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Microwave plasma activation of a polyvinylidene fluoride surface for protein immobilization

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Abstract

Polyvinylidene fluoride (PVDF) was modified by CO_2, N_2 or N_2/H_2 plasmas, which permitted the attachment of short carboxyl or amino groups. A variation of the discharge parameters was performed, for their optimization, as well as for minimizing degradation in favour of acidic, amphiphilic or basic functionalization, respectively. The optimum parameters of discharge for CO_2, N_2 or N_2/H_2 plasmas were $P = 50$ W, gas flow rate $Q = 16 \times 10^{-8}$ m^3 s^{-1}, exposure time $t = 30$–60 s, $d = 0.1$ m, pressure 15 Pa. The new surfaces were characterized by wettability measurements, attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR), x-ray photoelectron spectroscopy (XPS) and atomic force microscopy (AFM) methods. In a second step, the proteins (triglycine (TG) and protein A) were adsorbed or chemically grafted onto the carboxyl or amino functionalized surface, after EDC/NHS (1-ethyl-3-(-dimethylaminopropyl) carbodiimide/*N*-hydroxysuccinimide) activation of proteins. ATR-FTIR, XPS and AFM investigations confirmed the presence of protein on the surface. The XPS C1s core levels at 286.3 eV (C–N), 288 eV (amide bond) and 298 eV (carboxylic acid), together with variation of the O1s and N1s signals, illustrated the immobilization of proteins. It was established that TG was better attached on surfaces activated with N_2/H_2 plasma, while protein A was more tightly anchored on CO_2, N_2 plasma-activated surfaces. The former procedure allowed higher surface densities, while the latter permitted a better chemical control. The results proved that plasma-treated PVDF is a good substrate for protein coating, which can be further used for microorganisms' detection, as evidenced by the immunoassay test.

(Some figures may appear in colour only in the online journal)

1. Introduction

Recently, modification of the surface properties of polymers has become a topic of special interest for numerous researchers. The two usually applied procedures, referred to as physical and chemical methods, aim at modifying the surface properties of polymers. However, the chemical method is a water-wasting process and easily causes environment pollution. In contrast, physical methods have been preferred in recent years, due to their cleanness and high efficiency [1]. Therefore, plasma-surface modification can induce a specific surface chemistry,

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without altering the bulk properties of the material [2]. Since plasma is a dry process, there are no disposal or personnel safety issues commonly associated with wet chemistry or with other conventional methods, such as flame treatment. Inherent cleanliness and the ability to automate and control all critical functions of the processing make the treatment especially attractive for clean manufacturing environment.

Polymer-surface modification by a discharge plasma is expected to have increasingly important industrial applications [3]. Numerous experimental and theoretical studies have been devoted to this topic in recent years. For example, Liu *et al* [4] reported that examination of pre- and post-plasma-treated polymer surfaces by atomic force microscopy (AFM) revealed that only the outermost layer was affected by the dielectric barrier discharge (DBD) plasma treatment. Guruvenket *et al* [5] modified polystyrene and polyethylene (PE) using microwave (MW) electron cyclotron resonance plasma, to improve surface wettability. Bhowmik *et al* [6] studied the wettability and physico-chemical characteristics of a polypropylene (PP) film exposed to a dc glow discharge. Coen *et al* [7] and Akishev *et al* [8] modified PP, polymethylmethacrylate (PMMA), polytetrafluoroethylene (PTFE) and polyethyleneterephthalate (PET) under cold plasma conditions. Vesel [9] functionalized PET in both oxygen and CO_2 plasmas, finding differences only for extremely short treatment times. These surface modifications include surface cleaning and degreasing, topographical modification, oxidation, reduction, grafting, cross-linking (carbonization), etching and deposition. When trying to achieve targeted surface engineering mainly for biomedical applications, it is vital to gain full understanding of the mechanisms that cause these effects, for example, surface functionalization, adhesion promotion or multi-layer deposition [10, 11].

Reactive plasmas are often created in flowing gases (to ensure a fast removal of the plasma-surface reaction products), in electrodeless discharges, such as inductively coupled radiofrequency (RF) discharges and microwave discharges (MDs) [12]. A MW plasma can be generated at pressures from 10^{-3} Pa up to atmospheric pressure in pulse and continuum wave regimes, at incident powers ranging between several watts and hundreds of kW. Nowadays, MDs are widely used for the generation of quasi-equilibrium and non-equilibrium plasmas for different applications, such as generation of an active medium in gas discharge lasers, light sources, in plasma chemistry, analytical chemistry etc [13].

Use of a MW plasma offers some advantages. It typically takes place between ambient temperature and 50 °C. This phenomenon is due to the fact that, despite a low gas temperature, high electron temperatures are present, due to the increased path length [14]. This allows processing of very heat-sensitive plastics, such as balloon catheters or heat shrink tubing. The type of functionalization imparted can be varied by the selection of plasma gas (Ar, N_2, O_2, H_2O, CO_2, NH_3) and by the operating parameters (pressure, power, time, gas flow rate) [15]. Since treatments occur in vacuum in a precisely controlled environment, the results are reproducible.

