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BOVINE SERUM ALBUMIN ADSORPTION ONTO UV-ACTIVATED GREEN POLYURETHANE SURFACE

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The UV-irradiation is an alternative approach of the polymer surface functionalization that can be easily used in laboratory. This technique doesn't have an effect on the bulk properties of the green microporous polyurethane films. Thus, the UV-irradiation activates the surface of the green polyurethane by breaking some chemical bonds and generation of new functional groups on the surface which can be controlled by the irradiation time. The amount of bovine serum albumin (BSA) adsorbed on the UV-irradiated polyurethane surface increase as a function of the exposure time. The new structures of polyurethane-BSA conjugated with specific properties for each time of irradiation were formed. The polyurethane surface has been studied by ATR-FTIR, contact angle and fluorescence spectroscopy.

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Keywords: Polyurethane, UV irradiation, Surface functionalization, BSA adsorption.

1. Introduction

Polyurethanes (PUs) are a class of materials frequently used in a broad range of biomedical applications such as the heart valves [1] or patches [2]. The functionalization of these polymers is vitally for tissue engineering. There are many methods for surface activation, but the photo-functionalization is typically not accompanied by a reduction of polymer molar mass, and it is based on special photoreactive moieties (as side group or as part of the main chain of the macromolecule) having a distinct, selective and efficient reactivity [3]. Protein adsorption is generally considered to be the first step in directing the response of biological systems to implanted material [4]. In water, proteins and materials interact by multiple short-range (°A) interactions (H-bonding, electrostatic, van der Waals or dispersive forces, hydrophobic effect), which are individually rather weak but sum up and give important adsorption energy for large macromolecules. Conformational changes are likely to accompany protein adsorption on solid surfaces, but are difficult to evidence directly [5]. Polyurethane surface induced conformational changes of protein by complex mechanism [4, 6, 7]. On the other hand, on a charged surface, the electrostatic interactions between the polyurethane surface and functional groups along the polypeptide chain of the BSA molecules are the predominant driving forces. The adsorption density was higher at pH 7 than that at pH 3 or 11, and both α-helix and β-sheet structures were present in the adsorbed layer. BSA adsorbed onto these solids appears to adopt an extended conformation and the long axis of the molecule appears to lie in the plane of the interface [8]. The adsorbed protein layer will influence the subsequent biological reactions including platelet adhesion and activation [9]. Therefore, understanding the interaction between bovine serum

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albumin and polyurethane surfaces is critical, and control of protein–surface interactions continues to be an important factor for consideration in the design of biocompatible surfaces. We are not going to detail the progress that has been made in the understanding of BSA interaction with all biocompatible materials. Instead, we will focus on the interactions of BSA with UV- activated polyurethane surfaces.

2. Experimental

2.1. Synthesis of polyurethane

Polyurethane was synthesized from poly(ethylene-adipate)diol (PEA) having a molar mass of 2000 g/mol, hydroxyl number of 56 mg KOH $g^{-1}$and acidity number 0.2 mg g^{-1}KOH, isopropyl ricin-oleate diol (IRD), 98%,(synthesized in our laboratory), methylene diphenyl 4,4'-diisocyanate (MDI) freshly distilled, with isocyanate concentration of 99.96% and 1.4-buthane diol (BD), 99% (Sigma-Aldrich, anhydrous) in dimethylformamide (DMF) 99.8% (Sigma-Aldrich, anhydrous) as solvent. The synthesis was run at 60 ^{o}C according to a previously reported method [10]. The molar ratio between the components PEA: IRD: MDI: BD was 1: 0.1: 6: 4.9, the concentration of the solution PU-DMF was 33% dry weight and the viscosity 98 000 cP at 20 ^{o}C (Brookfield viscosimeter, spindle no 4). The molar mass of the resulting PU was 110 000 g^{-1} as determined by gel permeation chromatography. Elemental analyses calculated: C 61.28%, H 6.72%, N 4.22% and found: C 62.03%, H 6.86%, N 4.25%.

2.2. Sample preparation

Polyurethane solution was degassed for 8–12 min under vacuum (10–15 mmHg), and then the PU solution was cast onto a glass slide (220 - 240 mm) using a doctor blade with a gap of 0.6 mm. The films were precipitated in deionized and distilled water at 45 ^{o}C, for 1 h [11]. During this time, the resulted microporous films were detached from the glass plate and subsequently washed five times with 1 L of deionized and distilled water. The films were dried at room temperature and low pressure (1–2 mmHg) for 24 h. The thickness of the microporous film was 0.33 mm. The prepared PU microporous films have the water vapor permeability in the range of 280- 285 mg dm^{-2} 24 h at 25^{o}C.

