CHARACTERIZATION OF SOME PHYSICO-CHEMICAL PROPERTIES AND INTERACTIONS OF HUMAN AND BOVINE SERUM ALBUMINS WITH MITOMYCIN C

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Received March 10, 2017

Abstract. Physico-chemical properties, molecular interactions and thermodynamic stability of human and bovine serum albumins in complexes with mitomycin C were investigated. The pH and temperature influence on serum albumins properties and the binding mechanism of mitomycin C to these proteins have been monitored by spectrometry and spectrofluorimetry methods.

Key words: denaturation, fluorescence static quenching, fluorescent lifetime, affinity constants, interaction thermodynamic parameters.

1. INTRODUCTION

Serum albumins, including bovine serum albumin (BSA) and human serum albumin (HSA), act as circulatory carrier for many ligands (peptides, fatty acids, hormons, drugs, etc.). These albumin ligands bind reversibly to the mentioned proteins in order to be transported to the specific cellular targets. BSA and HSA are reported to possess different binding sites for their various exogenous and endogenous ligands, with moderate binding constants (10^4 M^{-1}– 10^6 M^{-1}) [1].

A special interest in the medical field is paid to the interaction and transport mechanisms of cytostatics by the serum albumins. One of these substances is mitomycin C (MMC), also called mutamycin, whose chemical structure is presented in Fig. 1.

MMC is a natural antitumour antibiotic, produced by *Streptomyces lavendulae* [2], used in the treatment of carcinomas, due to its very good antitumour activity and low toxicity. MMC also possesses a strong bactericidal action against Gram-negative and Gram-positive bacteria [3]. MMC is a DNA crosslinker, a single crosslink per genome being sufficient to kill a bacterium [5].

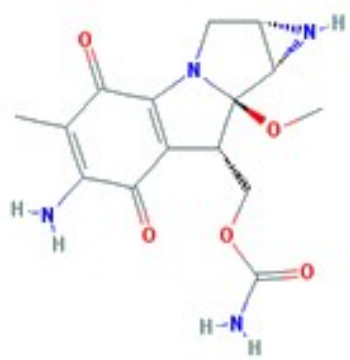

Fig. 1 – Chemical structure of the mitomycin C molecule [4].

In this paper, we report the results obtained from MMC binding both to BSA and HSA. The importance of such studies is connected to the action of this molecule on cancer cells in various tissues and organs (*e.g.*, breast, colon, head, and neck) [6]. There are evidences that MMC can be encapsulated in serum albumin microspheres in order to be delivered to the tumour sites and to maintain thus a prolonged antibiotic release [7]. Therefore, the investigation of the MMC action mechanism on serum albumins represents an important theme both in medical and pharmacological fields. However, *in vitro* studies showed that HSA induces changes in the biological activity of MMC. For instance, the MMC activity is reduced in the presence of HSA [8]. Also, the modification of biological activity of several anti-tumour drugs, including MMC, by binding to BSA was reported in the literature [9].

2. MATERIALS AND METHODS

Proteins. Bovine and human serum albumins (purity over 98 %) were purchased form SIGMA. Protein concentrations were measured using a *Perkin Elmer Lambda 2S* spectrophotometer in the ultraviolet range, on the basis of standard molar absorption coefficients at 280 nm (ε = 44,000 $M^{-1}cm^{-1}$ for BSA and ε = 36,000 $M^{-1}cm^{-1}$ for HSA).

Mitomycin C. Mitomycin C, MMC, with $C_{15}H_{18}N_4O_5$ molecular formula and MW = 334.33 Da was purchased from Fluka AG. MMC concentration was measured on the basis of standard molar absorption coefficient at 360 nm (ε = 23,000 M^{-1} cm^{-1}).

Buffer solution. Proteins were dissolved in 100 mM HEPES buffer, pH 7.4, using bi-distilled water. pH of the buffer and protein solutions was adjusted and measured using a *inoLab* 7110 pH-meter. The pH-dependence studies of the albumins-MMC interactions, were performed over a broad range of pH from 3 to 9.

UV-Vis Spectroscopy. The absorption spectra, both in the UV and visible (Vis) ranges, were recorded with a *Perkin Elmer Lambda 2S* spectrophotometer, in 10 mm x 10 mm quartz cuvette, with a rate of 500 nm/min.

Spectrofluorimetry. Fluorescence emission spectra were recorded by a *Perkin Elmer MS 55* spectrofluorometer, in the spectral range 300–500 nm, with 500 nm/min speed. Measurements were performed in 10 mm × 10 mm quartz cuvette. The excitation wavelength, for each albumin, was 290 nm. For the BSA-MMC interaction, the slit for the excitation monocromator was 5.0 nm and the slit for the emission monochromator was set at 4.5 nm. Titration quenching experiments were carried out by successive additions of the quencher in the protein solution (2 μM). The overall dilution did not exceeded 3.0 %. The solutions were mixed and kept 3 min. before the measurements. The binding mechanism was monitored at two temperature values: 25 °C and 35 °C.

