Digest Journal of Nanomaterials and Biostructures Vol. 6, No 3, July - September 2011, p. 1053 – 1064

ALBUMIN IMMOBILIZATION ON POLYVINYLIDENE FLUORIDE SURFACES

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Films of polyvinylidene fluoride (PVDF) were treated in a microwave plasma, using different discharge gases (N_2, N_2/H_2, and CO_2), for the treatment conditions established as being the most appropriate for functionalizing the PVDF surface, in order to further coat it with an albumin layer. To evidence the physical adsorption of protein, several methods were used (gravimetric measurements, contact angle measurements, AFM and FTIR spectroscopy). It was found that plasma treatment determines a drastic increase in the albumin quantity adhering to PVDF surfaces (especially when N_2/H_2 is used as discharge gas). After coating the plasma pre-treated PVDF film surface with proteins, the hydrophilic character of the polymer increased. As albumin-treated surfaces are resistant to platelet adhesion, an enhanced tolerance towards biological fluids and an increased haemocompatibility are expected. In the same time, the albumin increases the number of functional groups ($-NH_2$ and $-COOH$) on the polymer surface, for binding other bioactive molecules, thus extending the polymer applicability. Plasma treatment of PVDF in a microwave discharge, followed by coating with bovine serum albumin proved to be very useful for the appropriate modification of its surface properties, thus leading to a possible increase in the biocompatibility characteristics of the polymer.

(Recieved April 14, 2011; Accepted June 16, 2011)

Keywords: Polyvinylidene fluoride, Plasma, Albumin, Coating

1. Introduction

Polymeric supports to which bioactive compounds have been covalently immobilized may offer the potential of streamlining the capture, isolation, and detection of target analytes, and ultimately the miniaturization of bioanalytical devices. Since materials interact with the environment through their interfaces, both the kind and the strength of such interactions are largely dependent on the surface properties of the materials [1]. While a material is in contact with a biological environment, the surface chemistry and topography of the material are important parameters that may influence protein adsorption, cell interaction, and ultimately the host response (for biomaterials). However, materials that are polymeric, ceramic, or metallic with totally different surface properties may induce similar responses *in vivo*, and this has been attributed to non-specific protein adsorption on the surface [2]. Protein adsorption on the material surface is believed to be the initial event when a material comes into contact with a biological environment. The adsorbed protein layer will influence the subsequent biological reactions, including platelet adhesion and activation [3].

The strong hydrophobic character of PVDF restrained it from promotion and application. To make PVDF films hydrophilic, many researches focused on plasma treatment [4]. Plasma can provide modification of the top nanometer of a polymer surface without using solvents or

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generating chemical waste and with less degradation and roughening of the material than many wet chemical treatments. The type of the imparted functionalization can be varied by selection of the discharge gas (Ar, N_2, O_2, H_2O, CO_2, NH_3) and of the operating parameters (pressure, power, time, gas flow rate) [5]. Surface activation has been focused on creating functional groups capable of preferential adsorption of biologically active species (proteins, enzymes, cells, drugs etc.). Plasma exposure of the polymer surfaces led to a complex set of processes, such as enhanced roughness, increased surface wettability and modification of the balance between acidic and basic groups on the treated surfaces. All these changes could promote interfacial interactions between the specific groups of the biomolecules and the functional groups introduced onto the surface. Plasma exposure is useful for preactivation of polymer surface for immobilization of biologically active species such as proteins. Drobota M. et al [6] demonstrated that, for surface activation of PET and collagen immobilization, plasma-precursor and chemical treatment was more efficient than a simple chemical treatment with triethylenetetramine (TETA). N. Dumitrascu et al [7] used a dielectric barrier discharge (DBD) treatment in helium at atmospheric pressure, for the incorporation/immobilization of heparin and immunoglobulin (IgG) on the PA-6 treated surfaces. They demonstrate that DBD treatments had the ability to create active and ionized sites on the polymer surface of PA-6 foils, keeping the bulk properties of the material unaffected. The deposition of albumin and IgG layers could prevent cell attachment from blood on the surface [8].