2.3. UV Treatment

Films of the PU samples were irradiated in air at 25^{o}C with a UV-Philips lamp having a polychrome emission spectrum between 200-400 nm and light intensity of 30 mW/cm^2. The distance between the light source and samples was kept constant at 20 cm.

2.4. Analysis methods

The ATR-FTIR analysis was performed using a Bruker Vertex 70 type spectrometer (US), with a diamond crystal having a 6 mm^2 surface, provided with software for spectral processing. The sample surface was scanned in the 200–4000 cm^{-1} range, at 45^{o} angle. The ATR-FTIR spectra were recorded at a constant temperature of 25 ^{o}C.

Fluorescence spectra were measured using a Perkin-Elmer LS 55 luminescence spectrometer at room temperature. The excitation wavelength was 284 nm and emission within the region from 300 to 500 nm.

The static contact angles were measured by the sessile-drop method, with a CAM-101 (KSV Instruments, Helsinki, Finland) contact angle measurement system equipped with a liquid dispenser, video camera, and drop-shape analysis software, at room temperature. Double distilled water, ethylene glycol, diiodomethane and 1-octanol were used as solvents for the studies. For each kind of liquid, three different regions of the surface were selected to obtain a statistical result.

2.5. Bovine serum albumin (BSA) adsorption

All samples of polyurethane (10 mm x 10 mm) were made in duplicate and irradiated with UV light for 2, 4, 6, 8, 10 h respectively. Immediately after irradiation a series of polyurethane films was immersed in a solution of phosphate buffered saline (PBS), pH 7.4 and the other series in a solution of bovine serum albumin (BSA) to concentration 30 mg/ml in PBS. All samples were incubated at 37°C for 42 h. After certain periods, the films were removed and the amount of protein in the solution was determined by fluorescence using excitation at 284 nm and emission within the region from 300 to 500 nm. The adsorbed amount was calculated from the differences of initial and final values.

3. Results and discussion

3.1. ATR-FTIR analysis

The structure of the green polyurethane is illustrated in Fig.1.

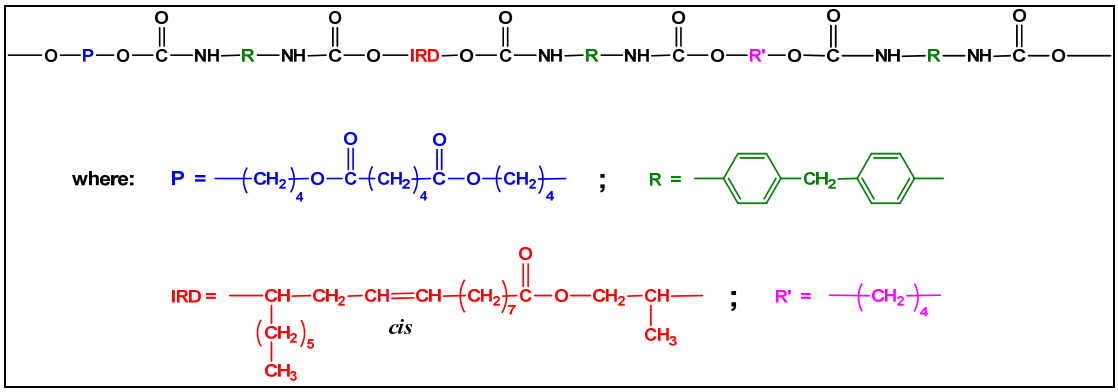

Fig. 1. Green polyurethane structure

The bovine serum albumin adsorption on the virgin polyurethane and on the polyurethane UV- activated surface as function of time was investigated. The level of surface activation was evaluated based on the ATR-FTIR data obtained by studying the polyurethane surfaces after exposure to UV light for 2, 4, 6, 8, 10 hours (Fig. 2).

