Acta of Bioengineering and Biomechanics
Vol. 20, No. 1, 2018

Original paper
DOI: 10.5277/ABB-00961-2017-03

Changes of albumin secondary structure after palmitic acid binding. FT-IR spectroscopic study

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Purpose: Albumin is an universal transport protein. Plasma pool of free fatty acids arising from triglyceride hydrolysis, critical in energy metabolism and etiology of metabolic disorders is transported by albumin. According to various studies albumin has from seven to nine binding sites with diverse affinity to long chain fatty acids. X-ray diffraction crystallography measurements have provided data only for pure human serum albumin or albumin with fully saturated binding sites. These results have shown that amount of α-helices is higher after fatty acids binding. Molecular mechanics simulations suggest that binding of fatty acids in two high-affinity sites leads to major conformational changes in albumin structure. The aim of this research was to investigate albumin secondary structure upon gradually increasing fatty acids to protein mole ratio. *Methods:* Fourier transform infrared spectroscopy was applied to study changes of bovine serum albumin (as an analogue of human serum albumin) α-helical structures after binding palmitic acid in a range of 0–20 palmitic acid: albumin molar ratios representing pure protein, partial, full saturation and excess binding sites capacity. *Results:* Amount of α-helices was increasing along with the amount of palmitic acid: bovine serum albumin molar ratio and reached maximum value around 2 mol/mol. *Conclusions:* Our studies confirmed molecular mechanics simulations and crystallographic studies. Palmitic acid binding in two high-affinity sites leads to major structural changes, filling another sites only slightly influenced bovine serum albumin secondary structure. The systematic study of fatty acids and albumin interactions, using an experimental model mimicking metabolic disorders, may results in new tools for personalized nanopharmacotherapy.

Key words: *palmitic acid, albumin, Fourier transform infrared spectroscopy, protein secondary structure*

1. Introduction

Albumin is a major plasma protein component. The dynamic properties of albumin, revealed with the use of some molecular approaches [15], enable fulfillment of biological functions, such as balancing plasma pH, delivery of nutrients to cells, maintaining colloidal osmotic pressure and solubilizing long chain fatty acids [13]. Plasma main carriers of esterified fatty acids (FA) are triglyceride-rich lipoproteins (TRL): chylomicrons, very low density lipoproteins and their remnants [10]. Plasma pool of free fatty acids (FFAs) arising from triglyceride hydrolysis, critical in energy metabolism and etiology of metabolic diseases is transported by albumin [11]. Factors potentially influencing the fabrication of human and/or bovine albumin-bound drug nanoparticles (ABDns) are widely discussed [3], [12]. FFAs are known to modify the unique ligand-delivery property of serum albumin. The presence of FA in experimental model that mimics metabolic disorders influences drug-albumin interaction. Along with glycation, palmitic acid (PAM) changes overall affinity and binding of drug [1].

According to previous studies based on nuclear magnetic resonance spectroscopy (NMR) and molecular

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Received: July 20th, 2017
Accepted for publication: December 30th, 2017

mechanics simulations, albumin molecule has from 7 to 9 binding sites of long fatty acids (i.e., FA1-FA9) with diverse affinity and binding energy [2], [13]. FA5 and FA4 show highest affinity to FA, while FA2 is a medium-affinity site. Under physiological conditions, FA to albumin molar ratio is 0.2–2 mol/mol and can increase to 6, for example, after extensive exercise [7], [14].

Structure of proteins can be determined by detection of X-ray diffraction on protein crystals. As identified by the X-ray crystallography, in five of HSA binding sites the carboxyl groups of FA are hold by hydrogen bonds and electrostatic interactions with polar amino acid residues present in these sites and FA acyl chains are placed within a hydrophobic cavities. These interactions induce conformational changes in protein molecule [9].

Molecular mechanics simulations indicate that conformational changes of albumin after FA binding are much more significant for high-affinity site FA5 than for other sites [14], [15]. That observation based on computational data should be evaluated experimentally.

Protein Data Bank (PDB) contains structural data of pure human serum albumin (HSA) and albumin with all binding sites saturated by fatty acids [2]. However, there is lack of information on albumin secondary structure for intermediate state between 0 and 9 FA to albumin molar ratio.

