^*$ ), 258 ( $\pi \rightarrow \pi^*$ ), 224 ( $n \rightarrow \pi^*$ ).

**MePE:** Yield 89%. Anal. calculated for  $\text{C}_{16}\text{H}_{18}\text{N}_4$  (M.W. = 266.4 g/mol): C, 72.14; H, 6.8; N, 21.03. Found: C, 71.17; H, 6.97; N, 20.65.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz, 298 K)  $\delta$ /ppm: 8.65 (d, 4H,  $\text{H}_{\text{Ar}}$ ), 7.62 (d, 4H,  $\text{H}_{\text{Ar}}$ ), 4 (s, 4H,  $\text{H}_{\text{en}}$ ), 2.29 (s, 6H,  $\text{H}_{\text{methyl}}$ ). UV–Vis:  $\lambda_{\text{max}}$  (nm): 282 ( $\pi \rightarrow \pi^*$ ), 221 ( $n \rightarrow \pi^*$ ).

### ***Preparation of stock solutions***

A Stock solution of HSA (5 mg/ml for fluorescence and UV-Vis studies) was made by dissolving HSA in phosphate buffer (50 mM, pH=7). The exact concentration of HSA was determined spectrophotometrically using molecular absorption coefficient of 35700 M<sup>-1</sup>cm<sup>-1</sup> at 278 nm. Schiff bases were completely dissolved in water and kept in the dark. All solutions were used freshly after preparation. UV-Vis spectra of compounds were recorded at different times and no changes were observed in these spectra.

### ***Fluorescence spectroscopy***

Fluorescence quenching is the best method to compute the binding constant and to quantify the binding affinities of macromolecules with drugs such as Schiff bases. Fluorescence of the proteins is created by three inherent fluorophores in the protein structure; tyrosine (Tyr), tryptophan (Trp) and phenylalanine(Phe) residues. Among them, phenylalanine has very low quantum yield, so normally fluorescence of protein was produced by tryptophan and tyrosine residues. When the excitation wavelength of HSA is set at 295 nm, fluorescence is created by the tryptophan residues. The band widths of the emission and excitation slits were set at 5.0 and 10 m. In generic experiments, emission spectra were recorded after addition of Schiff bases solutions (50 μM) at 298, 303 and 308 K to 2 ml of protein solution (5 μM). In these experiments, the observed fluorescence intensities were corrected for the dilution and inner filter effect. The excitation was performed at 295 nm and the emitted light was recorded from 300 to 450 nm. The solutions were incubated for 5 min before recording the emission spectra.

### ***Absorption spectroscopy***

The HSA solution was freshly prepared just before the measurements. The absorption study of HAS was performed in phosphate buffer (pH = 7) at 298 K. In typical experiments, 2 ml of HSA solution (10 μM) was placed in the cuvette and the absorbance spectra were recorded at 220-350 nm, after addition of the stock solutions (70 μM). In these experiments, the observed absorbencies corrected for the dilutions. The solutions were incubated for 5 min, before recording the absorption spectra. The absorption spectrum of HSA illustrate maximum absorption band at 279 nm assigned to  $\pi \rightarrow \pi^*$  transitions of aromatic amino acids and a higher maximum at 205 nm devoted to  $\pi \rightarrow \pi^*$  transitions of amide groups of peptide bonds (Shahabadi, Khorshidi, & Mohammadpour, 2014). Also, it should be noted that the UV-Vis spectra of these compounds have not overlap with the fluorescence spectrum of HSA in the same concentration. So, there is no energy transfer between the Schiff base ligands and HSA.

### ***Docking procedure***

For the docking calculations, the 3D structure of PE was provided by converting its crystallographic .cif file to .pdb file. However, the 3D structure of MePE was made using Gauss view 5.0. Calculation of structure optimization was carried out at the 6-31G\*\* level by employing the Becke three-parameter Lee-Yang-Parr (B3LYP) hybrid density functional theory using the quantum chemistry software Gaussian 03 (Frisch et al., 2009). The crystal structure of HSA (PDB ID: 1AO6) was taken from the Brookhaven Protein Data Bank (<http://www.rcsb.org/pdb>). The molecular docking software, AutoDock Version 4.2, using the implemented empirical free energy function and the Lamarckian Genetic Algorithm (LGA) was used for docking. The protein input file for docking was prepared by adding Gasteiger charges. Grids were calculated using AutoGrid. In the first step, for two compounds the dimensions of the grid box were set on the 126×126×126

points along with x, y and z axis. Then, the most numerous cluster with the lowest docking energy was selected for the second docking run using a grid map with 60×60×60 points and a grid-point spacing of 0.375 Å. 150 docking runs with 25,000,000 energy evaluations for each run were performed.