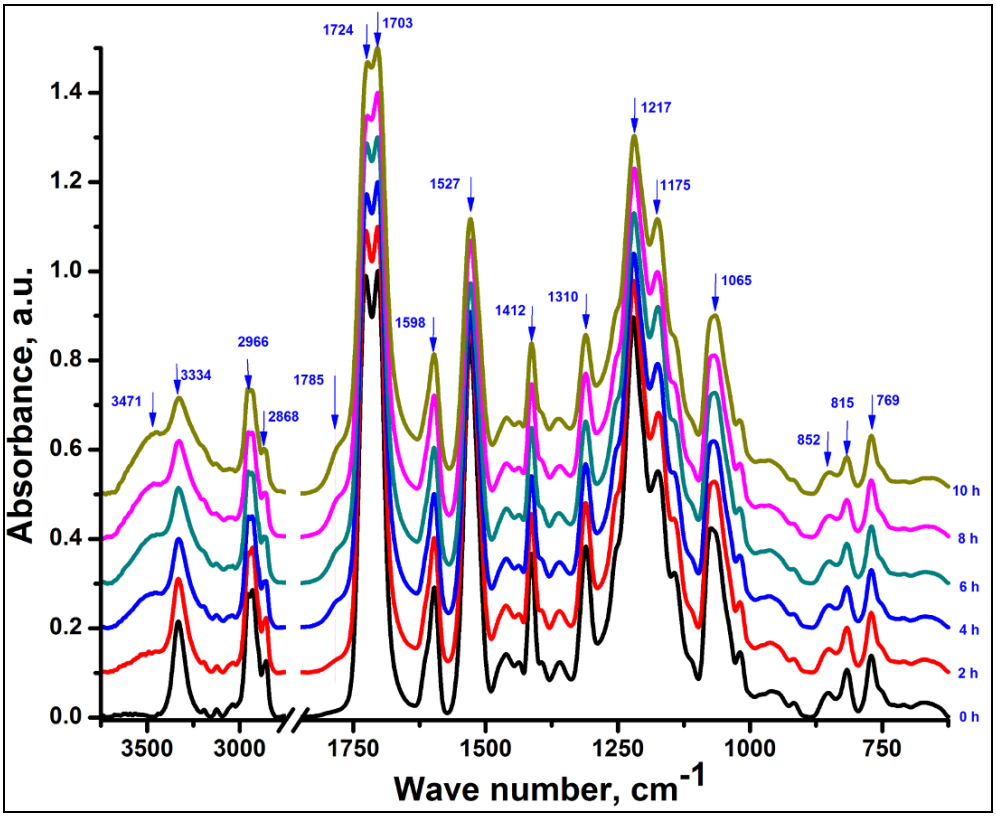

Fig. 2. ATR-FTIR spectra of the UV-activated polyurethane surfaces

In Fig. 2 it can be seen that appears a peak at 3471cm^{-1} corresponding to the hydrogen-free υ(N-H) stretching vibrations of free -NH-COO-, -NH_2, -OH and -COOH groups. Moreover, a characteristic shoulder for the υ(C=O) stretching vibrations of carboxyl group at 1750 cm-^{1} can be distinguished. The absorbance area is a function of the irradiation time for both peak and shoulder. The appearance of these peaks is the result of Photo-Fries rearrangement of the urethane groups [12-14] and photo oxidation of ester groups [15, 16]**.** The stretching vibration ν(N-H) at 3334 cm^{-1} can be attributed to the hydrogen bond interaction between the ester carbonyl (-CO-O-) and urethane (-NH-CO-O-) groups. It indicates that all the N-H of the hard segments participate to the formation of the hydrogen bonds. The peaks at 2966 cm^{-1} and 2868 cm^{-1} correspond to the ν(CH_2) anti-symmetric and symmetric stretch vibration of the soft segment. The strong peaks at 1724 cm^{-1} corresponds to the stretching vibration of the free carbonyl ν(C=O) from the urethane structure. The peak at 1703 cm^{-1} is characteristic to the stretching vibration ν(C=O) of the H-bonded urethane carbonyl group. At 1598 cm^{-1} is the peak due to the C=C bond vibration in the aromatic ring. Amide II is found at 1527 cm^{-1} and it is more complex than amide I. Amide II derives mainly from in-plane **δ** (N-H) bending with the greatest potential energy. The rest of the potential energy arises from the ν(C-N) and the ν(C-O) stretching vibrations; it is possible to take account the stretching vibration of the ν(C-O) of urethane if it analysis the second derivative minima of the peak at 1527 cm^{-1} and it is in concordance with the previous IR studies of amide II from polyamides [17,18]**.** The peak at 1412 cm^{-1} is due to the symmetric bent vibration of the **δ**(CH_2). At 1217 cm^{-1} is the peak of amide III (stretching vibration ν(N-C) and **δ**(N-H) bending). The stretching vibration ν(C–O–C) of the urethane group can be observed at 1175 cm^{-1}, and the symmetric stretching vibration ν(C–O–C) of ester group is at 1065 cm^{-1}. The peak at 769 cm^{-1} corresponds to the outside bent vibration zone of the adjacent hydrogen bonds on the aryl, which indicates there is a *para*-substituted aromatic ring in the final product.