Time resolved fluorescence. Fluorescence lifetimes of tryptophan (Trp) residues in BSA and HSA, and also of protein complexes with MMC, were measured into a home-made time resolved fluorimeter, built around a TimeHarp 200-Time Correlated Single Photon Counting acquisition board (PicoQuant, Germany). All opto-mechanical components were purchased from Thorlab (Thorlab, Germany). Fluorescence measurements were performed using the following optical filters: XHQA330 High Transmission Bandpass Filter (330 nm, FHWM 10 nm), XHQA340 High Transmission Bandpass Filter (340 nm, FHWM 10 nm), XHQA350 High Transmission Bandpass Filter (350 nm, FHWM 10 nm) and XUL0325 Longpass Filter (UV 325 nm). All filters where purchased from Asahi Spectra (Asahi Spectra, USA). As excitation source it was used PLS 8-2-363 (283 nm, FHWM 10 nm) LED, controlled by PDL 800-D at 10 MHz pulse repetition rate (PicoQuant, Germany). The protein samples were measured in a 10 mm × 10 mm quartz cuvette, in a thermostated sample holder equipped with a magnetic stirrer. The Peltier thermostat, type 5R7-001 Temperature Controller with RS-232 interface, was purchased from Oven Industries (Oven Industries, PA, USA). Fluorescence decays were recorded with TimeHarp 200 data acquisition software and analyzed with FluoFit software (PicoQuant, Germany). The fluorescence decays of Trp residues were obtained using a time-correlated single-photon-counting. Data obtained from fitting were processed using the OriginPro 2016 software package (OriginLab Corporation, Northampton, MA, USA).

3. RESULTS AND DISCUSSIONS

The influence of the environmental factors, such as pH, on a protein structure or a protein-ligand interaction can be detected by UV-Vis absorption spectroscopy,

by measuring the changes in the absorption of the protein molecules. The UV-Vis absorption spectrum of proteins exhibits a broad band (200–350 nm) in the UV region, with a maximum at 280 nm, due to the electronic transitions of Trp and tyrosine (Tyr) residues. Slight changes in the absorption maximum and molar absorptivity of proteins (alone or in molecular complexes) can occur with the variations of pH. Both protein structure changes and protein-ligand interactions can be investigated by steady-state fluorescence. In the case of proteins, the observed fluorescence signals, at the molecular level, can be interpreted by the assigment of the fluorescent properties (quantum yield, wavelength of fluorescence maximum, fluorescence lifetime) to Trp residues [10]. Both serum albumins possess the Trp amino acid in their sequences (Trp-134 and Trp-213 in BSA and respectively, Trp-214 in HSA). Because Trp residues are intrinsic chromophores of these two proteins, the fluorescence of the Trp, as a fluorescence marker, was monitored.

3.1. THE INFLUENCE OF ENVIRONMENTAL FACTORS ON BSA-MMC AND HSA-MMC STRUCTURES

Influence of pH on albumin-MMC complexes. Albumins have the ability to undergo reversible conformational transitions, with changes in pH [11]. This feature is very important for the mechanisms of the ligand/drug release and distribution. Both proteins undergo five structure modifications, at different pH values. Their expanded (E) form appears at pH < 3.5; the fast form (F) appears at pH ~ 4.0; the normal (N) form is predominant from pH 4.5 to pH 7.0; the B form appears between pH 8.0 and 9.0; the aged (A) form appears at pH > 9.0 [11, 12]. On the other hand, it is known that spontaneous activation of MMC at acidic pH has been reported in chemical systems [6]. The survival of MMC-treated cells is influenced by the pH of the environment and also by the intracellular pH [13]. Thus, the *in vitro* pH influence on the serum albumins-MMC complexes behaviour is necessary to be known.

The pH influence on the bovine and human serum albumins-MMC complexes, monitored by UV absorption spectroscopy, is presented in Fig. 2. One can see that unusual forms of pH dependence of BSA-MMC (■) and HSA-MMC (●) complexes were obtained. Increasing the pH from 3 to 12 led to a random variation of the absorption of albumins-MMC complexes (Fig. 2). It is interesting to note that a minimum absorption peak was found around pH = 7.4 for both MMC-albumin complexes. At pH = 7.4, serum albumins are found in the form (N) with normal physiological activities.

In the case of acidic pH, the absorption of the chromophores in proteins is greater than at basic pH values, for both albumin – MMC complexes. This can be due to the spontaneous activation of MMC at acidic pH.

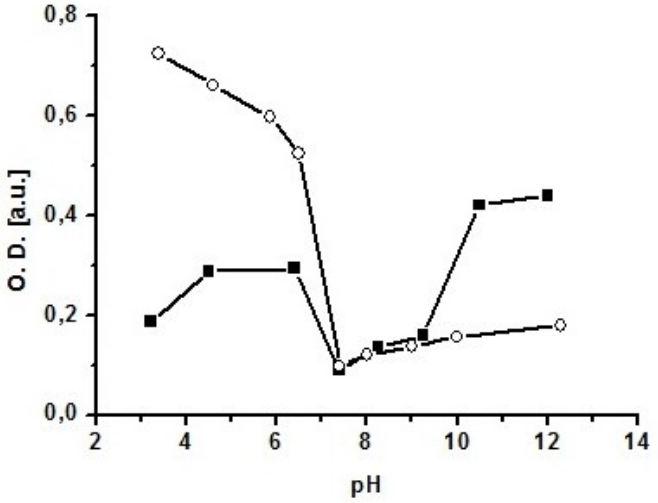

Fig. 2 – The effect of pH on BSA-MMC (■) and HSA-MMC (●) complexes, monitored at 280 nm. All samples were dissolved in 100 mM HEPES buffer.