The aim of this study was to investigate changes of albumin secondary structures related with PAM binding in a range of 0–20 PAM to albumin molar ratios, with bovine serum albumin (BSA) used as a model of HSA, due to functional similarity as a non-specific carrier protein [7]. NMR studies have shown that BSA has the amount of FA binding sites comparable to that of HSA [7]. We compared structural changes upon PAM binding in two commercially available types of BSA: regular BSA and essentially FA-free bovine serum albumin (BSA FA-free).

During our research we applied Fourier transform infrared spectroscopy (FT-IR), that enables measurement of absorbance in specific to proteins Amide I band. Shape of this bands is related with protein secondary structure. Contribution of α-helices, β-sheets, and turns in BSA structure can be determined [26].

2. Materials and methods

BSA, BSA FA-free and PAM were purchased from Sigma-Aldrich (Darmstadt, Germany). Ethanol was obtained from POCH (Gliwice, Poland). Experimental procedure of protein solvent preparation, titration with PAM, spectra registration and data analysis was identical for BSA and BSA FA-free.

BSA was dissolved in phosphate buffer saline of pH 7.4 to obtain concentration of 20 mg/mL and stirred for 2 hours in 30 °C. PAM was dissolved in ethanol to obtain solution of 15 mg/mL. PAM solution and ethanol were added to BSA solution to obtain bulk with 0, 0.1, 0.2, 0.4, 2, 10 and 20 PAM to BSA molar ratio and constant 10% ethanol concentration. Previous experiments proved that ethanol concentration below 25% does not affect albumin structure [6]. Bulk was stirred for 2 hours in 30 °C to enable PAM binding to BSA. After incubation, FT-IR spectra were registered.

Spectroscopic research was performed on FTIR Nicolet 6700 spectrophotometer (Thermo Scientific, USA) equipped with attenuated total reflection (ATR) accessory with diamond crystal (Pike Technologies, USA). Spectrophotometer was purged with nitrogen to carbon dioxide and water vapour removal. Sample volume of 30 µl was placed on ATR crystal and left to dry. In order to remove humidity from the sample, a silicon tube with o-ring gasket connected to vacuum pump was applied to ATR accessory. During each spectrum registration 256 scans were performed over the region of 4000–400 cm^{-1}, with a 4 cm^{-1} resolution and atmospheric correction, at 30 °C temperature. Liu et al. described comparable conditions for spectra registration [20].

Grams/AI software (Thermo Scientific, USA) was used for calculations. Spectra were smoothed with Savitzky–Golay polynomial filter (polynomial order 2, window width 21 points). Components related to secondary structures were calculated by curve fitting. Contribution of α-helices, short segment chains connecting α-helices and turns [21], [22], [26] can be determined by fitting of 3 Gaussian curves in Amide I band. Position of curves was determined by identification of minima in second-derivative spectra.

Partial least square (PLS) regression approach was used for the quantification of PAM added to BSA samples, based on their FT-IR spectra. To improve the accuracy of the method, second differentiated spectra were used for PLS models. PLS calibrations were carried out using Matlab R2009b software (MathWorks, USA).

For each molar ratio of PAM to BSA experiment was repeated from 3 to 5 times, including bulk and solution preparation, spectra registration and calculation of secondary structures contribution. Mean values and standard deviations (SD) were calculated for each molar ratio.

Structural information about ligand-free HSA and HSA saturated with PAM were obtained from protein data bank (PDB code: 4k2c and 1e7h, respectively).

3. Results

FT-IR spectrum of BSA FA-free dry film is shown in Fig. 1. Bands present between 2800–3000 cm^{-1} frequencies are related with CH_2 (methylene) and CH_3 (methyl) groups, present in amino acids (Table 1) [17]. Bands related with stretching vibrations (marked as ν), asymmetric (ν_{as}) and symmetric (ν_s) can be distinguished in this spectral region: $\nu_{as}(CH_3)$, $\nu_{as}(CH_2)$ and $\nu_s(CH_3)$. Strong CH_2 band is very characteristic for fatty acids as they arises from FA long tail and $\nu_{as}(CH_2)/\nu_{as}(CH_3)$ absorbance ratio can be used for FA quantification [4].