### ***Molecular dynamics (MD) simulation***

MD simulation on the compound–protein complex was used for further investigation on the structural changes of protein. A 20 ns MD study was carried out using GROMACS 4.5.6 software package (Berendsen, van der Spoel, & van Drunen, 1995). The GROMOS96 43a1 force field was used, and the SPC water model was employed for the water molecules. The system is required to be neutral, so charges were balanced with 15 sodium counter ions. A cubic box was chosen, with periodic boundary conditions. The number of the total atoms in the system were 43641 and 43638 for PE and MePE, respectively. The force field parameters were obtained from PRODRG web server. Gaussian 03 at the level of B3LYP/6-31G\*\* was used for calculating of atomic charges. Energy minimization was performed in the first step with the steepest descent method for the 3000 time steps (Hess, Bekker, Berendsen, & Fraaije, 1997). After energy minimization process, position restraint procedure was performed in association with NVT and NPT ensembles. An NVT group was adjusted at constant temperature of 300 K with 200 ps time duration. After stabilization of temperature an isothermal– isobaric ensemble (NPT) was performed at constant pressure of 1.0 bar with 200 ps time duration. Finally, a 20 ns MD simulation was carried out at 300 K and 1 bar. A berendsen thermostat (300 K) and the Particle-Mesh Ewald (PME) method were used to correct the long-range electrostatic interaction and the cut-off method (1.2 nm) was used to treat the van der Waals interactions. The leap-frog algorithm with the 2 fs time steps was used for integration of the equation of motion. For further analysis, the atomic coordinates were recorded to the trajectory file every 0.5 ps.

## **Result and Discussion**

### ***Absorption spectroscopy***

UV–Vis absorption study is the most usual technique to investigate the HSA structural changes through its interaction with other compounds. In this experiment, these studies were carried out using different concentrations of the synthesized Schiff bases.

The binding constant ( $K_a$ ) of these interactions have been determined using the Benesi–Hildebrand equation; (Eq. 1),

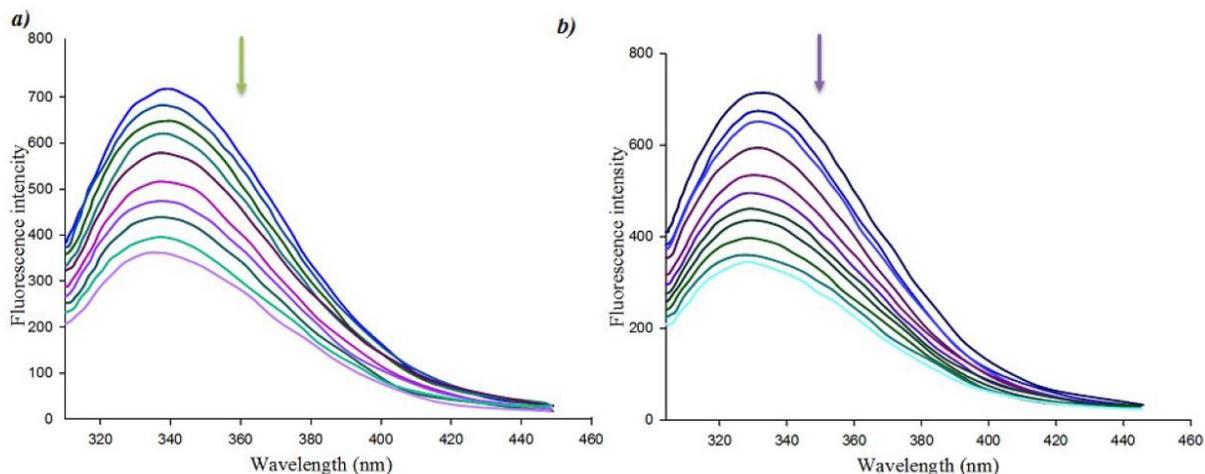
$$1/(A - A_0) = 1/(A_{\max} - A) + 1/K_b(A_{\max} - A)[M] \quad (1)$$