The coverage of the surface with biomolecules could be accomplished by preactivation/functionalization of the polymeric surfaces using other various physical and chemical techniques. Ionizing radiation allows endowing hydrophilic properties on hydrophobic PVDF films by grafting acrylic acid. Under certain conditions, poly(acrylic acid) totally covers the PVDF surface. All PAA carboxylic groups become consequently as many anchoring sites to immobilize peptides [9]. Li-Ping Zhu *et al.* modified poly(vinylidene fluoride) microporous membranes surface by the self-polymerization of DOPA (poly(3,4-dihydroxy-L-phenylalanine)) in aqueous solution. After this, heparin was immobilized covalently onto the obtained PVDF/poly(DOPA) composite membranes by the coupling between heparin and poly(DOPA) coating [10]. Dar-Jong Lin *et al* achieved the immobilization of heparin onto PVDF membranes surface in two steps: first, poly(acrylic acid) was grafted onto PVDF membranes with various surface porosities by plasma-induced polymerization, then heparin was covalently bonded to PAA using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDAC). The immobilized-heparin could effectively inhibit platelet adhesion on the PVDF membranes [11]. Among these techniques, the activation in cold plasma conditions appears to be a good approach because it offers some advantages such as it is a dry, surface localized process, is rapid, sterile, and keeping the bulk properties unaffected. Lee et al. [12] using Ar+ ion-beam irradiation and/or RF oxygen plasma treatment established that proper treatment conditions enhance the adhesion and retain the piezoelectric structure of PVDF.

In the immobilization processes, the surface topography and physico-chemical properties are very important, yielding good stable retention from chemical reactions between specific backbone groups. Protein adsorption is a complex multiparameter phenomenon. A number of factors are important in determining the amount of protein adsorbed on surfaces, including the protein structure, the surface chemistry, the magnitude and sign of charge of both protein and surface, and the degree of hydration of the protein. In some cases electrostatic effects are dominant, the protein showing the greatest tendency to adsorb when it has a net charge of opposite sign to the surface. In other cases the degree of hydration is most important. Maximum adsorption then occurs at the isoelectric point (pI) of the protein where the degree of hydration is minimum, allowing short range attractive forces to be involved in the process.

Albumin is clearly an extraordinary molecule of manifold functions and applications. Perhaps, the most outstanding property of albumin is its capacity to bind reversibly a numerous variety of ligands [13,14]. Bovine serum albumin (BSA) is constituted by 582 amino acid residues, and on the basis of the distribution of the disulfide bridges and of the amino acid sequence, it seems possible to regard BSA as composed of three homologous domains linked together [15]. Hence, BSA is a kind of hydrophobic globular protein. It is easily adsorbed onto synthetic surfaces such as the polymeric membranes polyethylene (PE), polytetrafluoroethylene (PTFE), PVDF, and polystyrene (PS) [16,17,18]. The adsorption process is dependent on the BSA

concentration. Some literature studies show that the adsorption isotherm conforms roughly to a Langmuir isotherm and the protein adsorption process could be irreversible, nearly irreversible, partly irreversible, and reversible [19]. Bowen et al studied the adsorption of bovine serum albumin at polyvinylidene fluoride membranes as a function of protein concentration, pH, electrolyte type, and flow rate through the membrane. Adsorption is initially rapid. Equilibrium is achieved after a period of 30 min to 3 h. Adsorption isotherms indicate two different adsorption sites, one of high affinity and the other of lower affinity for the protein. The numbers of such sites and the appropriate binding constants have been determined. The saturation coverage of the membranes by the protein occurs at substantially less than a monolayer the PVDF being considered as "low-protein-binding" [20]. To increase the amount of BSA adsorbed on PVDF surface supplementary treatment should be applied.

In this work, polyvinylidene fluoride was undergone to successive surface modification by microwave plasma treatment in different atmospheres, followed by coating with bovine serum albumin (BSA) by direct physiadsorption. The characterization of the modified surfaces, in respect with the unmodified one, was done by different investigation methods such as gravimetric method, contact angle measurements, FT-IR, and AFM.

2. Experimental

2.1. Materials

Non-piezoelectric films of polyvinylidene fluoride (PVDF) (0.25 mm in thickness) have been purchased from Goodfellow, England. This is a white, semi-crystalline, semi-opaque and fluorinated polymer. Its density is 1.76 g/cm^3 and its upper working temperature ranges between 135 and 150ºC.

Albumin from bovine serum (BSA) (minimum 98% electrophoresis – remainder mostly globulins, initial fractionation by heat shock) was purchased from Sigma-Aldrich. Water content was of 1.7%, the molecular weight 69,000, the molecular size 12 x 4 x 4 nm, and the isoelectric point 4.9. BSA was chosen, as it is readily available in highly purified form and as its adsorption on a range of materials has been extensively studied. Previous studies characterized these PVDF membranes as being low-protein-binding with respect to BSA, but results were only presented at one protein concentration at a single pH in a phosphate buffered saline solution [21].
Serum albumin was postulated to be an oblate ellipsoid with dimensions of 140 × 40Å. Each of the domains can be divided into 10 helical segments, 1 - 6 for subdomain A and 7 - 10 for subdomain B (Figure 1).