Influence of temperature on the complexes BSA-MCC and HSA-MCC. In order to understand the mechanism affecting BSA and HSA structures when temperature is changed, their denaturation studies were performed, both in the absence and presence of MMC. Intrinsic fluorescence of BSA and HSA (measured at 345 nm for BSA and 336 nm for HSA) was used to monitor the denaturation of the two albumins, in the presence and absence of the ligand (Fig. 3).

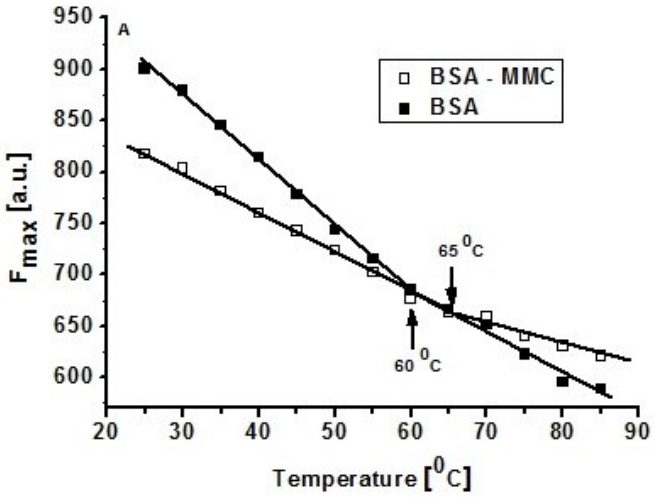

Fig. 3

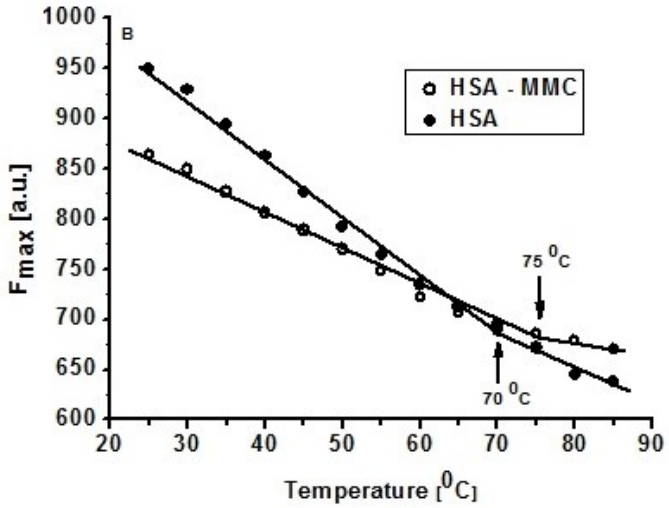

Fig. 3 (continued) – The denaturation process of BSA (■), HSA (●), BSA-MMC (□) and HSA-MMC (○) complexes, monitored by fluorescence emission. All samples contain 2 μM protein and were dissolved in 100 mM HEPES buffer, at pH = 7.4.

The denaturation process induces changes in the functional properties of albumins and of their complexes with MMC. The denaturation occurs slowly, in one step, for both albumins, with a transition temperature (marked by arrows for each denaturation process of the proteins or complexes) around 60 °C for BSA and 70 °C for HSA, and respectively, around 65 °C for BSA-MMC and 75 °C for HSA-MMC complexes.

3.2. INVESTIGATION OF THE INTERACTION MECHANISM OF BSA AND HSA WITH MMC

Any biological process, involving proteins, depends on the protein coupling with their specific ligands. At present, several methods (*e.g.*, analytical ultra-centrifugation, spectrofluorimetry, surface plasmon resonance, equilibrium dialysis, and titration isothermal calorimetry) enable the determination of the affinity constant of such molecular recognition processes. Most of the physical and chemical processes are accompanied by thermal effects that can be used as a starting point for a range of analytical methods and determination of thermodynamic parameters (changes in free energy, ΔG, enthalpy, ΔH, and entropy, ΔS).

The aim of this work was to characterize the fluorescence quenching mechanism and to calculate the binding parameters of the ligand MMC to BSA and

HSA. Intrinsic fluorescence intensities of BSA and HSA can provide information on conformational changes of proteins before and after ligand binding. The fluorescence emission spectra of BSA and HSA (excitation wavelength of 290 nm) in the absence and in the presence MMC (at different concentrations) show that these proteins exhibit strong fluorescence emission peaks (at 345 nm for BSA and 336 nm for HSA). MMC had no intrinsic fluorescence at excitation wavelength of 290 nm. The emission intensities of BSA and HSA decrease gradually with the increasing concentration of MMC. A blue shift was also observed with increase of MMC concentration, suggesting that MMC interacts with both BSA and HSA. Therefore, the albumin fluorescence quenching mechanisms by MMC was monitored.