In this equation,  $A_0$  and  $A$  are the absorbance of HSA in the absence and presence of other compounds, respectively.  $A_{\max}$  is the obtained absorbance at saturated concentration and  $[M]$  is the compound concentration.  $K_a$  was obtained from the ratio of the intercept to the slope in the plot of  $1/(A-A_0)$  versus  $1/[M]$ . A significant reduction in the maximum absorbance of two spectra was observed as a function of compound concentration (figures are not shown). This observation demonstrates that HSA interacts with the synthesized Schiff base compounds. The values of the binding constant ( $K_a$ ) were determined from Eq. 1 and were reported in **Table 1**.

### ***Fluorescence spectroscopy***

As mentioned above, the fluorescence of HSA is dominated by the residue Trp-214 in subdomain IIA. The binding of Schiff bases as small quencher molecule to HSA changes the environment of tryptophan residue. The intrinsic fluorescence intensity of HSA is induced by these changes.

The fluorescence emission spectra of HSA at various concentrations of Schiff bases at 298 K are shown in **Figure 1**. The experiment was repeated at 303 and 308 K and the related quenching plots are shown in **Figures S6** and **S7**. The HSA showed a strong fluorescence emission with a peak around 340 nm at  $\lambda_{ex}=295$  nm.



**Figure 1.** Fluorescence emission spectra of the HSA in the presence of various concentrations of Schiff base compound a) PE, b) MePE ( $[compound]/[HSA] = 0.7 - 7$ ,  $T = 298$  K and  $\lambda_{ex} = 295$  nm)

During the titration of a fixed concentration of HSA with various amounts of the Schiff bases, a remarkable decrease was observed in the fluorescence intensity with increasing the concentration of Schiff bases. The strong quenching of HSA fluorescence clearly indicated that both Schiff bases bound to HSA and changed its tertiary structure (Trynda-Lemiesz, Keppler, & Kozłowski, 1999). In these experiments, the observed fluorescence intensities were corrected for the dilutions. The absorbance of protein solution was less than 0.1 at both excitation and emission wavelengths. Hence, reabsorption effect can be ignored in our experiments.

The Stern–Volmer equation (Eq. 2) was used for analysis of HSA fluorescence quenching:

$$F_0/F = 1 + K_{sv}[Q] = 1 + \tau k_q[Q] \quad (2)$$

Where  $F_0$  and  $F$  are fluorescence intensities of HSA in the absence and the presence of the quenching agent (Schiff base compounds), respectively.  $K_{sv}$  is the Stern–Volmer quenching constant,  $[Q]$  is the quencher concentration,  $k_q$  is the bimolecular quenching constant and  $\tau$  is the average lifetime of protein in the absence of quencher ( $10^{-8}$  s) (Kazemi et al., 2016).  $K_{sv}$  and  $k_q$  were determined from the plot of  $F_0/F$  vs.  $[Q]$  at 298, 303 and 308 K (**Figure S8** and **Table 1**).

**Table 1.** The HSA-binding constant ( $K_b$ ), the number of binding site ( $n$ ), the Stern–Volmer constant (KSV) of the compounds and the quenching rate constant of HSA ( $k_q$ ),  $K_b^*$  is the binding constant from UV-Vis spectroscopy.

Name	T/K	$K_b^*/M^{-1}$	$K_b/M^{-1}$	$n$	$K_{sv}/M^{-1}$	$k_q/S^{-1}$
PE	298	$1.3 \times 10^4$	$1.7 \times 10^4$	1.3	$4.2 \times 10^4$	$4.2 \times 10^{12}$
	303		$2.1 \times 10^4$	1.2	$5.4 \times 10^4$	$5.4 \times 10^{12}$
	308		$3.6 \times 10^4$	0.98	$5.5 \times 10^4$	$5.5 \times 10^{12}$
MePE	298	$7.3 \times 10^4$	$5.4 \times 10^4$	0.93	$4.7 \times 10^4$	$4.7 \times 10^{12}$
	303		$5.48 \times 10^4$	1.0	$5.1 \times 10^4$	$5.1 \times 10^{12}$
	308		$5.7 \times 10^4$	0.9	$5.2 \times 10^4$	$5.2 \times 10^{12}$

If the Stern–Volmer plot is linear, the quenching fluorescence has only one mechanism, dynamic or static (In the static mechanism, the macromolecule and the quencher collide together in the ground state but they collide together at the excited state in the dynamic mechanism). For the synthesized compounds, the values of  $K_{sv}$  increase with increasing the temperature. This observation supports that quenching fluorescence of HSA by Schiff bases occurs *via* a dynamic mechanism .