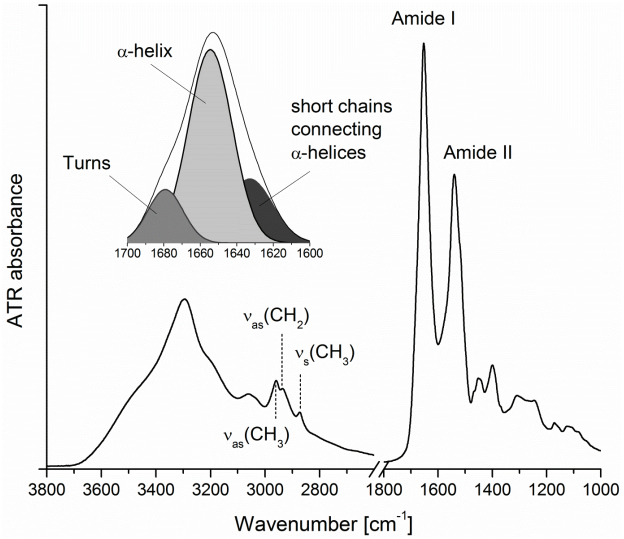

Fig. 1. FT-IR spectrum of BSA FA-free dry film; secondary structure determination – curve fitting under Amide I band (insert)

Table 1. Assignment of BSA absorbance bands; types of vibrations: ν – stretching, δ – bending, γ – wagging, *as* – asymmetric, *s* – symmetric [17]

Frequency [cm^{-1}]	Assignment
3294	ν(N-H): hydrogen-bonded N-H groups
3058	ν(N-H): Fermi resonance of hydrogen-bonded N-H groups
2960	$\nu_{as}(CH_3)$: methyl groups, side chains of amino acids
2934	$\nu_{as}(CH_2)$: methylene groups, fatty acids long chains, amino acids
2872	$\nu_s(CH_3)$: methyl groups, side chains of amino acids
1652	ν(C=O), Amide I: peptide bonds
1537	δ(N-H), Amide II: peptide bonds
1445	$\delta(CH_3)$ and $\delta(CH_2)$: amino acids
1401	$\nu_s(COO^-)$: hydrogen-bonded COO^- side groups
1248	ν(C-N) and δ(N-H), Amide III: protein bonds
1168	$\gamma(CH_2)$: amino acids
1117	ν(C-O): carboxyl COOH groups