A quencher is a small molecule that may penetrate the internal structure of a protein, diffusing and colliding with the aromatic groups of the protein amino acids, decaying the fluorescence [10]. The mechanism of a quencher binding to a macromolecule may be either static or dynamic (*i.e.*, by collision) [14]. The quenching is static when the distance between the excited fluorophore and a quencher is fixed. In the static quenching, the quencher-fluorophore complex is non-fluorescent. The dynamic (collisional) quenching depends on diffusion. In the dynamic quenching, the quencher diffuses to the fluorophore during the lifetime of the excited state, makes contact with it in this excited state and then, the fluorophore returns to the ground state, without photonic emission [15].

The BSA-MMC and HSA-MMC binding mechanisms were investigated using a constant concentration of protein (2 μM) and increasing concentrations of MMC, from 0 μM to 14 μM. The effect of adding different amounts of MMC to BSA and HSA was investigated at 25 °C and 35 °C, respectively.

The binding effect of MMC to proteins is revealed by the Stern-Volmer plot (Fig. 4) which allows the calculation of the Stern-Volmer constant, K_{SV}. The data obtained from the quenching of the Trp intrinsic fluorescence of BSA and HSA were used to estimate the quenching constant (K_{SV}) and the bimolecular constant (K_q) for the protein-ligand complex, using the Stern-Volmer equation:

$$F_0 / F = 1 + K_q \tau_0 [Q] = 1 + K_{SV} [Q], \quad (1)$$

where F_0 is the fluorescence intensity in the absence of quencher, F is the fluorescence intensity in the presence of the quencher, $[Q]$ is the concentration of the quencher, K_q is the bimolecular quenching rate constant and τ_0 is the average lifetime of the molecule in the absence of the quencher, observed at 350 nm. These values are listed in Table 1.

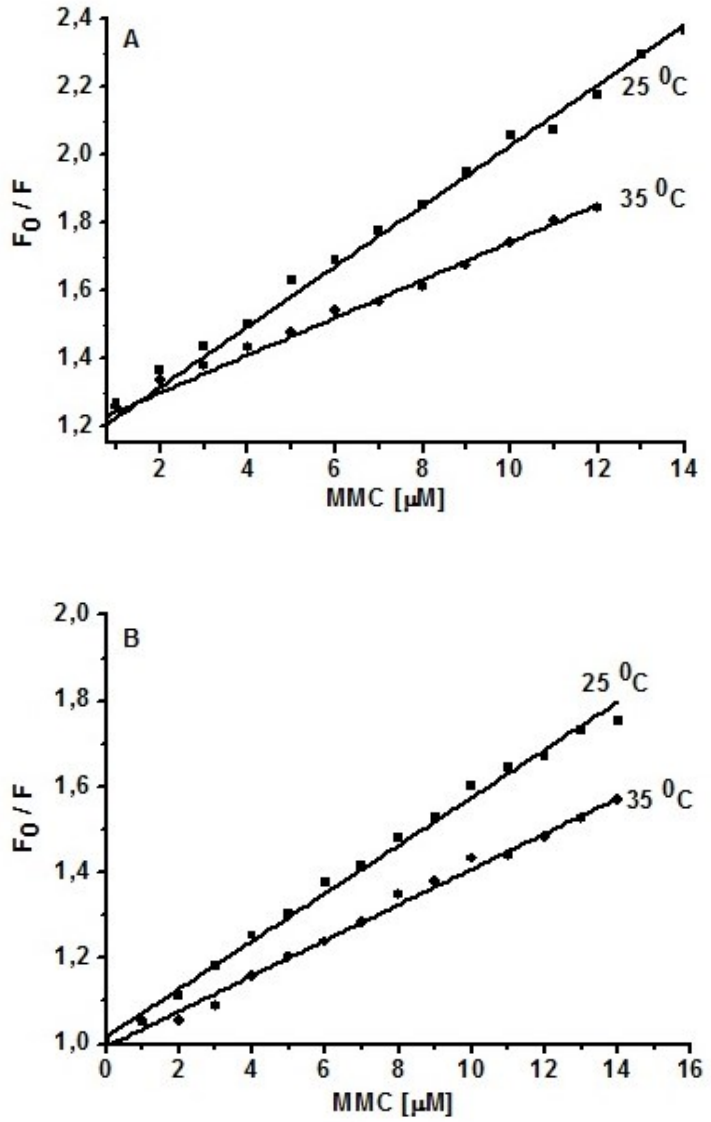

Fig. 4 – Stern-Volmer plots of BSA-MMC (A) and HSA-MMC (C) interactions in 100 mM HEPES buffer, at pH = 7.4 at two different temperatures: 25 °C and 35 °C.

The Stern-Volmer quenching constants for BSA-MMC and HSA-MMC, at 25 °C and 35 °C, are listed in Table 1. As one can see, the Stern-Volmer constant and the biomolecular constant values decrease with temperature increase for both albumins-MMC complexes. These results indicate that MMC is a static quencher.

Table 1

Stern-Volmer quenching constants, calculated at 25 °C and 35 °C

T [°C]	$K_{sv} \times 10^4$ [M^{-1}]	$K_q \times 10^{12}$ [M^{-1} s^{-1}]	r*
BSA			
25	8.91	1.96	0.99157
35	5.51	1.34	0.99570
HSA			
25	5.58	1.09	0.99682
35	4.14	0.90	0.99658

r* is the regression coefficient.

