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Maryam Sharifi
Jafar Ezzati Nazhad Dolatabadi
Farzaneh Fathi
Mohammad Rashidi
Behzad Jafari
Habib Tajalli
Mohammad-Reza Rashidi

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Maryam Sharifi,^a Jafar Ezzati Nazhad Dolatabadi,^b Farzaneh Fathi,^{b,c} Mohammad Rashidi,^b Behzad Jafari,^d Habib Tajalli,^a and Mohammad-Reza Rashidi^{b,*}

^aUniversity of Tabriz, Research Institute for Applied Physics and Astronomy, Tabriz, Iran

^bTabriz University of Medical Sciences, Research Center for Pharmaceutical Nanotechnology, Faculty of Pharmacy, Tabriz, Iran

^cTabriz University of Medical Sciences, Student Research Committee, Tabriz, Iran

^dTabriz University of Medical Sciences, Biotechnology Research Center, Tabriz, Iran

Abstract. The interaction of bovine serum albumin (BSA) with various drugs, such as antibiotics, due to the importance of BSA in drug delivery has attracted increasing research attention at present. Therefore, the aim of this study was investigation of BSA interaction with rifampicin using surface plasmon resonance (SPR) and molecular docking methods under the imitated physiological conditions (pH = 7.4). BSA immobilization on carboxymethyl dextran hydrogel chip has been carried out after activation with N-hydroxysuccinimide/N-ethyl-N-(3-diethylaminopropyl) carbodiimide. The dose-response sensorgrams of BSA upon increasing concentration of rifampicin were attained in SPR analysis. The high affinity of rifampicin to BSA was demonstrated by a low equilibrium constants (K_D) value (3.46×10^{-5} at 40°C). The process of kinetic values changing shows that affinity of BSA to rifampicin decreased with rising temperature. The positive value of both enthalpy change (ΔH) and entropy change (ΔS) showed that hydrophobic force plays major role in the BSA interaction with rifampicin. The positive value of ΔG was indicative of nonspontaneous and enthalpy-driven binding process. In addition, according to the molecular docking study, hydrogen binding has some contributions in the interaction of rifampicin with BSA. © 2017 Society of Photo-Optical Instrumentation Engineers (SPIE) [DOI: 10.1117/1.JBO.22.3.037002]

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1 Introduction

Serum albumin is the most abundant protein in the blood circulation and is well-known as transport protein. The most important function of albumins is transportation of various organic and inorganic compounds including drugs.¹⁻⁴ Serum albumin interaction with various drugs plays a major role in estimation of the small molecules distribution, elimination, and transport in biological systems. Being familiar with the molecular basis of drug-albumin binding is crucial in designing new therapeutic agents for improvement of drug activity. Therefore, albumin-drug binding study is important and may influence properties of typical drugs. Due to low cost, medical care, availability, and binding properties, bovine serum albumin (BSA) usually is selected for investigation of drug binding capability.⁵⁻⁷

Rifampicin (Fig. 1) is an example of antibiotics used for treatment of pulmonary tuberculosis.^{8,9} Despite the broad pharmacological application of rifampicin, its mechanism of action and effects on serum albumin is poorly understood and investigated using surface plasmon resonance (SPR). Therefore, study of BSA interaction with rifampicin could be a challenging issue and the development of a reliable analytical method, such as SPR that allows the measurement of rifampicin-albumin interaction kinetics, would be of great value.

SPR is a sensitive label-free technique that detects the changes in refractive index at surfaces of metals. It can be used for study of biomolecular interactions with various drugs and ligands with high sensitivity.¹⁰⁻¹² Label free, real-time and both qualitative and quantitative detection are examples of SPR advantages compared to other techniques. In addition, determination of kinetic constants and thermodynamic parameters is possible using this technique.¹²⁻¹⁴

To the best of our knowledge, interaction of rifampicin with BSA has not been reported using the SPR technique. Owing to the extensive application of rifampicin, investigation on BSA interaction needs to be addressed. Therefore, in the current study, we investigated the binding properties of rifampicin with BSA using SPR method and molecular docking analysis.

2 Materials and Methods

2.1 Materials

Rifampicin was purchased from Sigma-Aldrich (Steinheim, Germany). Phosphate-buffered saline (PBS) solution, acetate buffer, BSA, ethanolamine-HCl, N-hydroxysuccinimide (NHS), and N-ethyl-N-(3-diethylaminopropyl) carbodiimide (EDC) were purchased from Merck (Darmstadt, Germany). Carboxymethyl dextran (CMD) low-density-modified gold surfaces chip was prepared from Bionavis Company (Tampere-region, Finland).