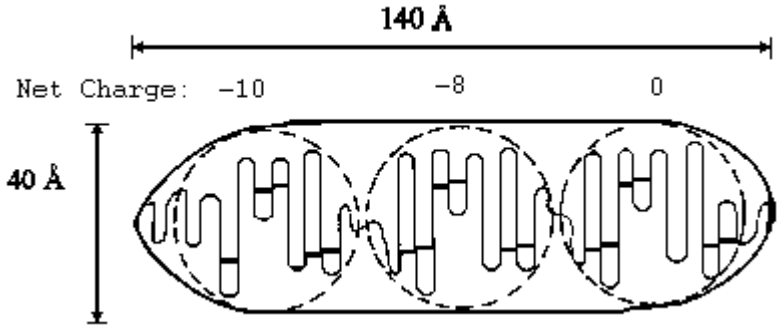

Fig. 1. Structure of serum albumin [22]

Albumins are characterized by a low content of tryptophan and methionine and a high content of cystine and of charged amino acids, aspartic and glutamic acids, lysine, and arginine. The glycine and isoleucine content of BSA are lower than in the average protein. The secondary structure contained about 68% - 50% alpha-helix and 16% -18% beta-sheet.

2.2. Microwave plasma treatment

Films of polyvinylidene fluoride (PVDF) have been treated in a microwave plasma, using different discharge gases (i.e. CO_2, N_2, and N_2/H_2 1:3), for the treatment conditions previously established as being the most appropriate for functionalizing the PVDF surface [23], in order to further coat it with protein layers. The experimental set-up is given in Figure 2.

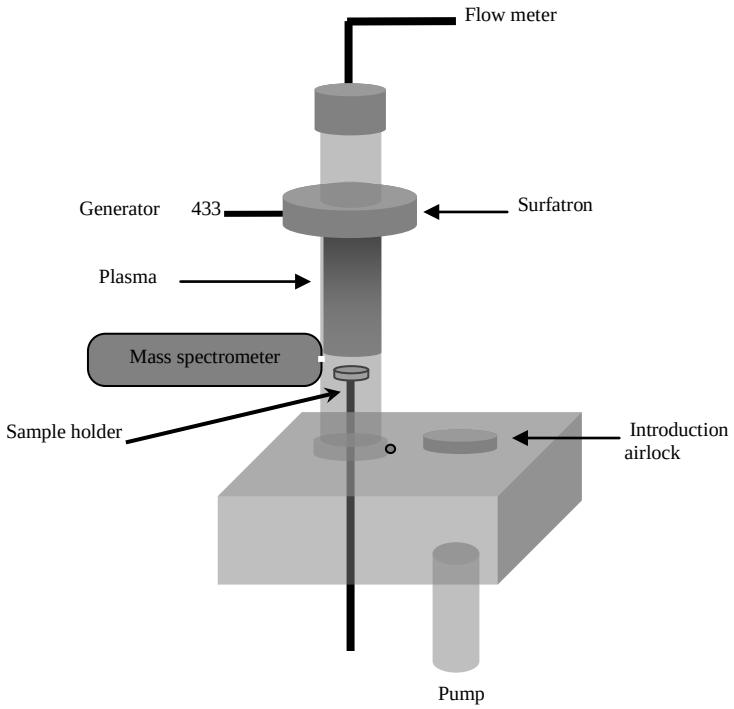

Fig. 2. The experimental set-up for microwave plasma treatment of the PVDF surface

A cylindrical MW plasma column is generated using a surfatron. The sample holder allows to locate the sample either in direct contact with a 2.45 GHz microwave plasma, or downstream, where only long-lived species (largely neutrals) in the plasma effluent contribute to the process chemistry. The optimal plasma parameters have been previously established [23,24]:
I) Plasma CO_2: Q = 10 sccm, P = 50W, t = 30 sec, d = 10 cm ,
II) Plasma N_2: Q = 10 sccm, P = 50 W, t = 60 sec, d = 10 cm;
III) Plasma N_2/H_2: in the ratio of 25/75: Q = 10 sccm, P = 50 W, t = 60 sec, d = 10 cm

Comparing our previously obtained results with the literature ones, we can conclude that three kinds of active surfaces obtained in the optima conditions of applied treatment are undergone to covering with BSA, namely acidic surfaces (obtained by CO_2 plasma activation, the surface roughness being increased and functionalization by implantation of oxygen functionalities mainly carboxyl groups being achieved) [25,26,27,28], basic surfaces (obtained by N_2/H_2 plasma activation [23]), and amphoteric surfaces (obtained by N_2 plasma activation [29,30]).