The morphological and chemical changes of the polyurethane surface are highlighted more clearly in Fig. 3 where each selective irradiated spectrum was subtracted from a control spectrum.

The subtracting spectra give us information about the arising and disappearing of the main polyurethane groups. The peaks from 3456, 3246, 1756, 1681 cm^{-1} which are characteristic of the stretching vibrations υ(N-H) free, υ(N-H) bond, υ(C=O) free and υ(C=O) bond respectively of urethane groups are attributed to the reorganization of the macromolecular chain at the UV-irradiated polyurethane surface. The stretching vibrations for -CH_2 (2926, 2854 cm^{-1}) and C=O (1732, 1703 cm^{-1}) groups consumed were induced by chemical reactions on the polyurethane surface, when the irradiation time is low.

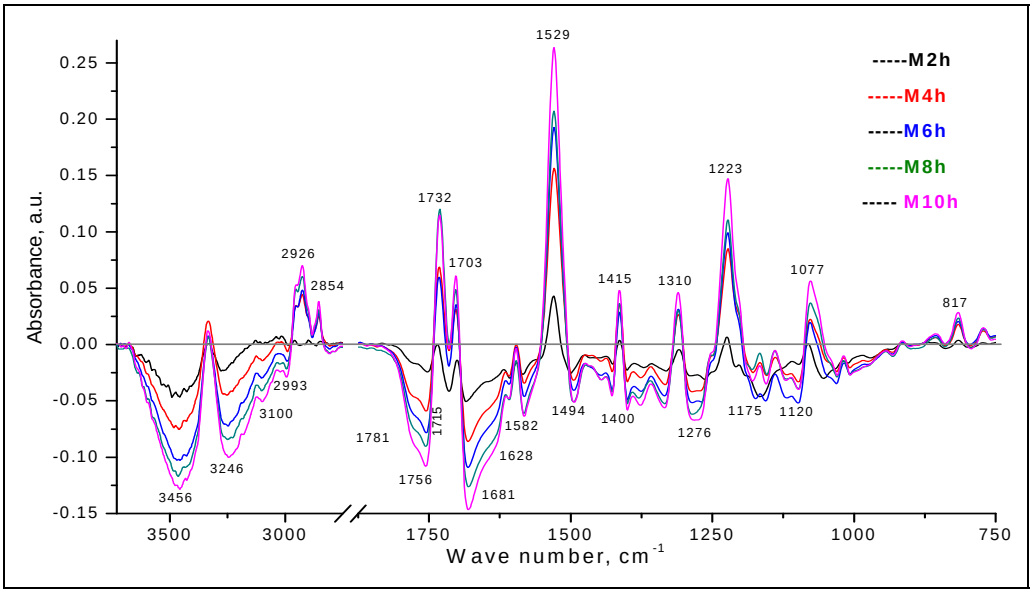

Fig. 3. The ATR-FTIR subtracting spectra

Also, in Fig. 3 is observed that the main peaks at 3456, 3246, 1756, 1681 and 1276 cm^{-1} are the same at all the irradiation times. Only the intensity of these peaks increases as a function of UV irradiation time. This suggests that occurs mainly photo-Fries rearrangement [12-14], Norrish I reaction [15-16] and α-H abstraction and radical reaction.

3.2. Bovine serum albumin absorption (BSA)

In Fig. 4 is illustrated the infrared spectra of the carbonyl groups (1800-1600 cm^{-1}) for the virgin and UV-irradiated polyurethane surfaces (Fig. 4A) and for the UV-irradiated samples with BSA (Fig. 4B). BSA is adsorbed on the PU surfaces in different proportions, phenomenon proved by increasing the peaks from 1658 cm^{-1} which is characteristic to the stretching vibrations ν(C=O) of protein [19-21] (Fig. 4B).