Immobilization of proteins onto polymer surfaces is of considerable interest for numerous applications, particularly in developing medical implant materials [16], bioseparators [17], biosensors [18], etc. Therefore, much effort has been made to develop methods of protein immobilization [19]. One of the most interesting ways is the modification of polymers by grafting techniques, with subsequent physical or covalent immobilization of proteins [20]. Methods for the immobilization of biological compounds include adsorption, cross-linking, covalent bonding, entrapment and encapsulation. The most common methods applied for the immobilization of proteins are adsorption and covalent bonding [21].

A gas discharge in CO_2 is characterized by an intense vacuum-ultraviolet irradiation, producing radicals in the surface layer of PTFE [22], which can effectively react with the active oxygen species during plasma treatment. This could be explained by the rapid dissociation of the CO_2 molecule to neutral oxygen atoms, which are fairly stable in glass discharge tubes and readily react with the surface of polymer materials and also with CO radicals [23].

Functionalization of PTFE for human thrombomodulin binding has been achieved by CO_2 plasma activation and subsequent vapour phase graft polymerization of acrylic acid [24]. In our previous papers, surface modification of high-density polyethylene by a CO_2 MW plasma with the aim of fixing carboxylic groups is described. Formation of carboxylic acids seems favoured mainly by the presence of the CO_2 active species [24, 25]. To minimize the degradation effect on the biomaterial surface (since the formation of a degraded layer gives a weaker bond layer, which may induce protein activation), the atomic species such as oxygen atoms [26] in the plasma should be eliminated. CO_2 is less aggressive than oxygen and safer under working conditions.

Some biomolecules were immobilized onto plasma-pretreated polyvinylidene fluoride (PVDF) surfaces using different methods. Lin *et al* [27] modified the surface of the PVDF membranes in two steps. First, poly(acrylic acid) (PAA) was grafted on PVDF membranes with various surface porosities by plasma-induced polymerization. Then, heparin was covalently bonded to PAA with the aid of 1-ethyl-3 (3-dimethylaminopropyl)-carbodiimide hydrochloride) (EDAC). Young *et al* [28] modified the PVDF surface by immobilization of L-lysine and 1,6-hexanediamine (HMDA). The membrane was first grafted with poly(glycidyl methacrylate) (PGMA), by means of plasma-induced free radical polymerization. Then, L-lysine or HMDA was reacted with the epoxy groups in the grafted PGMA.

In this work, PVDF was proposed as a support for protein immobilization. The high hydrophobicity of PVDF restrained it from promotion and application. To make the PVDF film hydrophilic, many studies focused on plasma treatment [29]. The surface activation technique was aimed at creating functional groups capable of preferential adsorption of the biologically active species (proteins, enzymes, cells, drugs, etc). To this end, PVDF was subjected to successive surface modifications by MW plasma activation (pretreatment) in different atmospheres (CO_2, N_2 and N_2/H_2), followed by coating with proteins by both direct adsorption and grafting.

The objectives of this study were to optimize the discharge parameters for surface functionalization using a CO_2 MW

plasma, and the immobilization procedures of some proteins on the treated surfaces. Study of protein immobilization on a surface functionalized by a CO_2 MW plasma was performed comparatively with the previously reported PVDF surface functionalization by N_2 and N_2/H_2 MW plasmas [30], to evidence the importance of the type of groups implanted on the surface for the protein coating stage. Characterization of the modified surfaces with respect to the unmodified one was done by different investigation methods, such as contact angle measurements, x-ray photoelectron spectroscopy (XPS), attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) and AFM. Triglycine and protein A have been selected for the study. The first one is a model protein with a short linear chain of three molecules of the simplest amino acid glycine with two functionalities (amino and carboxyl) [31], while protein A is originally found in the cell wall of the *Staphylococcus aureus* bacteria. Protein A is able to selectively bind IgG, antigen-bound IgG and IgM complexes, such as rheumatoid factors and circulating immune complexes [32].

2. Experimental

2.1. Materials

Polyvinylidene fluoride films (PVDF) (0.25 mm thick, purchased from Goodfellow, UK). As already mentioned, a non-piezo-PVDF was used for setting up the surface modification protocol, since it has already been demonstrated that the piezoelectric properties are not altered after treatment under optimal conditions [33].

Protein A (Sigma Chem) is a 42 kDa MSCRAMM and pI 5.3. Protein A is a single polypeptidic chain containing four repetitive domains rich in aspartic and glutamic acids free of cysteine [34].

Triglycine (glycil-glycil-glycine), obtained as a powder sample from Sigma Aldrich, has the molecular formula $C_6H_{11}N_2O_4$ and purity >99%.

2.2. Surface treatments

2.2.1. Plasma activation. Films of PVDF were treated in a MW plasma, using the experimental set-up presented in figure 1, and different discharge gases (i.e. CO_2, N_2 and N_2/H_2 1 : 3, purchased from Air Liquide France—Alphagaz; purity of N_2, CO_2 and H_2 was 99.999%).