If there are  $n$  substantive binding sites on the surface of protein to bind the compound molecules, the binding constant ( $K_a$ ) can be calculated as follows (Eq. 3) :

$$\ln(F_0 - F)/F = \ln K_b + n \ln[Q] \quad (3)$$

The values of  $K_a$  and  $n$  are reported in **Table 1**. The obtained results indicate that the  $K_a$  for MePE is larger than the obtained value for PE at all temperatures. Really, MePE is bound to HSA more tightly than PE and the affinity of HSA for binding of this compound is greater than the other one. Also, for two compounds at all temperatures the  $n$  value is about 1 which indicates that HSA and the Schiff base interact in a 1:1 ratio.

#### ***Determination of the thermodynamic parameters***

In order to find the nature of the interaction between Schiff bases and biomacromolecules, the thermodynamics parameters were determined at different temperatures (298, 303 and 308 K). The changes in both enthalpy ( $\Delta H^\circ$ ) and entropy ( $\Delta S^\circ$ ) were determined using Van't Hoff equation (Eq. (4)):

$$\ln K_b = -\Delta H^\circ/RT + \Delta S^\circ/R \quad (4)$$

Where  $K_a$  is the binding constant at the corresponding temperature, T is the absolute temperature in Kelvin and R is the gas constant. The values of  $\Delta H^\circ$  and  $\Delta S^\circ$  can be determined from the linear

relationship between  $\ln K_a$  and the reciprocal of absolute temperature. Moreover, according to Eq. (5), the binding free energy change ( $\Delta G^\circ$ ) was calculated as a function of temperature and the obtained data are listed in **Table 2**.

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \quad (5)$$

Thermodynamic parameters ( $\Delta H^\circ$ ) and ( $\Delta S^\circ$ ) provide significant information on the type of interactions. There are four main forces between biomacromolecule and small compounds: hydrogen bonding, van der Waals forces, electrostatic and hydrophobic interactions (Zolfagharzadeh, Pirouzi, Asoodeh, Saberi, & Chamani, 2014). The positive values for  $\Delta H^\circ$  and  $\Delta S^\circ$  imply a hydrophobic force but the negative values for both of them reflect the van der Waals force or hydrogen bond formation. Finally, the positive value for  $\Delta S^\circ$  and small amount of  $\Delta H^\circ$  ( $\Delta H^\circ \approx 0$ ) suggest the electrostatic forces. As shown in **Table 2**, the negative values for Gibbs free energy change ( $\Delta G^\circ < 0$ ) means that the binding process for HSA is spontaneous. Additionally, the positive values for enthalpy ( $\Delta H^\circ > 0$ ) and entropy ( $\Delta S^\circ > 0$ ) changes indicate that the binding is mainly entropy-driven, and the hydrophobic forces play a major role in this interaction. Also, for both Schiff bases, the negative value of  $\Delta G^\circ$  increased with raising the temperature, so that the interaction was done more spontaneous at higher temperatures. Besides, in each temperature the value of  $\Delta G^\circ$  for MePE is more negative than PE. Therefore, the affinity of HSA for binding to MePE is more than that of PE. Hence, replacing the hydrogen atom in PE with methyl group in MePE could have basic role in the stability of HSA-MePE complex. Really, the methyl group enhances the electron density on pyridine ring and increases the probability of  $\pi$  stacking interaction of MePE and HSA. This hypothesis should be examined by molecular docking calculation which can show the atomic details of macromolecule-ligand interactions.