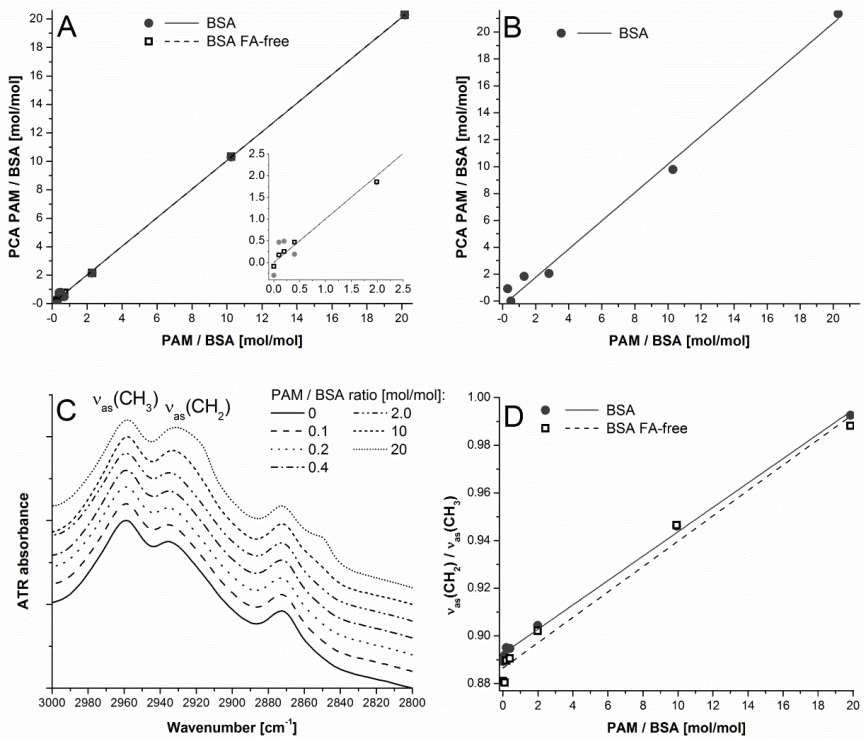

Fig. 2. PLS calibration for increasing PAM presence in BSA and BSA FA-free samples (A); predicted values of PAM/BSA molar ratios estimated by PLS model, $R^2 = 0.99$ (B); spectral changes in 2800–3000 cm^{-1} region related with increasing presence of PAM in albumin samples (C); increase of $\nu_{as}(CH_2)/\nu_{as}(CH_3)$ absorbance ratio due to PAM added to BSA ($R^2 = 0.99$) and BSA FA-free ($R^2 = 0.99$)(D)

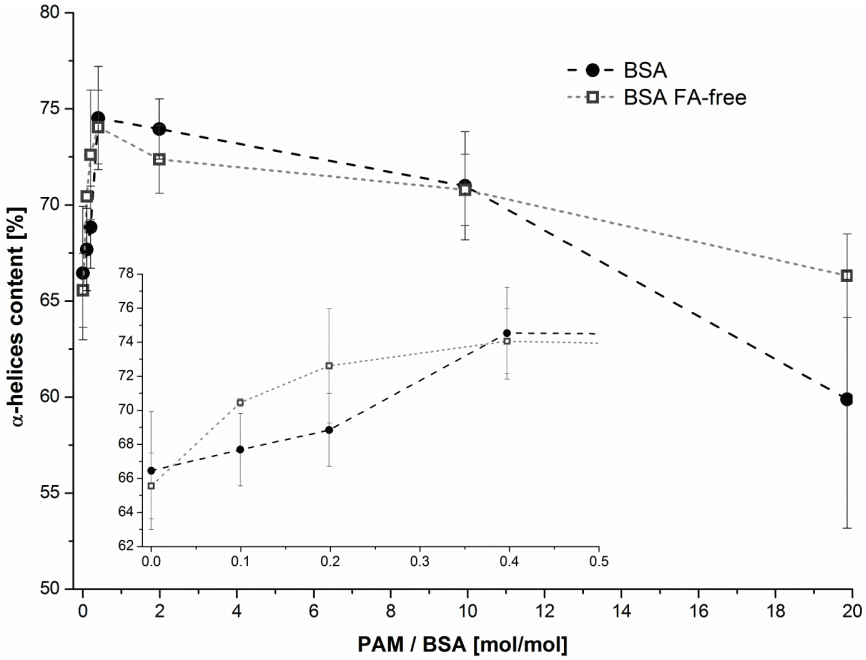

Fig. 3. Changes of α-helices content in BSA and BSA FA-free upon PAM binding, mean value ± SD, N = 3, 4 or 5

To document an increasing amount of PAM in albumin samples, we used 2800–3000 cm^{-1} area related with these bands. A PLS model was created based on the FT-IR spectra of the PAM/BSA and BSA FA-free mixtures, along with second derivation processing and three PLS factors (Fig. 2A). The relationship between predicted values of this PLS model and the PAM/BSA ratios in another set of samples is shown in Fig. 2B, with a correlation coefficient R^2 = 0.99. Figure 2C shows spectral changes in 2800–3000 cm^{-1} region related with increasing presence of PAM, what leads to an increase of $\nu_{as}(CH_2)$ absorbance around 2930 cm^{-1}, arising from fatty acids long chains. Relation between absorbance $\nu_{as}(CH_2)/\nu_{as}(CH_3)$ ratio and amount of PAM added to BSA and BSA FA-free is shown in Fig. 2D.

Amide I is a band present between 1600–1700 cm^{-1} frequencies and is related with stretching vibrations of C=O and C–N groups in protein bond. According to literature contribution of short segment chains connecting α-helices [21], [22], α-helices and turns [26] can be determined by fitting of 3 Gaussian curves in Amide I band with maxima at 1621, 1654 and 1679 cm^{-1}, respectively. Amount of each type of secondary structure is represented by area under one of Gaussian curves.