Plasma excitation is provided by a MW generator (SAIREM, 433 MHz), coupled to a resonant cavity (surfatron). A cylindrical MW plasma column is generated using this surfatron. The incident power (Pi) and the reflected power (Pr) are measured with a power meter (Hewlett–Packard no 435B). Impedance is adjusted until the reflected power is very low (Pr: 0.02 W). The glow is generated at the top of the reactor. The pumping system is composed of primary (CIT Alcatel no 2012) and oil diffusion (CIT Alcatel Crystal) pumps. A MKS mass flow meter (type 1259B) controlled the volume flow rate (Q). For a volume flow rate of 16×10^{-8} m^3 s^{-1}, the pressure is about 20–30 Pa. The reactor is a quartz cylinder 500 mm in length and 76 mm in diameter. The reactor is set up on a chamber

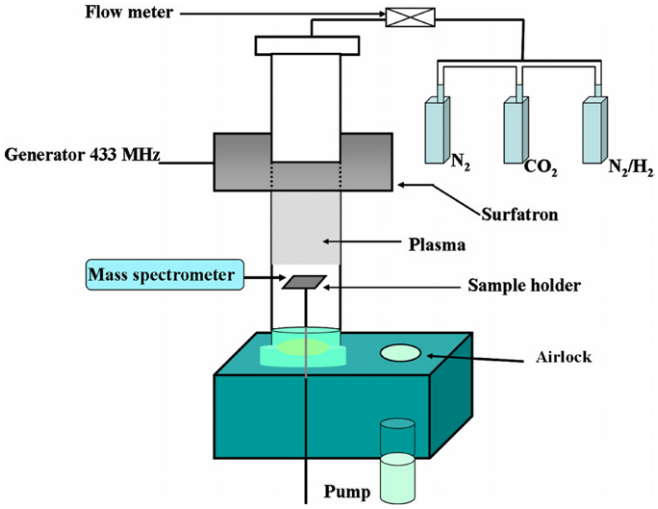


Figure 1. Experimental set-up for microwave plasma treatment of the PVDF surface.

used for sample introduction. The substrate could be moved in or out of the plasma volume, to vary the distance (d in m) between the bottom of the excitation source and the sample. The sample holder allows us to locate the sample either in direct contact with a 2.45 GHz MW plasma, or downstream, where only the long-lived species (largely neutrals) from the plasma effluent contribute to the process chemistry.

The following parameters are varied: exposure time (t, 5–60 s), discharge power (P, 10–70 W), volume flow rate (Q, 8×10^{-8}–50×10^{-8} m^3 s^{-1}), pressure (20–30 Pa), position of the samples with respect to the surfatron (d, 2.5×10^{-2}–15×10^{-2} m), allowing us to expose the samples both in discharge and post-discharge.

The optimal CO_2 plasma parameters will be established here, while those with N_2 or N_2/H_2 plasmas were previously determined [30]. In all cases, the composition of residual atmosphere, proved by optical emission spectroscopy (OES), was essentially constituted of water vapour and nitrogen, final pressure being 10^{-4} Pa.

2.2.2. Physisorption of protein A and triglycine. After rinsing with ethanol, the PVDF film was plasma-treated, after which a protein solution (10 μl of $c = 2.5$ mg ml^{-1}) made of protein A or triglycine was spread over the entire surface and stored at 4 °C overnight (for at least 15 h). The excess protein was removed by rinsing with phosphate-buffered saline (PBS).

2.2.3. Grafting of protein A and triglycine. The untreated and plasma-exposed surfaces were treated with 75mM EDC (1-ethyl-3-(-dimethylaminopropyl) carbodiimide) + 15mM NHS (*N*-hydroxysuccinimide) [35, 36] and protein (10 μl of $c = 2.5$ mg ml^{-1}) for 1 h, to convert the terminal carboxylic groups by generation of a stable acyl amino ester intermediate. After condensation of proteins and aminolysis of NHS, the adduct was formed. The excess of protein was removed by rinsing with PBS (pH 7.4). Prior to analysis, all films were stored at 4 °C.

2.3. Methods

2.3.1. Contact angle measurements. The contact angles for the polymer films were determined by the *sessile drop method,* at room temperature and controlled humidity, within 30 s after placing 1 μl drops of liquids on the film surface [37, 38], using a CAM-200 instrument from KSV, Finland. The contact angles between three different pure liquids (one non-polar: diiodomethane, and two polar: twice-distilled water and formamide) and the polymer surface were determined. For obtaining the components of the free surface energy from contact angle measurements, the Young–Good–Girifalco–Fowkes theory was used and the Young complete equation was applied [39]:

$$\gamma_L(1+\cos\theta) = 2[(\gamma_S^{LW}\gamma_L^{LW})^{1/2} + (\gamma_S^{+}\gamma_L^{-})^{1/2} + (\gamma_S^{-}\gamma_L^{+})^{1/2}] \quad (1)$$

where θ is the contact angle between the solid surface and the used liquid, γ^{LW} the Lifshitz–van der Waals component of the free surface energy, γ^{+} and γ^{-} the electron acceptor and the electron donor components, respectively, of the free surface energy; S and L stand for the solid surface and the liquid used, respectively.