**Table 2.** Thermodynamic parameters of PE and MePE with HSA.

Name	T/K	$\Delta G^\circ/\text{kJ.mol}^{-1}$	$\Delta H^\circ/\text{kJ.mol}^{-1}$	$\Delta S^\circ/\text{J. mol}^{-1}.\text{K}^{-1}$
PE	298	-24.3	37.8	206
	303	-25.5		
	308	-27.6		
MePE	298	-27.1	2.6	99
	303	-27.9		
	308	-28.7		

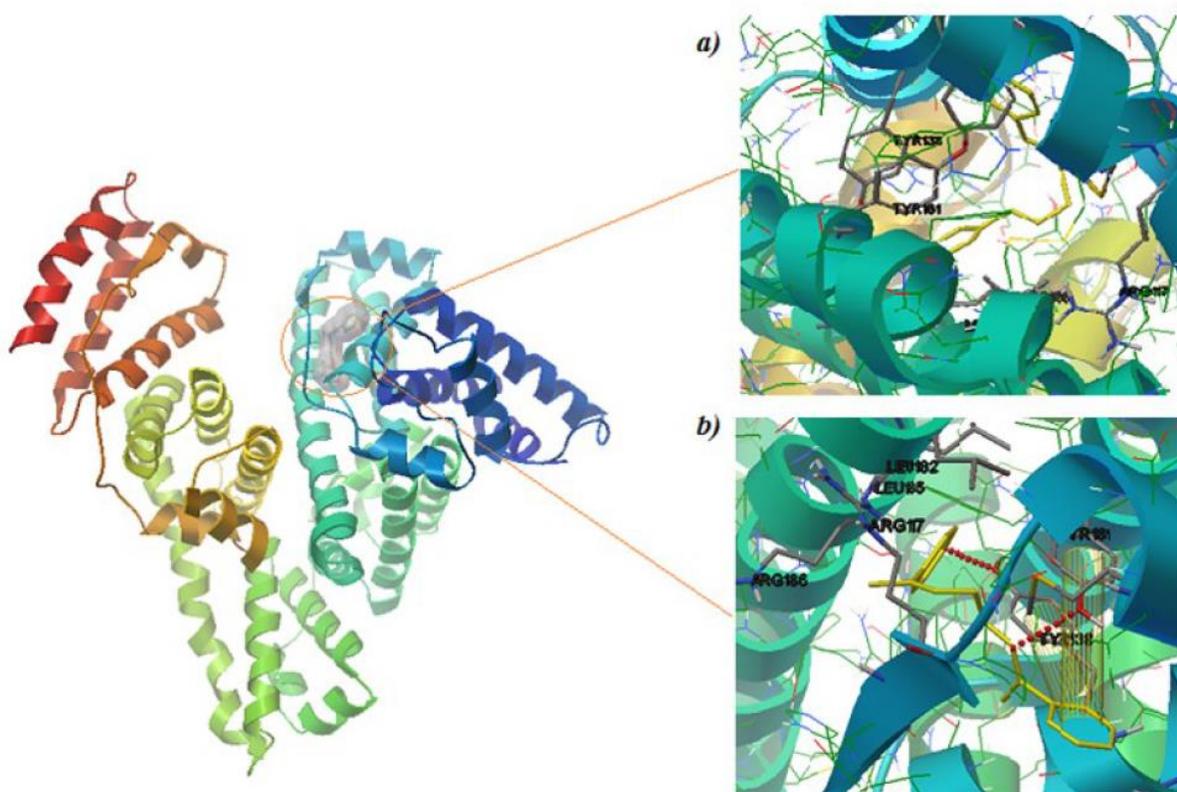
#### **Docking procedure**

HSA is a monomeric protein with three  $\alpha$ -helical domains that consists of 585 amino acids. These domains are divided into six-helix subdomains (A and B) (Gelamo, Silva, Imasato, & Tabak, 2002). The previous analyses showed that aromatic compounds can be located within two famous

hydrophobic sites known as Sudlow's Site 1 and 2 in sub-domains IIA and IIIA, respectively. Also, there is a D-shaped cavity in subdomain IB (termed as site 3) for binding of some compounds such as hemin (Assaran Darban, Shareghi, Asoodeh, & Chamani, 2016).