*Address all correspondence to: Mohammad-Reza Rashidi, E-mail: rashidi@tbzmed.ac.ir

Fig. 1 Chemical structure of rifampicin.

2.2 Apparatus

All kinetic parameters were recorded with dual-flow channels SPR device (MP-SPR Navi 210A, BioNavis Ltd., Tampere-region, Finland).

2.3 Bovine Serum Albumin Immobilization

After putting a CMD sensor chip on SPR instrument, the whole device is rinsed with acetate buffer. As soon as reaching steady baseline in sensorgram, the chip surface cleaned with NaCl (2 M) and NaOH (0.1 M) by injecting them to both channels for 3 min. To activate the chip surface EDC: NHS 1:1 (NHS 0.05 M + EDC 0.2 M) solution injected to the sensor chip for 7 min. For immobilization of BSA, the BSA solution injected (0.3 mg/ml) to channel 1, for 10 min and acetate buffer injected (running buffer) to channel 2 (reference channel). Finally, ethanolamine-HCl (1 M, pH 8.5) was injected for blocking the remaining activated surface groups. All of the injection processes were performed with flow rate of 30 μ l/min.

2.4 Kinetic Parameters of Bovine Serum Albumin Interaction with Rifampicin

Rifampicin solution in PBS buffer at pH 7.4 was injected using a flow rate of 30 μ l/min for 3 min at various concentrations (1 to 128 μ M). Both flow cells were used for sample injection and due to BSA immobilization at one flow cells, the flow cell without any BSA was used as reference. Rapid dissociation of rifampicin from BSA surface omits the need for regeneration process.

To estimate the affinity and kinetic of the BSA interaction with rifampicin, Trace Drawer™ for SPR Navi™ was used. Before calculations in Trace Drawer, data are extracted with SPR Navi™ Data viewer software.

2.5 Thermodynamic Analysis

The effect of temperature on binding of rifampicin to BSA was studied to estimate the thermodynamic parameters of rifampicin-BSA complex formation. Therefore, SPR measurements were investigated at four different temperatures (298, 303, 308, and 313 K).

2.6 Molecular Docking Simulation

The docking study of BSA with rifampicin was performed by the open-source windows version of Auto Dock Vina 1.1.2 software. Auto Dock Vina not only improves the accuracy of molecular docking between the ligand and receptor but also increases the speed of dock running.¹⁵ For study of the molecular interaction of BSA with rifampicin by in silico simulation,

Fig. 2 Schematic illustration of BSA immobilization by amine coupling: (a) carboxymethyl dextran sensor chip, (b) activation of COOH in CMD via EDC/NHS before BSA immobilization, (c) immobilization of the BSA through covalent amide bond formation, and (d) deactivation of the remaining activated surface groups by blocking reagents ethanolamine, which is the most commonly used agent.

the x-ray structure of BSA (PDB ID code 4F5S, 2.47 Å resolutions) was selected from the Research Collaboratory for Structural Bioinformatics protein data bank. The rifampicin molecular structure was prepared by drawing the two-dimensional chemical structure by Chem Draw Ultra 10.0 software and then for molecular energy optimization, the HyperChem 8.0.8 software was selected. Molecular structure optimization of rifampicin was done in two steps: at first the MM+ molecular mechanics was performed and after completed this step the AM1 semiempirical procedures was executed. To minimize rifampicin structure, the Fletcher-Reeves algorithm was selected in both optimization processes.¹⁶ For the docking study, the extracrystallographic structure, such as water molecules, was removed from the BSA structure. The BSA structure and compound were changed by adding the polar hydrogens. Also, the Kollman charges as well as Gasteiger charges were added to the receptor and ligand, respectively. After attaining the pdbqt files of BSA and rifampicin, drug was inputted in determined six different binding sites of BSA. The grid box dimension size at grid points in $x \times y \times z$ directions were set to 27 Å with a grid spacing of 1 Å in Vina docking. The energy range and exhaustiveness value were set to 4 kcal/mol and 40, respectively.

3 Results and Discussion

3.1 Bovine Serum Albumin Immobilization

Figure 2 shows the schematic illustration of BSA immobilization on the Au chip surface.