The adhesion work (W_a), a thermodynamic parameter relevant for the adsorption characteristics of the surface, controlling all interfacial events, was calculated using the Young–Dupré equation [38]:

$$W_a = \gamma_{LV}(1+\cos\theta) \quad (2)$$

where θ is the contact angle and γ_{LV} is the surface tension of the liquid used for measurements. The subscript LV denotes the interfacial liquid–vapour tension.

The interfacial tension between blood and the film surface was calculated using the following equation [37, 38]:

$$\gamma_{SL} = [(\gamma_L^{p})^{1/2} - (\gamma_S^{p})^{1/2}]^2 + [(\gamma_L^{d})^{1/2} - (\gamma_S^{d})^{1/2}]^2 \quad (3)$$

where γ^{p} and γ^{d} are the polar and the dispersive components of the free surface energy, respectively; L and S stand for the liquid and the solid, respectively.

2.3.2. ATR-FTIR. The ATR-FTIR spectra of the films were recorded at 4 cm^{-1} resolution, on a DIGILAB Scimitar Series FTIR spectrometer (USA), by the ATR technique, with a 45° ZnSe crystal. Penetration thickness was about 100 μm. For each sample, evaluations were made on the average spectrum obtained from three recordings. Spectra processing was done by a Grams/32 program (Galactic Industry Corp.).

2.3.3. X-ray photoelectron spectroscopy. XPS measurements were performed on a Kratos Axis Ultra spectrometer (Kratos Analytical Ltd, UK), using a monochromatic Al Kα x-ray excitation source (λ = 1486.6 eV), at 300 W. During each measurement, the pressure in the analysis chamber was kept at 4×10^{-6} Pa or lower. The polymer films were mounted on standard sample stubs by means of a double-sided adhesive tape, and a flood gun was used for charge compensation. Measurements were taken at a take-off angle of 15° with respect to the sample surface. Survey spectra for each sample over a binding energy range 0–1150 eV were the average of three scans acquired at a pass energy of 160 eV and resolution of 1 eV/step (lens in hybrid mode, which ensures maximum sensitivity). High-resolution spectra of C 1s, F 1s, N 1s and O 1s were the average of five scans acquired with a constant pass energy of 20 eV and 0.05 eV/step resolution. Quantitative analysis of the spectral data (surface chemical compositions, expressed as relative atomic percentage concentration (at%)) was obtained from the peak-area ratios corrected with the experimentally determined sensitivity coefficients for the most intense spectral line for each elemental species. The estimated uncertainty is ±1% for C and F, and ±2% for N and O. The CasaXPS software was used for background subtraction (Shirley-type), peak integration, fitting and quantitative chemical analysis. All binding energies were referenced to the C 1s (C–C) peak at 285 eV. The resolution for measurements of binding energy is about 0.2 eV.

The high-resolution spectra were curve-fitted using a mixed Gaussian–Lorentzian (70 : 30) function to input the required component contributions. Data from three replicates of each sample type were recorded and at least three separate areas on each individual sample were analysed.

2.3.4. Atomic force microscopy. AFM analysis was done by means of a Solver-Pro-M type instrument (NT-MDT company) using standard tips of Si_3N_4 with a small curvature radius of 10 nm. AFM images were obtained in the tapping (non-contact) mode, which is non-destructive for the surface, so that the biological layer is not damaged. Roughness of the PVDF surfaces was verified by statistical AFM estimations. Various ranges of the surface, of $40 \times 40\,\mu m^2$ and $5 \times 5\,\mu m^2$, were scanned. Special attention was devoted to phase detection. This piece of information is complementary to the topographic images, reflecting changes in the surface adhesion properties.

2.3.5. Immunofluorescence tests. The principle of the method is based on a precipitation reaction which can also occur in cells. By applying a solution of antibodies on a microscopic material, the antibodies will react with the corresponding antigens from the cells, leading to the formation of a microprecipitate. Following the position of the microprecipitate, conclusions on the intra- or inter-cellular distribution of the antigen can be drawn. If a fluorescent substance is attached on the molecules of the antibodies, then the microprecipitate will manifest a characteristic fluorescence in ultraviolet light. Following the distribution of fluorescence in the cells, localization of the studied antigen can be established. Direct and indirect procedures can be applied [40].

The *indirect procedure*, also named *the technique of the multiple layers*, consists of two stages. In the first stage, the microscopic material is treated with the unlabelled specific antibody. The formed immunoprecipitate is made visible in the second stage, by adding a fluorescent antibody, prepared for the unlabelled specific antibody [41].