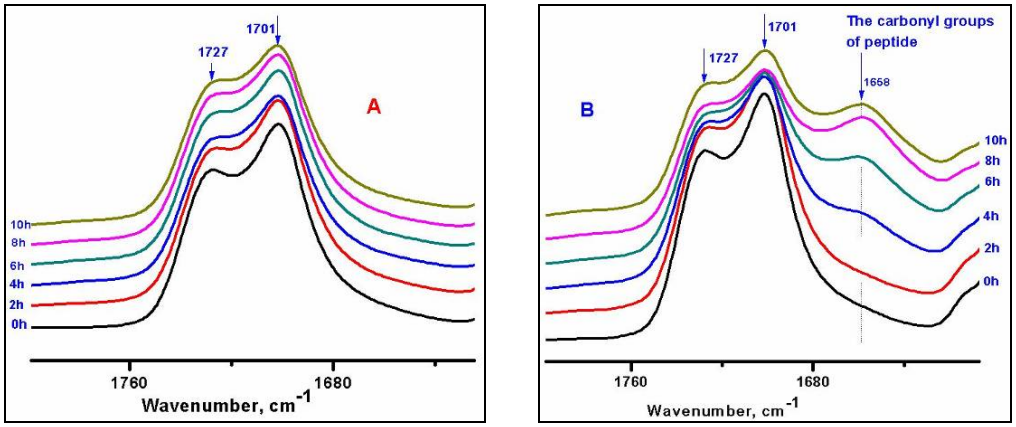

Fig. 4. The infrared spectra of the carbonyl bands (1800-1600 cm^{-1}) for:
A. UV-irradiated polyurethane surfaces and B. UV-irradiated samples with BSA

The amount of BSA adsorbed on surface is as a function of UV-irradiated time (Fig. 5). Thus, the virgin sample adsorbs 9 times less than the 10 hours treated sample. This can be explained by the fact that each polyurethane surface has a certain concentration reactive groups (-NH_2, -COOH,-NH-, free radicals) as a function of the UV treatment time [12-14]**.** These reactive groups interact with BSA to form a new polyurethane-BSA matrix with specific properties for each UV-irradiation time.

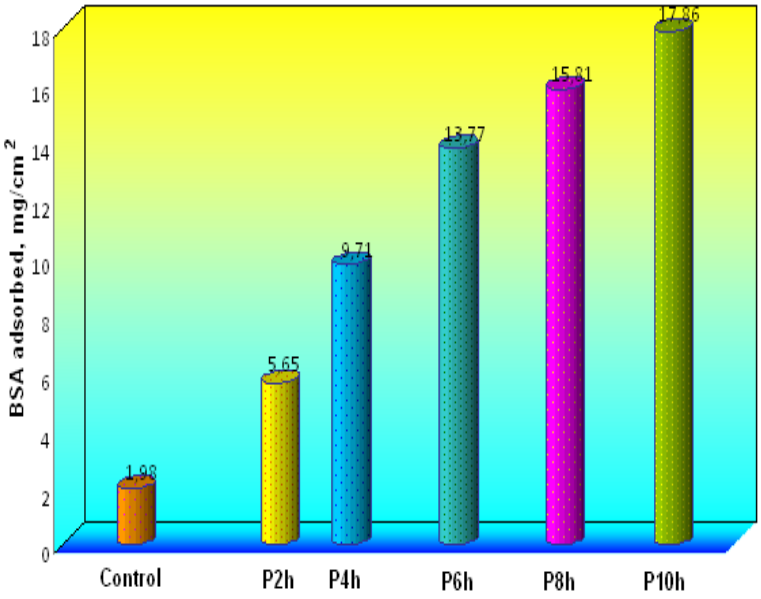

Fig. 5. The quantity of BSA adsorbed on the surface of the polyurethane UV activated for different times

If we are considering the literature data [12-16] and ATR-FTIR results, can formulate an interaction mechanism between the UV-activated PU surface and BSA (Fig. 6).