Fluorescence spectra were also used to study the interaction of BSA and HSA with MMC. Affinity constant, K_A, and the stoichiometry, n, were calculated from Scatchard plot of the binding of MMC to BSA and HSA (Fig. 5), using the double logarithmic representation, according to the equation:

$$\log(F_0 / F) = \log K_A + n \log[Q]. \quad (2)$$

As one can see from Table 2, MMC binds to the two albumins with 1:1 stoichiometry. The affinity constants (5.44×10^4 M^{-1} at 25 °C and 1.33×10^4 M^{-1} at 35 °C for BSA, 1.93×10^4 M^{-1} at 25 °C and 1.54×10^4 M^{-1} at 35 °C and for HSA) show a small to a moderate binding of MMC to the site of each albumin.

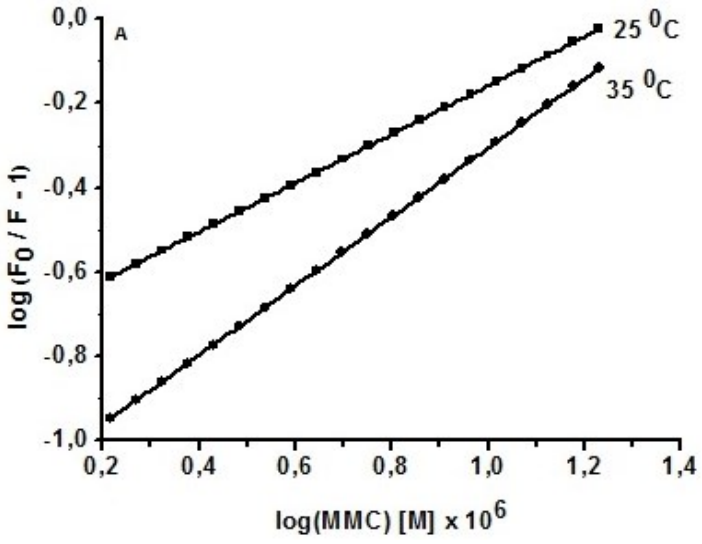

Fig. 5

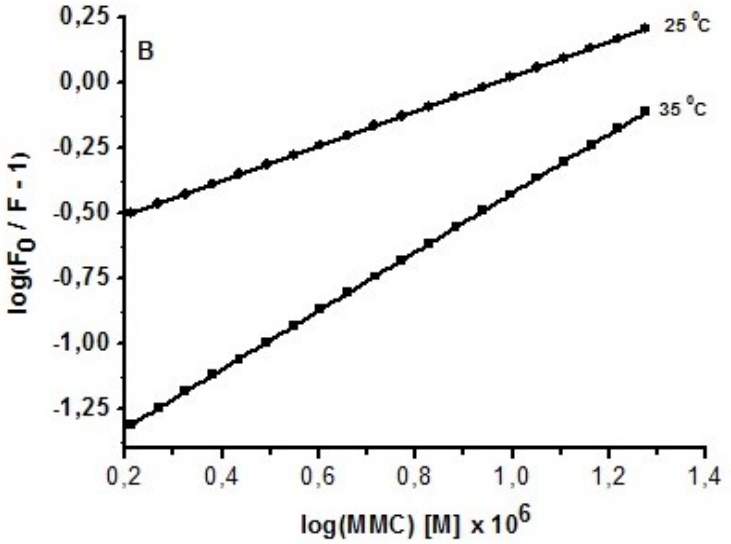

Fig. 5 (continued) – The linear fitting plots for Scatchard equation of binding of MMC to BSA (A, B) and HSA (C, D) showing the effect of increasing concentration of MMC (0–1.4) μM on the fluorescence emission of both proteins (2 μM). All samples were dissolved in 100 mM HEPES buffer, at pH = 7.4 and at two different temperatures: 25 °C and 35 °C.

Table 2

Affinity constant, stoichiometry, and thermodynamic parameters of the MMC binding to BSA and HSA, respectively at 25 °C and 35 °C

[°C]	$K_A \times 10^4$ [M^{-1}]	ΔG [kJ mol^{-1}]	ΔH [kJ mol^{-1}]	$T\Delta S$ [kJ mol^{-1}]	*n*
BSA - MMC					
25	5.44	–27.01	–1.04	25.97	0.98
35	1.33	–24.31		23.27	0.95
HSA - MMC					
25	1.93	–24.48	–17.22	7.26	1.07
35	1.54	–23.68		6.46	0.96