On the surface of the plasma-pretreated polymers, coated/grafted with different proteins, 5 μl of polyvalent serum anti *Escherichia coli* 0.1 mg ml^{-1} in PBS (pH = 7.2) were

dropped. The samples were incubated, for 6 h, at 4 °C, in humid atmosphere, and then washed with PBS. After adding 5 μl of fluorescent treponemal antibody absorption (FTA-ABS) antibody tracers and incubating for half an hour, the samples were again washed with PBS. For blocking the non-specific positions on the surface, 5 μl of BSA (bovine serum albumin) were added on each sample (concentration 5 mg ml^{-1} in PBS), after which the samples were washed with PBS and distilled water, and kept in the freezer, in humid atmosphere, in order to preserve the antibodies. Microscopic examination was done using an Olympus fluorescence microscope. Adding BSA in PBS on the polymers blocked the non-specific sites, fluorescence appearing due to the binding of antigen on the antibodies marked with fluorochrome [35].

As generally known, the criteria for judging a fluorochrome as a suitable dye are as follows [42]:

- The fluorochrome should possess chemical groups which will form covalent bonds with protein molecules.
- Easy removal of the unreacted fluorescent material is also important.
- The fluorescent colour of the conjugate should be different from that of the background.
- The conjugate should be stable under storage conditions.

The fluorescence emission of FITC (fluorescein isothiocyanate) conjugates is green, with the maximum wavelength at 529 nm. The fluorescence of microscopical preparations is subject to fading during illumination, and there may be a colour change. There should be minimum exposure to illumination during microscopic examination. All these conditions are fulfilled during the test, so that the obtained results are reproducible and trustful [43].

3. Results and discussion

3.1. CO_2 plasma treatment and surface characterization

Starting from the literature results [24, 25], the non-piezoelectric PVDF films are treated in a CO_2 MW plasma in order to create acidic groups on the surface. The plasma conditions, such as plasma power, the distance between the sample and the surfatron, treatment time and volume flow rate (Q), are varied for establishing the treatment parameters which constitute a good compromise between optimum functionalization and minimum degradation (figures 2(a)–(f)).

After the plasma treatment, the hydrophilic character of the PVDF samples significantly increases under all applied conditions, a behaviour proved by the decrease in the contact angle with water with respect to the untreated film (figures 2(a) and (b)); this was observed both for the distance between the sample and the surfatron (figure 2(a)), and for the treatment time (figure 2(b)), showing minimum values at 0.1 m distance and 30 s treatment time.

Improvement of this characteristic is necessary for applications in which adhesion with other macromolecules or metals is important [44].

After plasma exposure, the acid–base component of the free surface energy significantly increases when compared with the pristine sample, for all used discharge parameters. As polymer-surface functionalization under plasma exposure is a very rapid phenomenon that generally occurs within less than 1 min [45], the acid–base component of the free surface energy significantly increases during the first 5 s, after which its value remains almost constant for the entire period of plasma exposure (figure 2(c)). This indicates that the concentration of polar groups within the depth of the contact angle measurement (∼0.5 nm) reached the saturation level within 30 s of plasma treatment time, as also evidenced by other authors [46–48].

For an applied power of 10–50 W, γ_S^{ab} has a maximum value of ∼8–12 mN m^{-1} (figure 2(d)). When compared with the pristine sample, the increase in the acid–base component of the free surface energy under CO_2 plasma treatment evidences the incorporation of polar functions (oxygenated species), which agrees with the literature data [25, 30]. After plasma treatment, the atomic concentration of oxygen increased dramatically and small amounts of incorporated nitrogen were also observed [49]. Klomp *et al* showed that titration data revealed significantly higher surface concentrations of the carboxylic groups after CO_2 glow discharge [50].

The biocompatibility characteristics are also improved by plasma treatment, the interfacial tension with blood and tissues drastically decreasing from 27 mN m^{-1}, for the untreated PVDF, to values tending towards the biocompatibility zone (below 9 mN m^{-1}) (figure 2(e)).

In the meantime, the adhesion work changes from 72 mN m^{-1}, for the untreated sample, to a value around 100 mN m^{-1} (figure 2(f)) for all used discharge conditions, evidencing the obtainment of surfaces with improved adhesion properties. This behaviour could be a sign of a possible better adherence between the plasma-treated PVDF substrate and other materials.

Using UV spectroscopy (the absorbance of the solution was measured at 562 nm by UV–VIS spectroscopy), the amount of adsorbed protein was determined by comparison of the absorbance of the samples with a calibration curve. Three repetitions were performed for all samples. In the meantime, high-precision weighing of the samples was done. It has been appreciated that, after plasma treatment and subsequent air exposure, a decrease in weight of about 0.6–0.8 μg cm^{-2} was observed in all three cases.

Based on the results of contact angle analysis, and also on weight loss determinations for the PVDF samples exposed to MW plasma, using CO_2 as a discharge gas at different discharge parameters (figure 2), one may conclude that the optimal conditions which allow improvement of the surface properties of the polymer films (which are close to saturation, with minimum degradation) are as follows:

(I) Plasma CO_2: $Q = 16 \times 10^{-8}$ m^3 s^{-1}, $P = 50$ W, $t = 30$ s, $d = 0.1$ m, while for N_2 and N_2/H_2 plasmas, according to previous results [30], the optimal parameters of discharge are as follows:

(II) Plasma N_2: $Q = 16 \times 10^{-8}$ m^3 s^{-1}, $P = 50$ W, $t = 60$ s, $d = 0.1$ m;

(III) Plasma N_2/H_2 in the ratio 25/75: $Q = 16 \times 10^{-8}$ m^3 s^{-1}, $P = 50$ W, $t = 60$ s, $d = 0.1$ m.