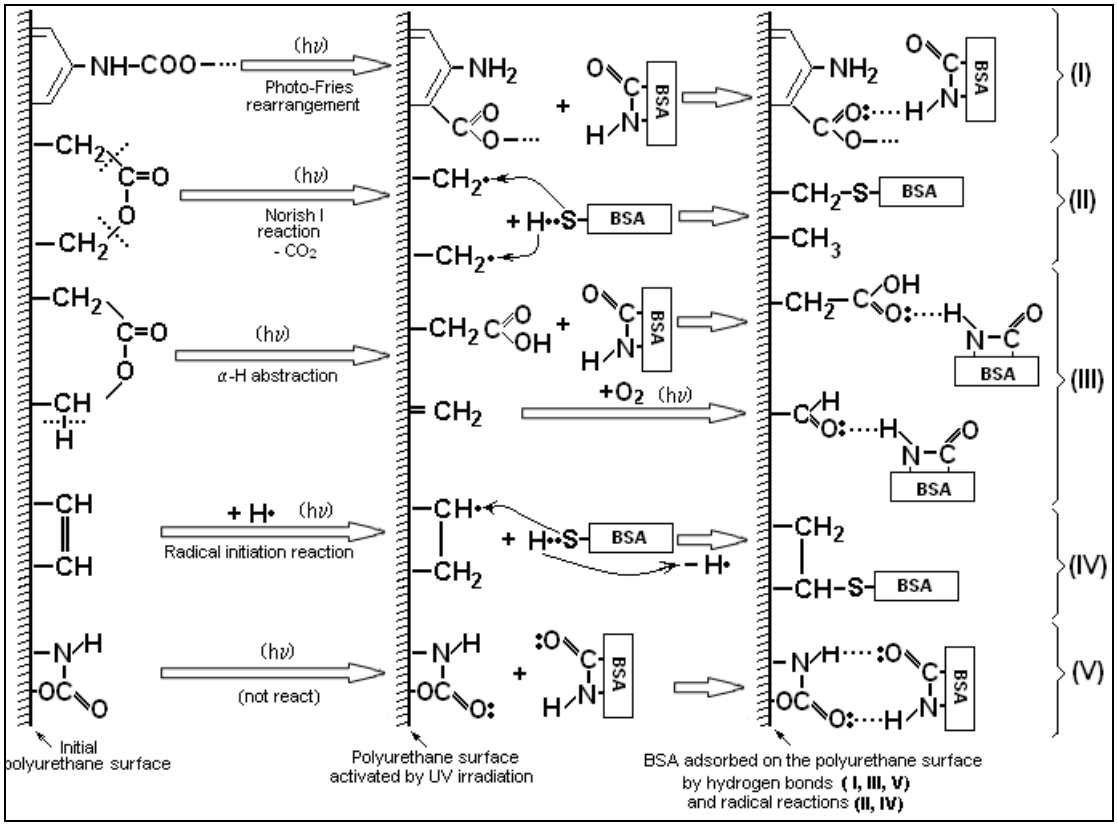

Fig. 6. A few reaction mechanisms of the polyurethane macromolecules from the polymer surface UV – irradiated

The urethane groups under the influence of the light give a Photo-Fries rearrangement in *ortho* position and forms an amino ester structure. Thus, after UV irradiation, polyurethane becomes a poly (urethane amino ester) copolymer (I). Another photo-chemical reaction at the UV-irradiated surface is Norrish I reaction which gives radicals that are reacting with BSA obtaining BSA-conjugate (II). The carboxyl and carbonyl groups which are obtained after the cleavage of the hydrogen from *alfa*-position and under the influence of oxygen from the air were formed H-bonds with peptide groups (III). The exposure of green polyurethane surface to ultra violet light alters the physical behavior and chemical composition of the green polyurethane surfaces. Irradiated surfaces of green polyurethane become hydrophilic and unstable radicals together with stabile carboxyl, carbonyl and amino groups are formed. The carboxyl, carbonyl and urethane groups react with amide group from BSA and form hydrogen bonds. The radicals interact with BSA and form a BSA- green polyurethane conjugates.

Some proteins, such as BSA, act as permanent blocking agents that attach to the surface and thereby inhibit nonspecific protein-surface interactions and protein-protein interactions. BSA is used as a traditional blocking agent to prevent nonspecific protein-surface binding on both hydrophobic and hydrophilic surfaces, in addition to many surfaces activated [22].

3.3. The fluorescence spectrum analysis

Most of proteins have intrinsic fluorescence and absorbance in UV region due to the aromatic amino acids. In particularly, BSA, the most used protein in the research studies display fluorescence properties because of the different chromophores of tryptophan, tyrosine and phenylalanine residues. Although the number of phenilalanines is larger, the fluorescence is dominated by tryptophan and tyrosine residues [23]. In this paper, the intrinsic fluorescence of the

proteins was applied to study the interactions of BSA with UV activated microporous polyurethane membrane.