The results of the interaction between Schiff bases and protein in docking procedure are shown in **Figure 2**. As it can be seen in this figure, PE and MePE are situated within subdomain IB in site 3 with the binding energy of  $-6.69$  and  $-7.08$  kcal.mol<sup>-1</sup> for PE and MePE, respectively. Furthermore, these compounds were adjacent to some hydrophobic residues in subdomain IB of HSA (Tyr 161, Tyr 138, Tyr 133, Phe 149, Leu 185, Arg 186, Gly 189 and Met 123). Also, there is a high binding affinity between HSA and MePE compared to PE. This result is in agreement with UV-Vis spectroscopy and fluorescence quenching results. MePE has two hydrogen bonds between imine and pyridine nitrogen with the oxygen atoms of Tyr 161 and Tyr 138 residues and there are two  $\pi$ - $\pi$  interaction between the pyridine ring of this compound and the aromatic rings of Tyr 161 and Tyr 138 residues. This observation is in consistent with thermodynamic results (see previous section).

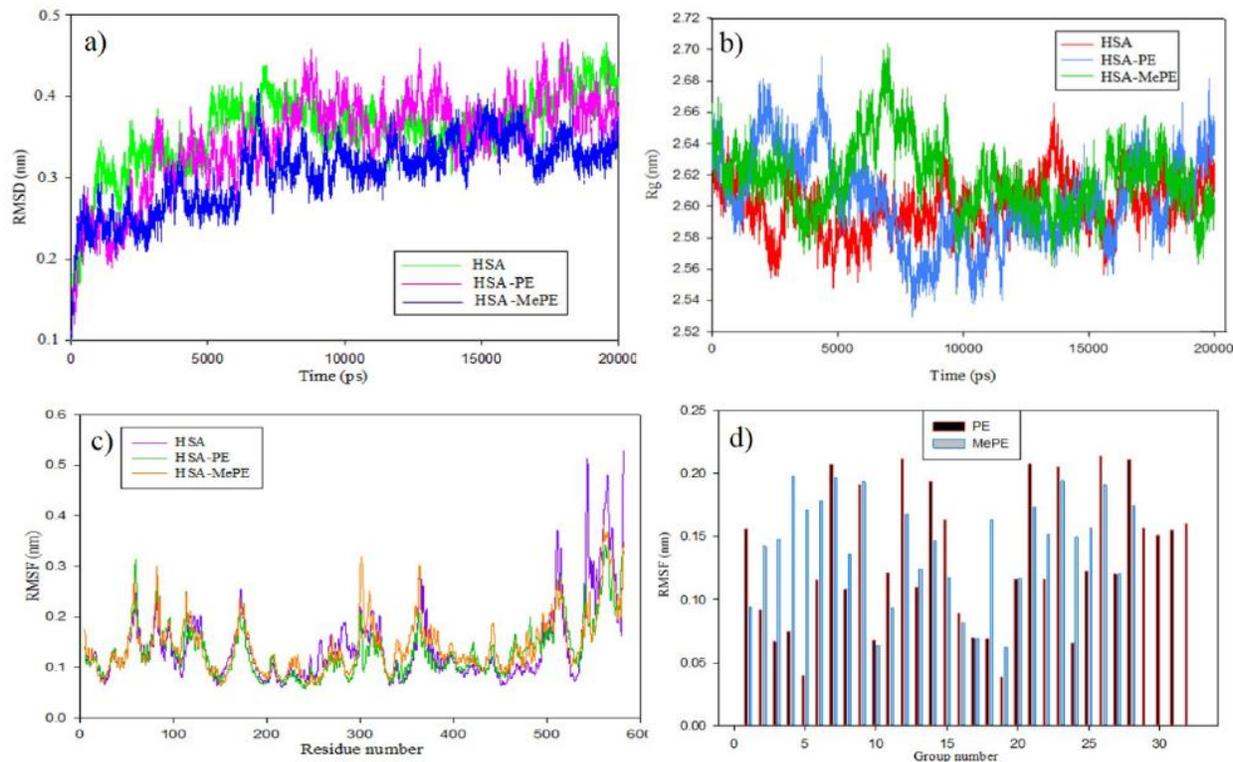
As it mentioned before, the methyl group increases the electron density of pyridine ring and causes stabilizing the HSA-MePE complex with  $\pi$ -stacking interactions. As a result, these bonds play an important role in stabilizing MePE compared to PE in IB binding site of HSA.