2.3. Immobilization procedures

The albumin adsorption was done by dip-and-dry procedure by immersing the polymer films, at room temperature, in an aqueous solution of albumin of 2 wt%. The ratio polymer / albumin was kept approximately constant of 1/2 w/w [31]. Albumin fraction V from bovine serum (Sigma-Aldrich for biochemistry) has been used. The quantitative determination of the immobilized albumin on the film surface was made by gravimetric measurements. Before and after immersion of the samples in albumin solution, they were dried at 60 °C, for 1.30 h and kept in a medium with constant relative humidity. Even if the dip-and-dry method has a poor reproducibility, it was found to be useful for the study of the binding ability [32,33].

The following samples have been studied: untreated and uncoated PVDF film (noted as PVDF), PVDF untreated in plasma, coated with albumin (noted as MII), and PVDF films treated in different plasmas and coated with albumin (noted as CO_2, N_2 and N_2/H_2).

2.4. Investigation methods

Contact angle measurements. The static contact angles for the polymer films were determined by the *sessile drop method*, at room temperature and controlled humidity, within 30 s, after placing 1μL drops of liquids on the film surface [34], using a CAM-200 instrument from KSV- Finland. Contact angles of double distilled water were measured on the PVDF films to estimate wettability. Contact angle measurements were taken at least fifteen times at different locations on the surface. The average values were used in contact angle analysis. Measurements showed a standard deviation of less than 2°.

Dynamic contact angles (DCA) and contact angle hysteresis were measured on PVDF films by using a KSV Sigma 700 tensiometer system – a modular high-performance, computer controlled surface tension/contact angle meter. The measurement parameters were: advancing–receding speed, 5 mm/min; start depth, 0 mm; immersion depth, 8 mm; number of cycles, 3; the average values were taken into consideration. The dynamic contact angles were divided into an advancing angle ($\theta_{advancing}$) and a receding angle ($\theta_{receding}$). The contact angle hysteresis (ΔH) was determined by the difference between the advancing contact angle and the receding one. The contact angle hysteresis is a function of surface roughness, chemical heterogeneity, surface polarity, and molecular rearrangement of the surface during wetting and drying. A lower value of the contact angle hysteresis indicates the homogeneity of the surface [35,36]. The contribution of the hydrophobic (non-polar) component is large for the advancing angle, whereas the contribution of the hydrophilic (polar) component is large for the receding angle.

To obtain reproducible results for contact angle measurements, several conditions have to be fulfilled, such as: constant temperature during determinations; the same volume of solvent drops (not higher than 1μL); evaluation of the contact angles in different points of the studied surface, the final result being the average of the obtained values. The errors involved in contact angle determinations are mainly caused by the surface roughness and the chemical heterogeneity of the polymeric systems. The largest accepted variation in the values of the contact angles was of ± 1–2 degrees, in the case of the most heterogeneous surfaces. A limitation of this approach is the heterogeneity of the active surface and of the ionized sites, which may not be uniformly distributed on the surface.

The FT-IR spectra were recorded by means of a spectrometer Bruker VERTEX 70, in transmittance mode. Background and the sample spectra were obtained in the 600 to 4000 cm^{-1} wave number range. The processing of spectra was achieved using SPECVIEW program.

AFM investigation was done by means of a Solver-Pro-M type instrument (Russia) under ambient conditions, using standard tips of Si_3N_4 with small curvature radius of 10nm. AFM images were obtained in the tapping (non-contact) mode which is nondestructive for surface and the biological layer is not damaged. Various ranges of the surface have been scanned namely of 60 x 60 microns and 40x40 microns. Images have been recorded on different zones in order to be representative for the total sample surface state. Roughness of the PVDF surfaces was verified by statistical AFM estimations. The root mean square roughness (RMS or Sq) has been calculated on the total image sample after a second order flatness treatment of the raw data.

3. Results and discussion

3.1. Gravimetric results

The quantitative determination of the immobilized albumin on the film surface was made by gravimetric measurements. The obtained data are presented in Figure 3.