3.3. INVESTIGATION OF THE THERMODYNAMIC FINGERPRINT OF THE INTERACTIONS OF MMC WITH HSA AND BSA

The thermodynamic parameters (ΔG, ΔH, ΔS) of the ligand-albumins interactions, were also determined, using the following equations:

$$\ln \frac{K_{A2}}{K_{A1}} = \frac{\Delta H}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right) \tag{3}$$

$$\Delta G = -RT \ln K_A = \Delta H - T\Delta S \,. \quad (4)$$

The enthalpy of binding was –1.04 kJ mol^{-1} for the BSA-MMC complex, and –17.22 kJ mol^{-1} for the HSA-MMC complex (Table 2), according to the data in the literature [4, 9]. The variation of the Gibbs free energy, ΔG, was found to be –27.01 kJ mol^{-1} at 25 °C and –24.31 kJ mol^{-1} at 35 °C for the BSA-MMC complex, and –24.48 kJ mol^{-1} at 25 °C and –23.68 kJ mol^{-1} at 35 °C for the HSA-MMC complex. The enthalpic term, $T\Delta S$, was 25.97 kJ mol^{-1} at 25 °C and 23.27 kJ mol^{-1} at 35 °C for the BSA-MMC complex, and 7.26 kJ mol^{-1} at 25 °C and 6.46 kJ mol^{-1} at 35 °C for the HSA-MMC complex. The thermodynamic parameters, listed in Table 2, show that formation of the complex, MMC-HSA, is more exothermic than that of MMC-BSA formation with a strong contribution of the hydrophobic interactions and hydrogen bonding [16].

3.4. FLUORESCENCE LIFETIME OF BSA AND HSA Trp RESIDUES

Structure and dynamics of proteins may be studied by observation of fluorescence decay kinetics, followed with ps and ns resolutions. In this study, BSA and HSA-mitomycin C interaction was monitored by means of Trp fluorescence decay kinetics. The fluorescence response of the most common intrinsic fluorescent amino acid in proteins, Trp, provides information on the conformational changes of proteins. These changes are usually reflected by maximum shifts in fluorescence spectra, changes in quantum yield, and alterations in fluorescence decay kinetics [13, 17]. A very common method to put in evidence the fluorescence emission properties of each Trp residue in proteins is the time-resolved fluorescence. Some studies put in evidence the existence of two or three lifetimes for Trp in serum albumins [18, 19, 20], in different experimental conditions. In this study, the fluorescence decays of Trp was monitored, for both BSA and HSA without any other ligand, and for their complexes with MMC. Fluorescence lifetime parameters were observed at 330 nm, 340 nm, and 350 nm and also above 325 nm, in order to obtain the entire Trp fluorescence emission [10]. Examples of the fluorescence lifetime decay measurements for the Trp residues in BSA and HSA, at 350 nm, are presented in Fig. 6. The blue curves are the fitted fluorescence decay of Trp while the red one is the instrument response function (IRF), recorded with the stray light signal of a dilute colloidal silica suspension – Ludox®. In the bottom of these graphics there are the weighted residuals resulting from the fitting. One can see that the fluorescence intensity decay with χ^2 was found to be around 1. As the value of this parameter is closer to 1, the lifetime fitting is considered a good one [10, 13, 21].

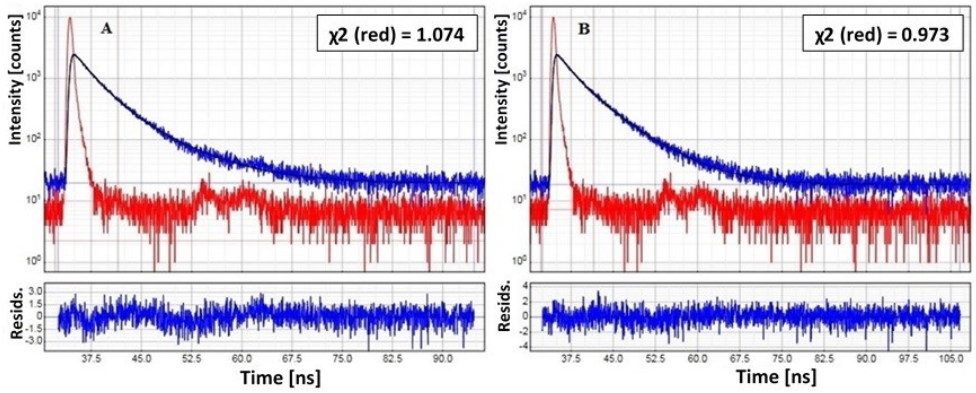

Fig. 6 – Fluorescence intensity decays of Trp residues of BSA (A) and HSA (B), measured at λ_{ex} = 283 nm and λ_{em} = 350 nm.

The fluorescence lifetimes of Trp from the two albumins are shown in Table 3, for two temperatures. Also, in this table, there are presented the lifetimes of the albumin-MMC complexes in 1:1 molar ratio.

Table 3

Fluorescence lifetime of Trp residues in albumins, measured at 25 °C and 35 °C

Compound	T [°C]	330 nm			340 nm			350 nm			325 nm		
		τ_1	τ_2	τ_0	τ_1	τ_2	τ_0	τ_1	τ_2	τ_0	τ_1	τ_2	τ_0
BSA	25	6.35	2.18	3.95	6.94	2.41	4.31	7.34	2.63	4.54	7.29	2.48	4.68
BSA-MMC		6.48	2.23	3.92	6.81	2.43	4.24	7.54	2.67	4.54	7.38	2.45	4.68
HSA		5.67	1.74	4.37	6.20	2.16	4.82	6.66	2.59	5.10	6.51	2.27	5.06
HSA-MMC		5.54	1.64	4.31	5.98	2.03	4.73	6.66	2.55	5.07	6.36	2.10	5.02
BSA	35	5.23	1.71	3.52	6.37	2.16	3.94	6.01	2.19	4.10	7.26	2.30	4.47
BSA-MMC		5.40	1.79	3.56	5.81	1.97	3.88	6.64	2.34	4.17	7.10	2.31	4.48
HSA		5.37	1.72	4.04	5.89	2.12	4.39	5.88	2.22	4.59	6.32	2.21	4.69
HSA-MMC		5.32	1.69	3.99	5.63	1.97	4.32	6.14	2.41	4.60	6.12	2.08	4.67