**Figure 2.** Detailed view of the interactions of a) PE, b) MePE with the binding pocket in subdomain IB. Red lines and yellow cones shows the Hydrogen bonding and  $\pi$ - $\pi$  interaction

### *Molecular dynamics simulation*

The trajectories of free HSA and HSA-ligands systems were investigated based on Root Mean Square Deviations (RMSDs) of back bone atoms of protein, Radius of gyration ( $R_g$ ), and Root Mean Square Fluctuation (RMSF) of protein residues. The stability of the system (protein, Schiff base, ions, water, etc.) was examined by RMSD. RMSD values as a function of simulation time for free HSA and HSA–ligand complexes during 20 ns MD simulations (**Figure 3a**). It is observed that for all molecular systems, the RMSD remains stable with some fluctuations after 6 ns of simulation, so all of the following analysis was carried out based on the stability of the simulation after the 14 ns of equilibration and the stability of the whole protein structure makes it possible to obtain reliable analysis on compound binding and its effect on HSA structure through MD simulation.



**Figure 3.** Obtained diagram of MD simulation for unconjugated HSA and HSA-Schiff base complexes, a) time dependence of RMSD, b) radius of gyration ( $R_g$ ), c) root mean square fluctuation (nm) of HSA residues, d) RMSF(nm) of PE and MePE atoms.

Also, the compactness of protein was specified by radius of gyration ( $R_g$ ) values of the HSA and HSA–ligand complexes. The results were plotted versus simulation time (**Figure 3b**). Stabilization of  $R_g$  values about 8 ns indicated that the MD simulation could achieve equilibrium after this time. There is no conformational changes during the simulation, because the radius of gyration of all systems are approximately similar to each other. The mobility of protein was also investigated by calculating the time averaged root mean square fluctuation (RMSF) (Jana, Ghosh, Dalapati, & Guchhait, 2012) of free HSA and HSA-ligand complexes based on the last 14 ns of trajectory data (**Figure 3c**). The general profiles of atomic fluctuations were found to be very similar to each other and were showed that the fluctuations of residues in the IB cavity are lower than the others which indicates that during the MD simulation, the binding site remains approximately rigid. In addition, the RMSF of the atomic positions of the Schiff bases (**Figure 3d**)

indicated that the atoms of the Schiff bases showed limited fluctuations (<0.25 nm). Hence, it can be concluded that the interactions of HSA and the Schiff bases were stable during the simulation time.

## Conclusions

In the present study, two Schiff base ligands, PE and MePE, were synthesized and characterized using elemental analysis, UV-Vis and <sup>1</sup>H NMR spectroscopies. The crystal structure of PE was also determined using single crystal X-ray analysis. Furthermore, Binding of the Schiff base ligands to HSA was investigated using fluorescence spectroscopy at three temperatures and full thermodynamics study was done on the fluorescence results. Also, UV-Vis spectroscopy method was used to approve the fluorescence results. Furthermore, computational molecular docking study was done to investigate the atomic details of these bindings and to obtain the theoretical binding constants. All of the obtained results by the used experimental methods were in good agreement with molecular docking results. The obtained results from both experimental and theoretical methods indicated that the MePE binds to HSA more tightly than PE. Also, the thermodynamics parameters for binding of two synthesized Schiff bases to HSA were determined at 298, 303 and 308 K. The negative values of  $\Delta G^\circ$  increased with raising the temperature for both Schiff bases and the values of  $\Delta G^\circ$  for MePE is more negative than PE at all temperatures. Moreover, based on the enthalpy and entropy changes of interaction, the binding is mainly entropy-driven, and the hydrophobic forces play a major role in the stability of ligand-HSA complexes. Molecular docking results revealed that both of the ligands bind in the IB domain of HSA. Also, it can be concluded that methyl group in MePE increases the electron density of pyridine ring and causes stabilizing the HSA-MePE complex with  $\pi$ -stacking interactions. As a result,  $\pi$ -stacking interactions play important role in stabilizing MePE in its binding site. Finally, analysis of MD trajectories suggested that these Schiff base ligands can interact with HSA without any modification of protein structure.

## Appendix A. Supplementary data

CCDC 1536516 contains the supplementary crystallographic data for **PE**. These data can be obtained free of charge via <http://www.ccdc.cam.ac.uk/conts/retrieving.html>, or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: (+44) 1223-336-033; or e-mail: [deposit@ccdc.cam.ac.uk](mailto:deposit@ccdc.cam.ac.uk).

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