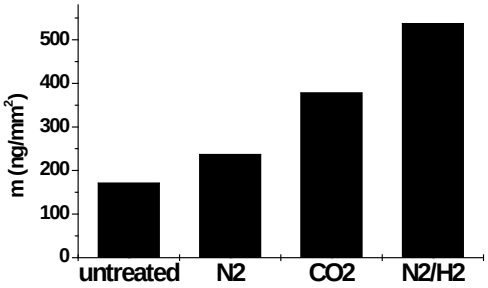

Fig. 3. The quantity of adsorbed albumin on PVDF surfaces, untreated and plasma treated using different gases

It can be observed that plasma treatment determines a drastic increase in the albumin quantity adhering to PVDF surfaces (especially when N_2/H_2 is used as discharge gas), possibly due to the polar character, induced during plasma exposure (behavior evidenced in our previous studies, by contact angle measurements [23]). As albumin-treated surfaces are resistant to platelet adhesion, an enhanced tolerance towards biological fluids and increased haemocompatibility are expected. At the same time, the albumin increases the number of functional groups (−NH_2 and −COOH) on the polymer surface for binding other bioactive molecules, thus extending their applicability.

3.2. Contact angle measurements

Usually, water contact angle measurements are the most convenient way to assess the hydrophilicity (or hydrophobicity) and the wetting characteristics of the polymer surfaces.

Figure 4a shows a water droplet formed on the untreated PVDF film. The surface contact angle of the film is 87°, in agreement with the strong hydrophobicity of PVDF material to water. The image in Figure 4 b-e shows a significant decrease in the contact angle on the treated and albumin coated PVDF film (≈ 67-77°), which may be ascribed to the increase of PVDF hydrophilicity after plasma treatment and to the BSA adsorption. The decrease of the contact angle after BSA adsorption on the surface and this decrease is much pronounced after plasma treatment and mainly in the case of N_2/H_2 plasma.

Surface chemical feature is not the only factor influencing the surface contact angle. The Wenzel equation [37] indicates that the water contact angle of the surface decreases with increasing surface roughness when the surface is composed of hydrophilic substances. Roughness, however, is so complicated that it is difficult to develop a general method for the roughness measurement.

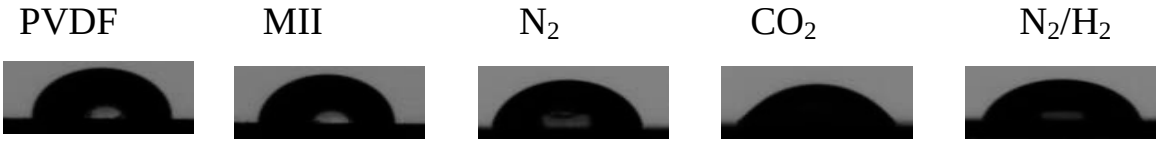

Fig. 4. Images of the water drop on the untreated and plasma treated PVDF surfaces after albumin immobilization

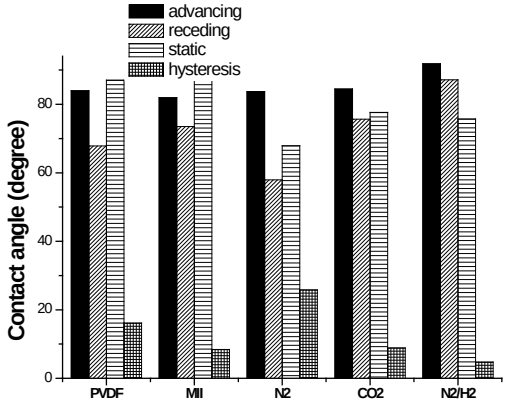

Fig. 5. Variation of the advancing, receding and static contact angle, and of the hysteresis of PVDF surfaces untreated and uncoated with albumin (PVDF), untreated and coated with albumin (MII) and plasma treated with discharge gases

The advancing, receding and static contact angles show the same variation for all the studied samples, that is a small decrease after each kind of treatment (Figure 5). A significant decrease of static and receding contact angles for N_2 sample is observed. At first, it seems surprising that only the receding contact angle is affected, while the advancing angle remains more or less constant. It can be expected that the advancing contact angle is characteristic for the most hydrophobic component of the surface, while the receding contact angle is more determined by the hydrophilic components of the surface. Surface roughness appears to have much more influence on the receding contact angle.

From contact angle measurements, the contact angle hysteresis was determined. The contact angle hysteresis provides information on surface energetics, roughness, and heterogeneity. The larger the hysteresis, the greater is the impediment to flow [38]. A lower value of contact angle hysteresis indicates the homogeneity of the surface and the ability of the drop to roll off or move easily on the rough surface [39,40].