Our results have shown the following presence of α-helices: 66.5 ± 3.0% in BSA, 65.6 ± 1.9% in BSA FA-free. For albumin fully saturated with PAM (albumin to PAM molar ratio equal 10 mol/mol) the amount of α-helices increased: 71.0±2.8% in BSA, 70.8 ± 1.8% in BSA FA-free (Fig. 3).

4. Discussion

Results show linear growth of absorbance ratio as a result of PAM presence for both BSA and BSA FA-free (similar slope and interception). Both PLS analysis and calculation of $\nu_{as}(CH_2)/\nu_{as}(CH_3)$ ratio is an evidence of increasing presence of PAM in albumin samples (Fig. 2).

NMR and *X*-ray diffraction crystallographic studies show that structure of albumin is purely helical, without any β-sheet element [13]. Nevertheless, in many FT-IR studies of albumin secondary structure, one of fitted bands is assigned to β-sheets in Amide I curve fitting [16], [23], what is inconsistent with recent knowledge. It has been proposed that this fitted peak arises from short segment chains connecting α-helices [21], [22].

There is no data on PDB referring to structure of BSA-PAM complex. Based on structural and functional similarity between HSA and BSA [7], we compared our results obtained for changes in BSA secondary structure due to PAM binding with data available in PDB referring to HSA and HSA-PAM complex. According to PDB, an increase of α-helices amount is observed in HSA after PAM binding (Table 2). Our

Table 2. Content of α-helices upon FA ligand binding by albumins; values obtained from PDB (X-ray crystallography) and this study; *data referring to PAM:BSA molar ratio equal 10.0 (fully saturated binding sites)

Ligand	FA length	α-helix content [%]			
		PDB data		Our study	
		HSA	PDB code	BSA	BSA FA-free
Ligand-free	**N/A**	**69**	**4k2c**	**66.5 ± 3.0**	**65.6 ± 1.9**
Decanoic acid	10:0	71	1e7e	N/A	
Lauric acid	12:0	71	1e7f		
Myristic acid	14:0	71	1e7g		
Palmitic acid	**16:0**	**72**	**1e7h**	**71.0 ± 2.8** *	**70.8 ± 1.8** *
Stearic acid	17:0	72	1e7i	N/A	

results are in accordance with PDB data for HSA: 69% of α-helices in ligand-free HSA and 72% in HSA-PAM complex. Such structural changes upon PAM binding occur for other FA molecules as well.

For PAM to BSA molar ratio from 0 to 0.4 mol/mol rapid increase of α-helices was observed. The maximum content of α-helices is achieved for 0.4 mol/mol (74.5 and 74.0% for BSA and BSA FA-free, respectively). In this range, the changes were more significant for BSA FA-free than BSA. Between 2 and 10 mol/mol ratio secondary structures showed stability for both types of albumin. These results indicate that binding of FA molecules in high-affinity binding sites leads to structural changes while filling another sites with lower affinity does not affect protein structure. Our findings are consistent with molecular mechanics simulations indicating that conformational changes after FA binding in high affinity sites are much more remarkable than binding in low affinity sites [14], [15]. FA5 consist of three α-helices and one turn. Conformational changes in this area may be related with increased amount of helical structures presented in PBD.

A decrease in amount of α-helices was observed for higher PAM concentration, faster for BSA than BSA FA-free. Fatty acid binding causes displacement between two domains, opening up the central crevice and increasing the width of the molecule from 80 to 90 Å [8]. Change in the geometry of the molecule may cause the reduction in α-helices content. This decrease may be also explained by micelle effect on protein structure. It has been documented that protein exposure on micellar structures lead to protein unfolding [24] and decrease of α-helices content in albumin [25].

Another step should be to repeat this procedure with HSA. Results could be used in reagent-free determination of FFA binded to albumin in lipoprotein deficient serum (LPDS) – a product of sequential flotation and ultracentrifugation of lipoproteins.