The fluorescence lifetime measurements for the two albumins and for their complexes with MMC, increasing the concentration of MMC in the range 0–14 μM, were also made, in order to confirm the mechanism of quenching in the steady-state fluorescence experiments. The results are presented in Figs. 7 and 8. One can see that, in all cases, at 330 nm the lifetime values are smaller than those obtained at 340 nm and 350 nm. One explanation for this could be that the fluorescence observed at 330 nm is due to a more hydrophobic environment than that at 340 nm and 350 nm. These data are confirmed by the results obtained in the literature for BSA [18] and HSA [20]. When fluorescence emission is recorded with the long-pass filter (cut-off wavelength 325 nm) a slight increase in average lifetime for BSA-MMC complex is observed. This increase is explained by the fact

that BSA contains two Trp residues and this type of filter helps to record the fluorescence emission from all Trp residues.

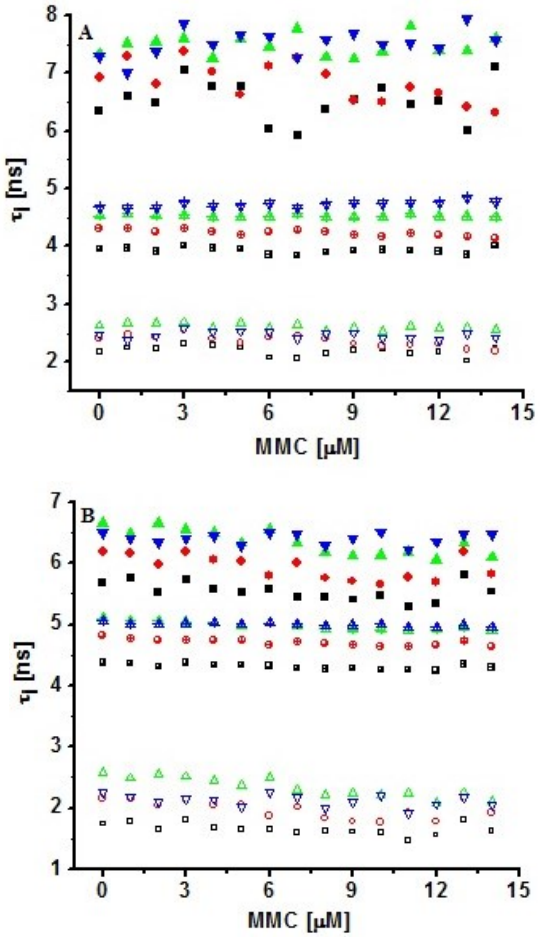

Fig. 7 – Fluorescence lifetime for BSA-MMC (A) and HSA-MMC (B) at 25° C; closed symbols – τ_1, open symbols – τ_2 and open symbols with cross – τ_0; black symbols – λ_{em}= 330 nm, red symbols – λ_{em}= 340 nm, green symbols – λ_{em}= 350 nm and blue symbols – λ_{em}= 325 nm.

The differences between long, short and average lifetimes (τ_1, τ_2, and τ_0), recorded at different wavelengths, are due to different orientation of the Trp residues inside the spatial conformation of the molecule [10]. Modifying the 3D conformation of the two albumins, by increasing temperature, as shown in Fig. 8, leads to a decrease of all three lifetimes above mentioned.

The free (uncomplexed) fluorophores, eliciting normal excited state properties, can emit fluorescence light after excitation pulse. In the case of pure

static quenching, the fluorescence lifetime of the sample is not affected, because the Trp residues contained in different conformational states of BSA and HSA form a nonfluorescent complex with MMC. Therefore, the fluorescence of the sample is reduced since the quencher essentially decreases the number of free fluorescent molecules, by forming non fluorescent stable ground state complexes. This leads to an increased acquisition time of fluorescence decays (data not shown). When a pure static quenching occurs, the value of the ratio τ_0/τ is approximately 1 (where τ_0 is the average lifetime of the sample in absence of quencher and τ is the average lifetime corresponding to every concentration of the quencher) [10, 14].