Some researchers make correlation between the hysteresis and the surface roughness: contact angle hysteresis decreases with decreasing roughness [41,42,43]. In the case of the studied samples, the hysteresis takes the lowest value of 4.7 degrees for N_2/H_2 sample and the highest for N_2 sample of 25.8 degrees.

3.3. AFM results

The surface topography was examined using Atomic Force Microscopy (AFM). Even though the adsorbed BSA molecules could not be observed visually and the thickness of the BSA layer could not be obtained directly from AFM images, some important information can be deduced. The AFM images of the PVDF surface untreated and uncoated and treated and coated with albumin are shown in Figure 6. The surface of the film is uneven and the topographical variation is about 35 nm. Since the long axis of BSA molecules is only 12 nm, it is very difficult to distinguish BSA molecules from the uneven surface. A decrease (not an increase) in variation after adsorption is due to the uneven surface of the naked as was found also for PE anion-exchange membrane [16] and thus proves that BSA is actually adsorbed on the film surface. Phase imaging provides clearer observation of BSA adsorption, since it emphasizes grain edges and is not affected by large-scale height differences. By comparing the phase images before and after adsorption, it was found that the plasma treatment and the coverage with BSA makes the membrane surface more smooth and uniform (Figure 7).

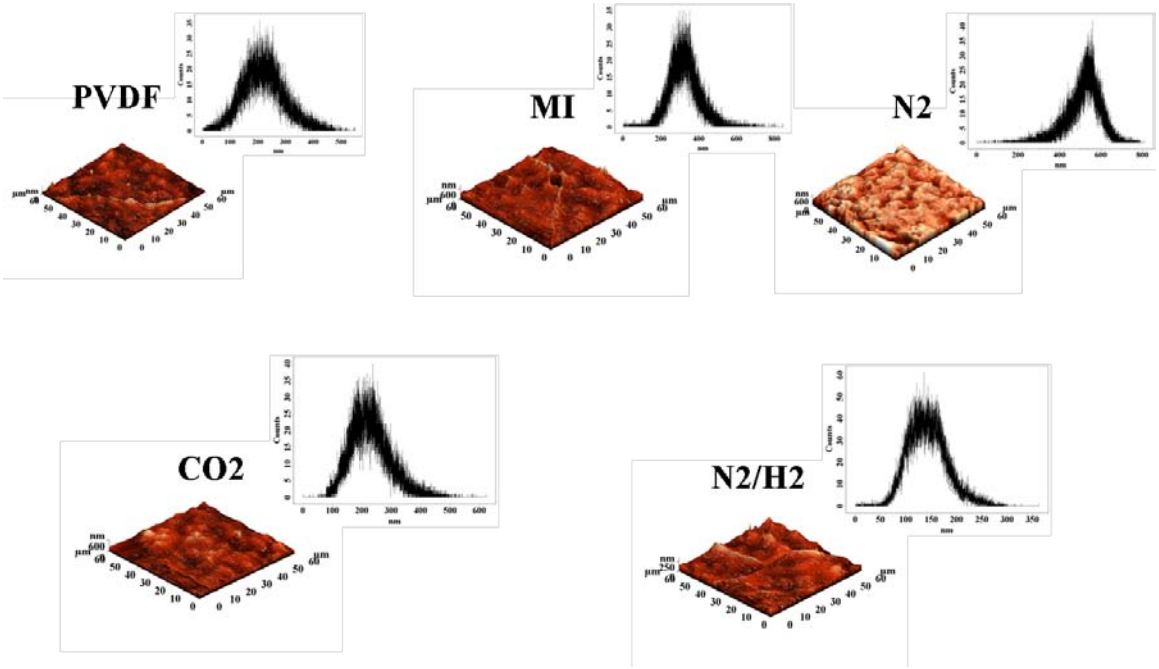

Fig. 6. AFM images and histograms of: PVDF-native film, MI-untreated; N2, CO2, and N2/H2-films treated in plasma using N2, CO2 and N2/H2 discharge gases, after immobilization of albumin

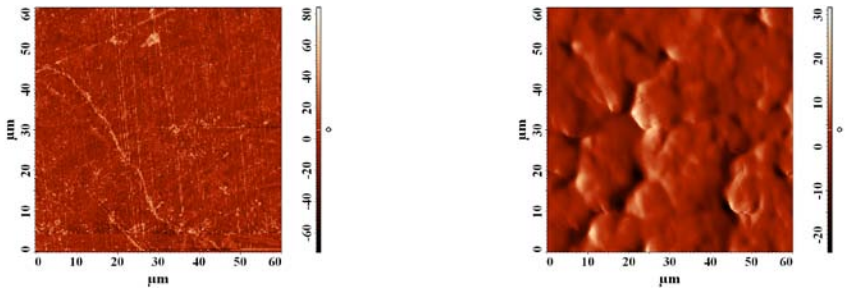

Fig. 7. Phase images before and after BSA adsorption

The surface topography of the films, as observed by AFM, undergoes significant changes as a result of PVDF treatments. Defects such as vacancies and irregularities were observed in all PVDF films.