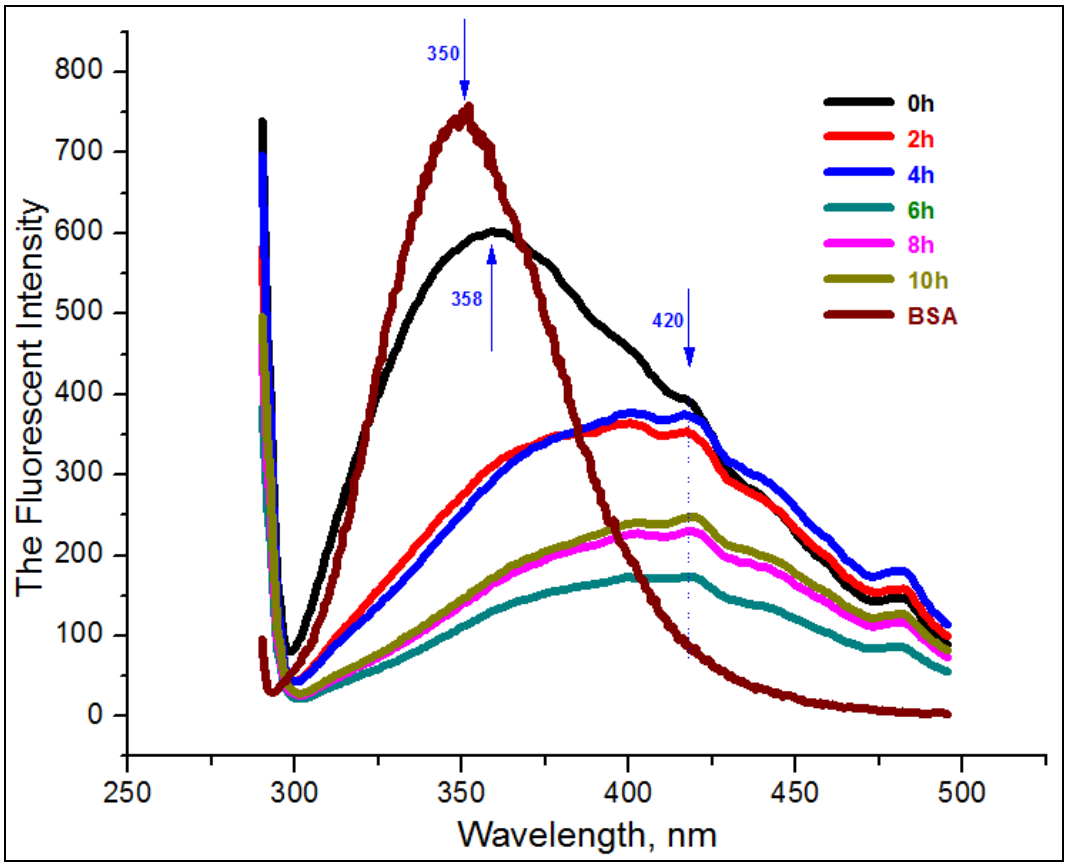

Fig. 7. The fluorescence spectra of free and adsorbed BSA on the UV-activated surface

The emission spectra of free and adsorbed BSA are illustrated in Fig. 7. The fluorescence intensity of the adsorbed BSA onto non-irradiated polyurethane is slightly decreased and maximum shifts with 8 nm towards higher wavelengths. For the UV-irradiated samples, the intensity is much lower, the peak is broader and its maximum moves with 60 nm to the higher wavelengths.

These phenomena suggested that BSA adsorption on UV-activated surfaces occurs in two main ways: i) a physical process that takes place in the presence of the C=O and NH groups of the polyurethane surface and BSA and that interact and form intermolecular hydrogen bonding and ii) -SH of BSA react with the free radicals on the surface and form the BSA-polyurethane conjugates.

The fluorescence intensity of the adsorbed BSA on the PU surfaces is characteristic for each sample and not decrease as a function of UV- treatment time.

3.4. Contact angle and surface free energy

The surface energy of the films was calculated using Young equation:

$$\gamma_{SV} = \gamma_{SL} + \gamma_{LV} \cos\theta \quad (1)$$

where γ_{SV} is the energy of the surface, γ_{SL} is the interfacial tension between the solid and the drop, γ_{LV} is the liquid-vapor surface tension, and cos θ is the contact angle of the drop with the surface [24,25].

The surface-free energy values (γ_{SV}) as well as the polar (γ^p_{SV}) and dispersive (γ^d_{SV}) components were obtained according to the Owens-Wendt-Rabel, and Kaelbe method [26,27]:

$$\gamma_{SV} = \gamma^p_{SV} + \gamma^d_{SV} \tag{2}$$

$$W_a = 2\left(\sqrt{\gamma^d_{SV}\gamma^d_{LV}} + \sqrt{\gamma^p_{SV}\gamma^p_{LV}}\right) \tag{3}$$

where W_a is the work of adhesion.

Critical surface tension of solids was calculated using Zisman method by extrapolation to $cos\ \theta = 1$, when the liquid wet the surface perfectly.