Plasma pool of FFA is an effect of cooperation between esterified FA, i.e., triglyceride (TG) depots in adipose tissue, TG transporters: TG-rich lipoproteins, chylomicrons postprandially and VLDL in fasting state and TG hydrolysis by lipoprotein lipase anchored to vessel wall. If this cooperation fails (leading to metabolic disorders, e.g., dyslipidemia, insulin resistance, diabetes and obesity), albumin FFA cargo increases.

5. Conclusions

Our study confirmed that albumin structure is sensitive to amount of binded fatty acids particles, but only during high-affinity binding filling. Relation between PAM-to-BSA molar ratio and the amount of secondary structures is not linear. Structure is relatively stable for 2–10 mol/mol ratios. Only if amount of PAM highly exceeds BSA, binding capacity of helical structures decreases. No significant differences between BSA and BSA FA-free were observed. Our FT-IR spectroscopic results can be confirmed by data available in PDB.

It is remarkable that for PAM:BSA molar ratios corresponding to normal, physiological conditions of FA:albumin (0.4–9.0 mol/mol) the protein showed maximum content of α-helices and structural stability. Moreover, recent studies indicate that FA:albumin complex has higher denaturation resistance than defatted albumin [19].

Cardiovascular diseases are still a significant cause of death [5]. Beyond cholesterol, TG-rich lipoproteins and plasma FFA level are known to be Coronary Artery Disease risk factors [18]. In metabolic diseases, e.g., dyslipidemia, insulin resistance, diabetes and obesity, FFA cargo of albumin is huge. The systematic study of FA and albumin interactions, using an experimental model mimicking meta-

bolic disorders, may result in new tools for personalized nanopharmacotherapy.

Conflict of interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgements

This work was partially supported by statutory founds of Wrocław University of Science and Technology and is part of project "WroVasc – Integrated Cardiovascular Centre", co-financed by the European Regional Development Fund, within Innovative Economy Operational Program, 2007–2013, realized in Regional Specialist Hospital, Research and Development Center in Wrocław. "European Funds – for the development of innovative economy".

References

[1] BASIAGA S.B., HAGE D.S., *Chromatographic studies of changes in binding of sulfonylurea drugs to human serum albumin due to glycation and fatty acids*, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci., 2010, 878, 3193–3197.

[2] BHATTACHARYA A.A., GRÜNE T., CURRY S., *Crystallographic analysis reveals common modes of binding of medium and long-chain fatty acids to human serum albumin*, J. Mol. Biol., 2000, 303, 721–732.

[3] BHUSHAN B., DUBEY P., KUMAR S.U., SACHDEV A., MATAI I., GOPINATH P., *Bionanotherapeutics: niclosamide encapsulated albumin nanoparticles as a novel drug delivery system for cancer therapy*, RSC Advances, 2015, 5, 12078–12086.

[4] BOIX M., ESLAVA S., COSTA MACHADO G., GOSSELIN E., NI N., SAIZ E., DE CONINCK J., *ATR-FTIR measurements of albumin and fibrinogen adsorption: Inert versus calcium phosphate ceramics*, J. Biomed. Mater Res. A, 2015, 103, 3493–3502.

[5] CARUSO MV., SERRA R., PERRI P., BUFFONE G., CALIÒ FG., DE FRANCISCIS S., FRAGOMENI F., *A computational evaluation of sedentary lifestyle effects on carotid hemodynamics and atherosclerotic events incidence*, Acta Bioeng Biomech, 2017, 19, 42–52.

[6] CHARBONNEAU D.M., TAJMIR-RIAHI H.A., *Study on the interaction of cationic lipids with bovine serum albumin*, J. Phys. Chem. B, 2010, 114, 1148–1155.

[7] CISTOLA D.P., SMALL D.M., HAMILTON J.A., *Carbon 13 NMR studies of saturated fatty acids bound to bovine serum albumin. I. The filling of individual fatty acid binding sites*, J. Biol. Chem., 1987, 262, 10971–10979.

[8] CURRY S., BRICK P., FRANKS N.P., *Fatty acid binding to human serum albumin: new insights from crystallographic studies*, Biochim. Biophys. Acta, 1999, 1441, 131–140.