Surface roughness is an important parameter for biomedical materials, as it may affect cell adhesion [5]. A RMS value equal to 38.42 nm was found for N_2/H_2 plasma treated and albumin coated film, value lower than that of the native PVDF and treated in plasma using CO_2 and N_2 discharge gases (where a roughness of about 60 nm was found – Figure 8).

The height histogram of topographical images shows the statistical distribution of z-values (heights) within the image. The histograms for PVDF films treated in plasma show that after immobilization of albumin on PVDF film treated in plasma using N_2/H_2 discharge gases, the surface becomes more homogenous. Combining these results with those of Figure 5, it can be remarked that is a direct correlation between the contact angle hysteresis and the roughness as measured by AFM.

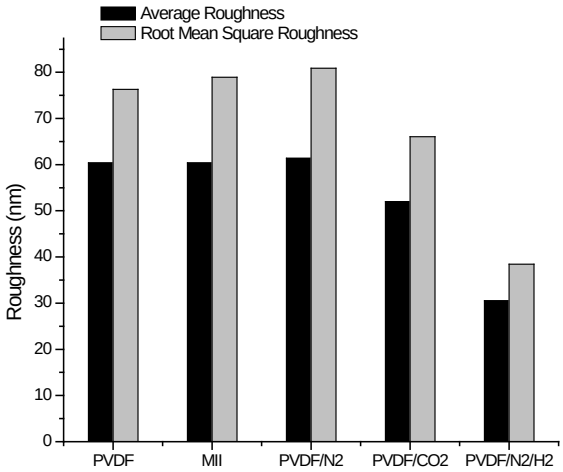

Fig. 8. Compared roughness of the PVDF pristine film, untreated and treated in plasma and coated with albumin

3.4. FTIR results

The FTIR spectra of the PVDF films treated in microwave plasma using different discharge gases are given in Figure 9 and the bands assignment are shown in Table 1.

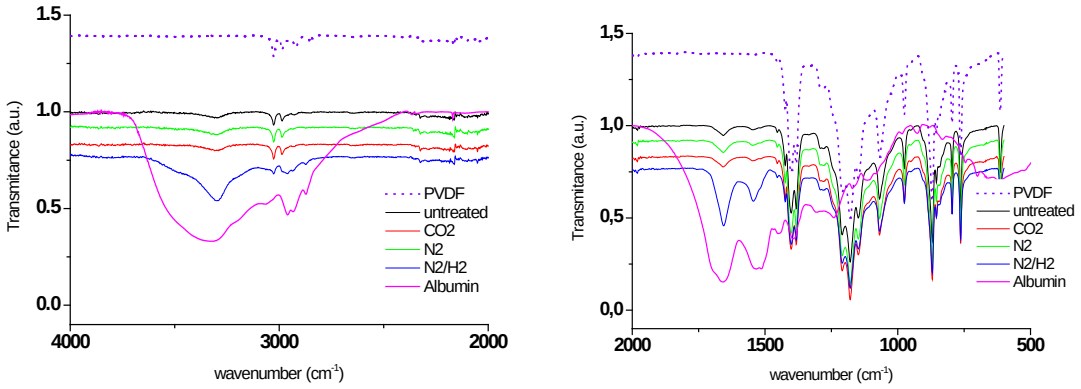

Fig. 9. FT-IR spectra for plasma treated PVDF surfaces after coating with albumin

Table 1. The FTIR bands assignment for PVDF films treated in microwave plasma using different discharge gases and coated with albumin [44]

Wavenumber (cm^{-1})	Band assigmement	PVDF	MII	CO2	N2	N2/H2	Albumin
2500-3300	νOH-from COOH group and combination bands, ν_{NH}	-	3026 2987	3026 2987	3026 2987	3289 3027 (broad band)	3322 (broad band)
1610-1660	δNH amide region I	-	1655	1656	1651	1655	1658
1485-1550	δNH amide region II	-	1545	1540	1547	1544	1534
1100-1400	ν_{CF2} stretching	1068 1402	1148 1401	1148 1423	1148 1401	1149-1389	-
~3050	ν_{CH2} asymmetric	3026					