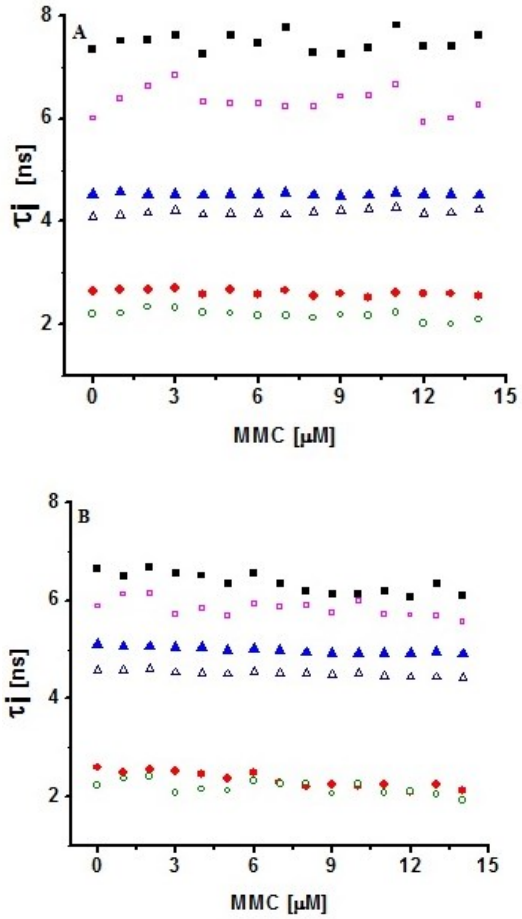

Fig. 8 – Fluorescence lifetime for BSA-MMC (A) and HSA-MMC (B) at λ_{em} = 350 nm; upper symbols (black and magenta) – τ_1, lower symbols (red and olive) – τ_2 and middle symbols (blue and navy blue) – τ_0; closed symbols – 25 °C and open symbols – 35 °C.

From the the average lifetime values of Trp residues in the case of the two albumins (middle section of Fig. 7 – open symbols with cross and Fig. 8 – blue and navy blue symbols) we conclude that the fluorescence quenching induced by MMC is a purely static process.

4. CONCLUSIONS

The binding propreties of mitomycin C to bovine and human serum albumins, in various conditions, were investigated by steady-state and time resolved fluorescence.

The fluorescence of both serum proteins are quenched by MMC, by a static mechanism.

MMC spontaneously binds to a single site both of BSA and HSA with very similar and moderate affinity constants.

The thermodynamic parameters of the MMC-albumin interaction suggest that the main role in the MMC binding to serum proteins is played by the hydrophobic interactions and hydrogen bonding.

This study is a step to a better understanding of the binding mechanism of cytostatics to serum albumins known to be important drug carriers.

REFERENCES

1. M. Dockal, M. Chang, D. C. Carter, F. Rüker, Protein Science, **9**, pp. 1455–1465, 2000.
2. T. Hata, Y. Sano, R. Sugawara, A. Matsumae, K. Kanamorei, T. Shima, T. Hoshi, J. Antibiot., **9**, 141–146, 1956.
3. J. Jang, H. Liu, W. Chen, G. Zou, J. Molec. Struct., **928**, 1, pp. 72–77, 2009.
4. www.sigmaaldrich.com/catalog/product/sigma/m4287?lang=en&region=RO
5. M. Tomasz, Chemistry & Biology, **2**, 575–579, 1995.
6. K. A. Kenedy, J. D. McGurl, L. Leondaridis, O. Alabaster, Cancer Research, **45**, pp. 3541–3547, 1985.
7. N. Hideshi, S. Kenji, J. Kazuhiko, M. Yasunori, S. Toshikatsu, F. Sigeru, Intl. J. Pharmaceutics, **58**, 1, pp. 79–87, 1990.
8. I. Takahashi, T. Ohnuma, S. Kavy, S. Bhardwaj, J. F. Holland, Br. J. Cancer, **41**, pp. 602–608, 1980.
9. S. Bi, Y. Sun, C. Qiao, H. Zhang, C. Liu, J. Luminescence, **129**, 5, pp. 541–547, 2009.
10. J. R. Lakowicz, *Principles of Fluorescence Spectroscopy*, Plenum, New York, 1999.
11. C. Chilom, G. Barangă, D. Găzdaru, A. Popescu, J. Optoelectron. Adv. Materials, **15**, 3–4, pp. 311–316, 2013.
12. D.C. Carter, J. X. Ho, Advanced in Protein Chemistry, 45, 153–203, 1994.
13. P. B. T. Hill, A. S. Bellamy, *Antitumor Drug Radiation Interactions*, CRC Press, 1989.
14. B. Valeur, *Molecular Fluorescence*, Wiley-VCH Verlag GmbH, 2001.
15. S. Deepa, A. K. Mishra, I. Pharm. Biomed. Anal., **38**, pp. 556–563, 2005.
16. C. Chilom, A. Popescu, C. T. Crăescu, Rom. J. Phys., **51**, 3–4, pp. 443–457, 2006.
17. O. J. RolinskI, A.Martin, D. J. S. Birch, Ann. N.Y. Acad. Sci., **1130**, pp. 314–319, 2008.

18. N. Tayeh, T. Rungassamy, J. R. Albani, J. Pharmaceutical and Biomed. Analysis, **50**, pp. 107–116, 2009.
19. M. Amiri, K. Jankeje, J. R. Albani, J. Pharmaceutical and Biomed. Analysis, **51**, pp. 1097–1102, 2010.
20. S. Gorinstein, I. Goshev, S. Moncheva, M. Zemser, M. Weisz, A. Caspi, J. Prot. Chem., **19**, 8, pp. 637–642, 2000.
21. J. R. Lakowicz, *Topics in Fluorescence Spectroscopy*, vol. III: *Biochemical Application*, Plenum Press, New York, 1992.