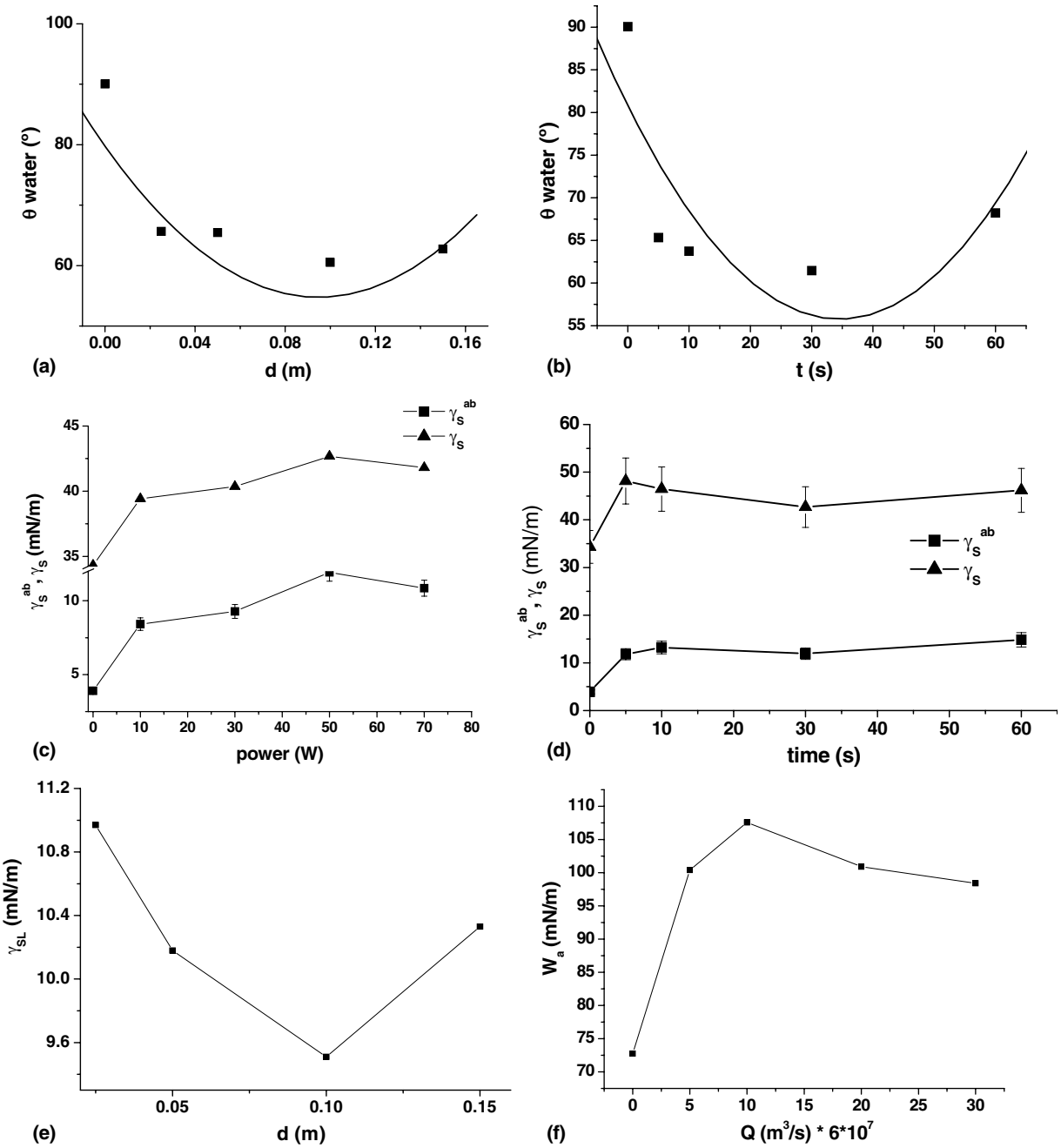


Figure 2. Surface properties of the PVDF film, obtained after CO_2 plasma exposure for different discharge parameters. Water contact angle versus the distance between the sample and the surfatron (*a*) (for constant values of $Q = 10$ sccm, $P = 50$ W, $t = 30$ s) and the exposure time (*b*) (for constant values of $Q = 10$ sccm, $P = 50$ W, $d = 10$ cm); the acid–base component and the free surface energy versus the discharge power (*c*) (for constant values of $Q = 10$ sccm, $d = 10$ cm, $t = 30$ s) and the treatment time (*d*) (for constant values of $Q = 10$ sccm, $P = 50$ W, $d = 10$ cm); the interfacial tension between blood and the surface versus the distance between the sample and the surfatron (*e*) (for constant values of $Q = 10$ sccm, $P = 50$ W, $t = 30$ s); the adhesion work versus the gas flow (*f*) (for constant values of $P = 50$ W, $t = 30$ s, $d = 10$ cm).