The spectrum of original PVDF (Figure 9) exhibits some intensive bands at 1400, 1280, 1076, 835 cm^{-1}, which are assigned to the characteristic vibration of C–H and C–F. Compared to the pristine PVDF membrane, there is no obvious change on the film surface after plasma treatment, as illustrated in Figure 9. This step only attributes to the formation of activated radicals on PVDF surface. For coated film, the appearance of representative bands at 3380 cm^{-1}, as shown in Figure 9, attests the presence of the –O–H groups. From the spectra, stretching vibration of O=C–O– at 1727 cm^{-1} is well observed, as well as the symmetric stretching vibration of –C=O at 1695 cm^{-1}. It can be concluded that a thin layer of BSA was adsorbed on the PVDF film, typical vibration of C–F and C–H from 800– 1400 cm^{-1} obviously appearing weakened on the FTIR spectra of coated film.

After the treatment, new functional groups are found on the PVDF surface, these being assigned to amide, amine and carboxyl groups of BSA; so FTIR spectra evidence the albumin immobilization on PVDF surface. This affirmation is also demonstrated by the presence in the FTIR spectra of the albumin characteristic bands in the 2500-3300 cm^{-1} region. Plasma exposures induce a polar character to the PVDF surface and make possible the retention of albumin. The bands corresponding to the BSA have highest intensities in the case of N_2/H_2 plasma treatment, indicating a high quantity of BSA immobilized (in accordance with all the other used investigation methods: contact angle measurements, AFM and gravimetric data).
To explain these results, the structure of albumin (Figure 1) should be considered.

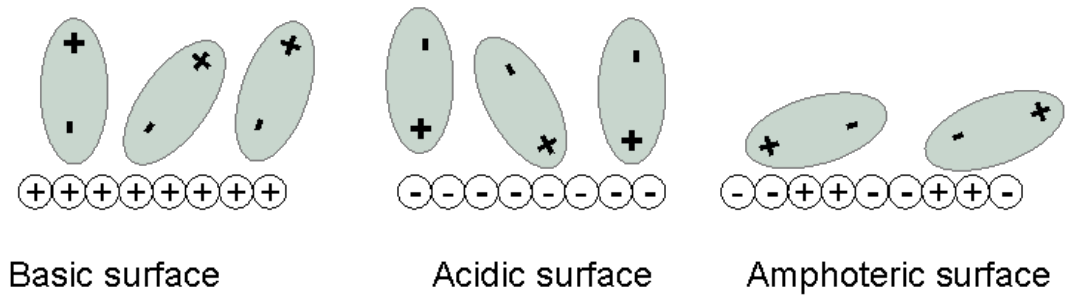

Fig. 10. Schematic representation of BSA interaction with different PVDF surfaces

BSA is a kind of hydrophobic globular protein of ellipsoidal revolution with long axis 12 nm and short axis 4 nm. Its adsorption on hydrophobic surface is nearly irreversible or partly irreversible in a side-on or end-on way, and its aggregation on film surface gives rise to more than a single layer. BSA contains a high number of carboxyl groups available to interact with the

surface and preference will be for the basic groups, which are found mainly after N_2/H_2 plasma treatment (basic surface), then amino groups will interact with carboxyl groups implanted on surface by CO_2 plasma discharge (acidic surface), and finally both amine and carboxyl groups will interact with complementary groups found on amphoteric surface created after N_2 plasma treatment, where probably the number of contacts will be greater and the surface more stable, but the BSA adsorbed quantity – smaller - (Figure 10).

4. Conclusion

A two step procedure has been developed for BSA immobilization on PVDF surfaces, consisting in plasma treatment followed by physiadsorption of protein. In this way, the quantity of adsorbed albumin was significantly increased, depending on the discharge gas used in microwave plasma. By using ATR-FTIR spectroscopy, the presence of functional groups of BSA protein on PVDF surface was proved. Water contact angle measurements and AFM analysis showed an increase in the hydrophilicity in these two steps of modification and a decrease in heterogeneity, mainly in the case of the microwave plasma treatment using N_2/H_2 discharge gases, which is more convenient for BSA immobilization.

Plasma treatment of PVDF in a microwave discharge, followed by coating with BSA proved to be very useful for the appropriate modification of its surface properties, thus leading to a possible increase in the biocompatibility characteristics of the hydrophobic PVDF. The purpose of these coatings is to create biocompatible surfaces for medical applications.

Acknowledgements

The authors would like to thank CNCSIS for funding their researches in the framework of the grant ID_2541.

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