The initial curve of the aforementioned process before and after BSA immobilization was shown in Fig. 3. The blue curve shows channel 1, in which BSA has been immobilized and showed angle shift around 0.98 deg. The resonance curves shift to higher incident angles and become wider due to new

Fig. 3 Initial SPR curve (a) before and (b) after BSA immobilization.

Fig. 4 Sensorgram of rifampicin interaction with immobilized BSA at various concentration 1 to 128 μM at different temperatures (a) 298, (b) 303, (c) 308, and (d) 313 K.

layer formation on Au sensor slide and increase in the thickness of the adsorbed layer on the surface. From the aforementioned results, it can be concluded that the BSA attachment was performed properly on the Au sensor slide through covalent amide bond formation.¹⁷

3.2 Kinetics Binding of Bovine Serum Albumin and Rifampicin

The interaction between the analyte (A) and the immobilized BSA (B) can be demonstrated through simple Langmuirian mode of binding for complex formation (AB). For reversible associations and dissociations in a chemical equilibrium, it can be defined by the following equation:¹⁷

where k_a is the association rate constant and k_d is the dissociation rate constant. To attain the kinetic features, calculation of

the k_a and k_d rate constants and the equilibrium constants (K_D) for the bindings of BSA to rifampicin is required.^{17,18} The K_D is a measure of ligand affinity to various biomacromolecules, such as BSA and defined as $K_D = k_d/k_a$. Figure 4 shows the SPR sensorgrams of the reaction between BSA and rifampicin after reference subtraction at concentrations ranging from 1 to 128 μM at four temperatures (298, 303, 308, and 313 K).

Table 1 shows values of kinetic parameters at four temperatures. According to the Table 1, k_a and k_d of BSA interaction with rifampicin decreased regularly upon rising temperature. It shows that high temperature led in the reaction rate decrease. The mean K_D for rifampicin binding increased almost 2.30 from 15 to 34.6 μM over this temperature range. The temperature dependence of the K_D was due to an increase in the k_d value rather than the k_a value. About 15-fold increase in the k_d value was observed when temperature increased from 298 to 313 K. However, there was only sixfold increase in the k_a value when temperature rose to 313 K. As it is clear in Table 1, K_D of BSA interaction with rifampicin increased upon rising temperature,

Table 1 Association rate constant (k_a), dissociation rate constant (k_d), and equilibrium constants (K_D) values of rifampicin interaction with BSA.

Temperatures (K)	k_a (1/M×s)	k_d (1/s)	K_D (M)
298	1.48×10^2	2.22×10^{-3}	1.50×10^{-5}
303	7.70×10^2	1.46×10^{-2}	1.90×10^{-5}
308	6.83×10^2	1.71×10^{-2}	2.50×10^{-5}
313	9.07×10^2	3.14×10^{-2}	3.46×10^{-5}

which showed that affinity between two materials decreases upon rising temperatures.

3.3 Thermodynamic Parameters Analysis of Binding Bovine Serum Albumin with Rifampicin

To investigate the effect of temperature and thermodynamic features, the reaction between BSA and rifampicin was carried out at different temperatures. The type of interaction between BSA protein and rifampicin may consist of the following forces: hydrogen bond, Van der Waals, electrostatic, and hydrophobic forces. The value of thermodynamic parameters, such as enthalpy change (ΔH) and entropy change (ΔS), is used for the estimation of interaction types. The positive value of ΔH and ΔS shows that the hydrophobic forces play major roles in the binding of two molecules; the negative values represent

Van der Waals interactions and hydrogen bonds occurring and finally, the negative value of ΔH and positive amount of ΔS is indicative of electrostatic interaction happening. These parameters can be calculated through the van't Hoff Eq. (1) by the plot of $\ln K_D$ versus $1/T$ ¹⁹

$$\ln K_D = -\frac{\Delta H}{RT} + \frac{\Delta S}{R}, \quad (1)$$

where R and T are the universal gas constant and temperature, respectively.

BSA binding study was performed at four different temperatures (298, 303, 308, and 313 K). Figure 5 shows the plot of $\ln K_D$ versus $1/T$.

The total energy released by the reaction is calculated with the Gibbs–Helmholtz

$$\Delta G = \Delta H - T\Delta S. \quad (2)$$

The positive values have been attained for both ΔH and ΔS according to Table 2 for the binding of BSA to rifampicin, which indicates that the binding processes are mainly enthalpy-driven and the rifampicin–BSA interaction can be explained in terms of hydrophobic forces. Although Yu et al⁷ showed that electrostatic interactions play a major role in the BSA binding to rifampicin using fluorescence spectroscopy; according to some other authors,⁶ the interaction between rifampicin and BSA occurs through hydrophobic binding processes. Taking into account the lipophilicity of rifampicin, the involvement of the latter forces in the binding process of this drug with BSA

Fig. 5 Van't Hoff plot for the interaction of BSA and rifampicin.**Table 2** Thermodynamic parameters of BSA binding with rifampicin at four different temperatures (298, 303, 308, and 313 K).