The hydrophobic and hydrophilic interactions play an important role in the surface chemistry and biochemistry of the polymers and biological molecules such as proteins and cellular membranes [27]. Solid surface dynamics can be described by contact angle measurements. Based on these measurements, the parameters who characterized the solid surface and its absorption capacity can be calculated. The most used parameters are the surface-free energy, solid-liquid interfacial tension, work of adhesion or critical surface tension. The resulted parameters are given in Table1.

Table 1: Parameters values who characterise the polyurethane surface

Sample	θ (°)	W_a (mN/m)	γ_c (mN/m)	γ_{SV} (mN/m)	γ^p_{SV} (mN/m)	γ^d_{SV} (mN/m)	γ_{SL} (mN/m)
M 0h	**86.64**	**77.06**	**24.09**	**25.18**	**6.66**	**18.51**	**20.91**
P 0h	74.83	91.85	21.13	29.21	17.30	11.30	10.16
M 2h	**85.55**	**78.44**	**20.37**	**23.60**	**8.92**	**14.67**	**17.95**
P 2h	72.34	94.88	18.69	30.99	20.24	10.75	8.91
M 4h	**81.54**	**83.50**	**17.56**	**24.16**	**14.74**	**9.42**	**13.46**
P 4h	68.64	99.30	13.41	34.91	29.49	5.42	8.40
M 6h	**80.55**	**84.74**	**16.80**	**24.93**	**14.99**	**9.93**	**12.98**
P 6h	64.71	103.9	10.19	40.59	37.49	3.10	9.49
M 8h	**78.66**	**87.11**	**13.93**	**26.09**	**19.09**	**6.99**	**11.77**
P 8h	62.58	106.32	5.93	42.39	39.05	3.30	8.87
M 10h	**73.47**	**93.51**	**12.20**	**30.33**	**23.92**	**6.41**	**9.62**
P 10h	58.75	110.56	2.38	49.74	48.27	1.47	11.97

From the experimental data can be observed that the non-irradiated sample (M0h) has a contact angles with water of about 86°, while the irradiated sample (M2h-10h) decreases according with the irradiation time [28,29] up to 73° for the 10 h irradiated sample, Table 1. These results demonstrate that with the increasing of the UV-irradiation time, the surfaces become more hydrophilic. Therefore, during the surface irradiation some polar groups, such as -NH_2, -OH, -COOH [14, 30, 31] arise as demonstrated by increasing the polar component of the surface free energy, Table 1. After BSA adsorption on the irradiated surfaces, we observed that the contact angle values decrease. This behavior is because the UV-irradiated polyurethane surface that contain hydrophilic groups can interact with the adsorbing protein molecules via hydrogen bonding and van der Waals interactions [14,30,31] leads to the formation of a new polyurethane matrix. Also, the free radicals from the surface can interact with the proteins. Therefore, the polar groups such as –NH_2, -OH, -COOH, are linked to a „rigid" matrix where the macromolecules changes their conformation and in contact with the air they have a minimum energy, like at the hydrophobic protein gels [32,33]. Because of this, the groups remain on the surface, in contact with the air, making polyurethane surface more hydrophilic. Therefore is observed that the sum of the polar and disperse component of the free surface energy of the polyurethane samples (γ_{SV}), increase only at the samples with BSA. The polar component (γ^p_{SV}) includes two types of coulomb

interactions: dipole-dipole and dipole-induced dipole, while the dispersive component (γ^{d}_{SV}) contains the interactions based on the electron charge of the molecules [34]. The values of the adhesion work are increased only for the sample with BSA while the values of their contact angle decrease. The critical surface tension, calculated with the Zisman method [24-26] has lower values at the sample with BSA, like the interfacial tension solid-liquid. In conclusion, after irradiation, the increment of surface energy is significantly higher for samples with BSA, which shows that between BSA and the polyurethane surface was created strong links.

4. Conclusions

The UV treatment proved to be a suitable surface modification technique to functionalize/activate the polyurethane films without affecting the bulk properties. After UV irradiation of the polyurethane surfaces, the wettability, surface energy, and water adhesion work increase for all the samples. Moreover, UV- irradiation introduced some chemical structures and morphological changes on the surfaces. BSA adsorption is enhanced on UV-irradiated PU films. The carboxyl, carbonyl and urethane groups react with amide group from BSA and form hydrogen bonds and the radicals interact also with BSA and form a BSA- green polyurethane conjugates. The new PU-BSA structures have specific properties for each time of irradiation.

Acknowledgments

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