The surface chemical environment and the atomic concentrations (%) present on the surface of the pristine PVDF films, plasma-pretreated and modified with proteins, were obtained using XPS. The XPS survey spectra of untreated and plasma-treated/modified samples are shown in figure 3, and associated data are given in table 1.

A comparison between the survey spectra presented in figure 3 reveals that C and F are the predominant species (usually found on virgin PVDF surface) and, as expected, new characteristic O and N emission peaks appeared in the modified PVDF samples spectra, due to plasma and/or subsequent treatment. An analysis of the data presented in table 1 shows some increase in the percentage of carbon, a drastic decrease in fluorine percentage, as well as an important increase in the percentage of oxygen and nitrogen atoms for each treated sample, comparatively with the reference sample (PVDF). This situation suggests that the F content at the sample surface was reduced after this treatment, probably as a result of fluorine loss by chain breaking in the polymer, and of the chemical reorganization induced by the electrons

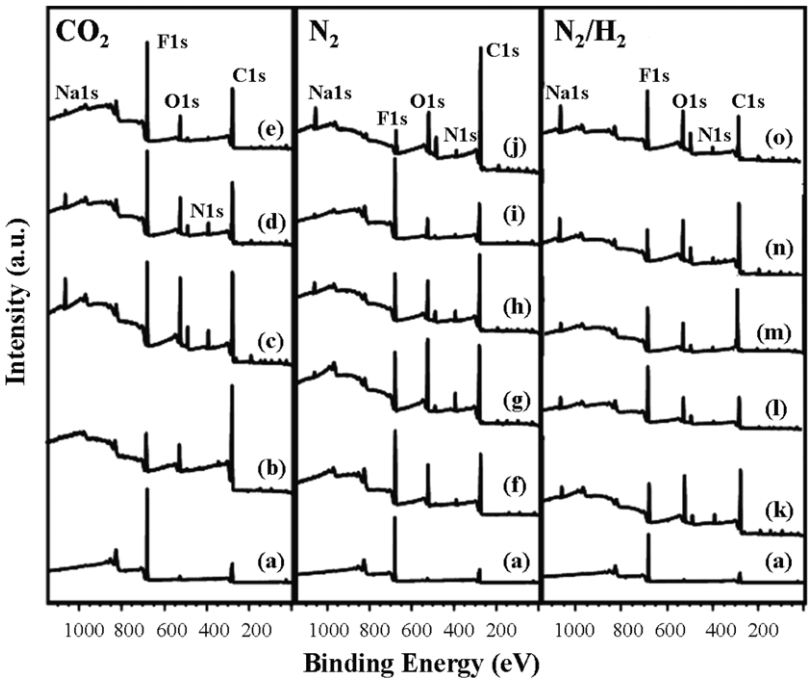


Figure 3. XPS survey spectra of (a) pristine PVDF film, (b) CO_2 plasma treated, (c) CO_2 plasma treated and coated with protein A, (d) CO_2 plasma treated and grafted with protein A, (e) CO_2 plasma treated and coated with triglycine, (f) N_2 plasma treated, (g) N_2 plasma treated and coated with protein A, (h) N_2 plasma treated and grafted with protein A, (i) N_2 plasma treated and coated with triglycine, (j) N_2 plasma treated and grafted with triglycine, (k) N_2/H_2 plasma treated, (l) N_2/H_2 plasma treated and coated with protein A, (m) N_2/H_2 plasma treated and grafted with protein A, (n) N_2/H_2 plasma treated and coated with triglycine, (o) N_2/H_2 plasma treated and grafted with triglycine.

Table 1. Experimental atomic composition (%) and different atomic ratios obtained by XPS analysis for CO_2 plasma-activated and protein-modified PVDF surfaces.