[9] CURRY S., MANDELKOW H., BRICK P., FRANKS N., *Crystal structure of human serum albumin complexed with fatty acid reveals an asymmetric distribution of binding sites*, Nat. Struct. Biol., 1998, 5, 827–835.

[10] DEMIGNOT S., BEILSTEIN F., MOREL E., *Triglyceride-rich lipoproteins and cytosolic lipid droplets in enterocytes: key players in intestinal physiology and metabolic disorders*, Biochimie, 2014, 96, 48–55.

[11] EBBERT J.O., JENSEN M.D., *Fat depots, free fatty acids, and dyslipidemia*, Nutrients, 2013, 5, 498–508.

[12] ERUKULA S.V., SRIVARI Y., CHATTERJEE P., *Factors influencing the fabrication of albumin-bound drug nanoparticles (ABDns): Part II. Albumin-bound carbamazepine nanoparticles (ABCns)*, J. Microencapsul., 2016, 33, 524–534.

[13] FANALI G., DI MASI A., TREZZA V., MARINO M., FASANO M., ASCENZI P., *Human serum albumin: from bench to bedside*, Mol. Aspects Med., 2012, 33, 209–290.

[14] FUJIWARA S.I., AMISAKI T., *Identification of high affinity fatty acid binding sites on human serum albumin by MM-PBSA method*, Biophys. J., 2008, 94, 95–103.

[15] FUJIWARA S.I., AMISAKI T., *Fatty acid binding to serum albumin: molecular simulation approaches*, Biochim. Biophys. Acta, 2013, 1830, 5427–5434.

[16] GIACOMELLI C.E., NORDE W., *The adsorption-desorption cycle. Reversibility of the BSA-silica system*, J. Colloid Interface Sci., 2001, 233, 234–240.

[17] GRDADOLNIK J., MARÉCHAL Y., *Bovine serum albumin observed by infrared spectrometry. II. Hydration mechanisms and interaction configurations of embedded H_2O molecules*, Biopolymers, 2001, 62, 54–67.

[18] JIN J.L., GUO Y.L., LI J.J., *Plasma free fatty acids in relation with the severity of coronary artery disease in non-diabetics: A Gensini score assessment*, IJC Metab. Endocr., 2017, 14, 48–52.

[19] LEGGIO C., GALANTINI L., KONAREV P.V., PAVEL N.V., *Urea-induced denaturation process on defatted human serum albumin and in the presence of palmitic acid*, J. Phys. Chem. B, 2009, 113, 12590–12602.

[20] LIU K.Z., SHAW R.A., MAN A., DEMBINSKI T.C., MANTSCH H.H., *Reagent-free, simultaneous determination of serum cholesterol in HDL and LDL by infrared spectroscopy*, Clin. Chem., 2002, 48, 499–506.

[21] LU R., LI W.W., KATZIR A., RAICHLIN Y., YU H.Q., MIZAIKOFF B., *Probing the secondary structure of bovine serum albumin during heat-induced denaturation using mid-infrared fiberoptic sensors*, Analyst, 2015, 140, 765–770.

[22] MURAYAMA K., TOMIDA M., *Heat-induced secondary structure and conformation change of bovine serum albumin investigated by Fourier transform infrared spectroscopy*, Biochemistry, 2004, 43, 11526–11532.

[23] NAIK P.N., NANDIBEWOOR S.T., CHIMATADAR S.A., *Non-covalent binding analysis of sulfamethoxazole to human serum albumin: Fluorescence spectroscopy, UV–Vis, FT-IR, voltammetric and molecular modelling*, J. Pharm. Anal., 2015, 5, 143–152.

[24] OTZED D.E., *Protein unfolding in detergents: effect of micelle structure, ionic strength, pH, and temperature*, Biophys. J., 2002, 83, 2219–2230.

[25] PARKER W., SONG P.S., *Protein structures in SDS micelle-protein complexes*, Biophys. J., 1992, 61, 1435–1439.

[26] SCHWINTÉ P., BALL V., SZALONTAI B., HAIKEL Y., VOEGEL J.C., SCHAAF P., *Secondary structure of proteins adsorbed onto or embedded in polyelectrolyte multilayers*, Biomacromolecules, 2002, 3, 1135–1143.