Temperatures (K)	K_D	ΔH (kJ mol ⁻¹)	ΔS (J mol ⁻¹)	ΔG (kJ mol ⁻¹ k ⁻¹)
298	1.50×10^{-5}			27.58735684
303	1.90×10^{-5}	43.10066	52.05806822	27.3270665
308	2.50×10^{-5}			27.06677616
313	3.46×10^{-5}			26.80648582

Table 3 The lowest binding free energy values (ΔG) calculated by Auto Dock Vina from rifampicin docking with six different binding sites of BSA.

Binding site	Site IA	Site IIA	Site IIIA	Site IB	Site IIB	Site IIIB
E_{lowest} (kcal/mol)	-4.3	-6.0	-4.2	-6.5	-7.0	-6.5

Fig. 6 The best docked conformation poses of rifampicin in six various binding sites of BSA.

seems to be more logical. In addition, the positive value of ΔG means that the binding process was nonspontaneous and enthalpy driven and the formation of rifampicin–BSA complex was an endothermic process.^{2,20,21}

3.4 Molecular Docking Study

Twenty mode runs were defined in our docking procedure. After molecular docking process, the best conformation of each rifampicin–BSA complexes relative to the lowest free binding energy (ΔG) according to the Vina cluster was selected and summarized in Table 3 and the lowest free binding energy conformer of docking for each binding site has been shown in Fig. 6. The lowest free binding energy (ΔG) for the sites A and B of albumin molecule was found and presented in Table 3. The subdomain IIB and IIA of albumin molecule has some amino acids with functional groups that can make hydrogen binding as well (Fig. 7). Our docking analysis revealed some H-bondings between rifampicin and BSA (Table 4). Rifampicin has also several functional groups, such as hydroxyl with hydrogen binding capability. Therefore, the possibility of some hydrogen bindings between rifampicin and BSA cannot be totally excluded, although based on the results obtained from thermodynamic studies, this is the hydrophobic forces that act as the

Fig. 7 H-bonding between rifampicin and different amino acids in two binding subdomains of BSA. Green spheres are H-bonding and wireframe spheres are close contact between rifampicin and residues.**Table 4** Amino acids that contacted with rifampicin from the analyzing of all runs of each docking procedure and the number and type of amino acids that join together in formation of H-bond in selected binding site of BSA (IIA and IIB). The number of phrases indicated the repeating of H-bonding with dependent amino acid.

Binding site	Amino acids that contacted with rifampicin	Amino acids company in H-bonding formation	Number of H-bonding
Site IIA	Arg10-His67-Ile202-Glu207-Arg208-Lys211-Lys232-Thr235-Lys239-Lys242-Glu243-His246-Asp248-Asp255-Asp258-Lys261-Tyr262	Glu207-Lys211-Lys232-Lys239-Lys242(6)-Asp248	11
Site IIB	Phe205-Arg208-Ala209-Lys322-Lys350-Glu351-Glu353-Ala354-Glu357-Glu358-Lys362-Thr371-Lys375-His378-Leu379-Glu382-Asn482	Lys131(2)-Lys159(5)-Asn161-Lys180-Lys187(5)	14

major driven forces in the interaction of rifampicin with the protein. Therefore, it seems that rifampicin interacts with BSA through hydrophobic interactions as the major driven forces.^{1,21}

4 Conclusion

In this paper, we studied the interaction between BSA and rifampicin using SPR method and molecular docking analysis. The experimental results revealed that BSA has high affinity to rifampicin, which confirmed with low value of K_D . However, K_D of BSA interaction with rifampicin increased upon rising temperature, which showed that affinity between two material decreases upon rising temperatures. The results of thermodynamic parameters showed that the main force between rifampicin and BSA is hydrophobic due to the hydrophobic nature of drug. Also positive value of ΔG confirmed that the binding between BSA and rifampicin is enthalpy-driven and non-spontaneous. In addition, molecular docking study showed the involvement of hydrogen binding in the formation of rifampicin-BSA complex.

Disclosures

The authors declare no competing interests.

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Biographies for the authors are not available.