Sample	% C	% F	% O	% N	O/C	O/F	N/F	N/C
PVDF	53.8	43.2	2.4	0.2	0.045	0.06	0.005	0.003
(A) CO_2 plasma								
PVDF/CO_2 plasma	81.3	8.8	7.5	0.4	0.093	0.85	0.05	0.005
PVDF/CO_2 plasma/coated protein A	62.9	11.7	13.8	5.6	0.219	1.18	0.50	0.089
PVDF/CO_2 plasma/grafted protein A	63.8	16.3	10.9	5.2	0.171	0.67	0.32	0.081
PVDF/CO_2 plasma/coated triglycine	68.1	11.1	7.8	1.0	0.163	0.70	0.090	0.015
(B) N_2 plasma								
PVDF/N_2 plasma	66.1	16.6	5.62	2.3	0.085	0.34	0.14	0.03
PVDF/N_2 plasma/coated protein A	63.7	9.4	16.7	6.2	0.262	1.78	0.66	0.097
PVDF/N_2 plasma/grafted protein A	70.6	9.5	11.3	4.4	0.160	1.19	0.46	0.06
PVDF/N_2 plasma/coated triglycine	48.7	7.19	6.04	2.9	0.124	0.84	0.403	0.06
PVDF/N_2 plasma/grafted triglycine	57.5	7.93	7.88	4.6	0.137	0.994	0.58	0.08
(C) N_2/H_2 plasma								
PVDF/N_2/H_2 plasma	69.8	21.55	9.05	1.25	0.129	0.42	0.058	0.0179
PVDF/N_2/H_2 plasma/coated protein A	57.6	20.1	14.2	1.7	0.247	0.71	0.084	0.0295
PVDF/N_2/H_2 plasma/grafted protein A	45.2	7.82	8.84	1.9	0.195	1.13	0.243	0.042
PVDF/N_2/H_2 plasma/coated triglycine	67.0	8.7	13.0	2.7	0.194	1.49	0.310	0.040
PVDF/N_2/H_2 plasma/grafted triglycine	56.4	7.2	15.1	3.6	0.267	2.09	0.5	0.064

generated in the plasma. Plasma exposure led to weight loss and changes in the chemical composition on the polymer surface. Selective surface modification of fluoropolymers introduces various functional groups, without altering the bulk properties. The results may be summarized as follows: the remote hydrogen plasma ensured the most effective abstraction of fluorine [51]. The loss of surface fluoride was attributed by Ryan and Badyal [52] to vacuum ultraviolet photons, ion-beam treatment or electron impact dissociation. Charbonnier *et al* used XPS to assess the effects of RF plasma treatments on PTFE substrates in various gaseous atmospheres [53]. In all cases, short treatment times (<1 min) were efficient, a long time creating a certain surface roughness. N_2 plasma provokes moderate defluorination, associated with oxygen and nitrogen uptake. N_2 originates from plasma treatment itself and also, very likely, from the post-reaction with the venting gas. The oxygen atoms are both in the form of alcohol (C–OH) or/and carbonyl (C=O) groups and of epoxy radicals, while the nitrogen atoms appear as amino (C–NH_2) groups.

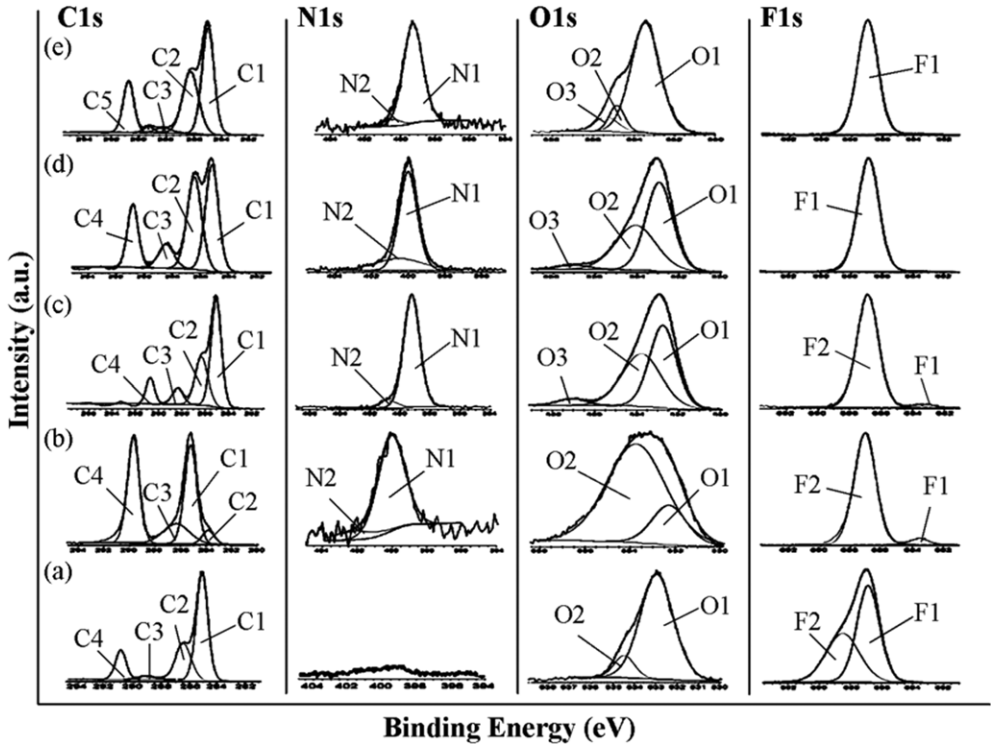


Figure 4. Fitted C 1s, N 1s, O 1s and F 1s envelopes for (*a*) pristine PVDF film, (*b*) CO_2 plasma treated, (*c*) CO_2 plasma treated and coated with protein A, (*d*) CO_2 plasma treated and grafted with protein A, (*e*) CO_2 plasma treated and coated with triglycine.

Subsequent exposure of the plasma-treated surface to air causes oxygen incorporation onto the PVDF surface, leading to surface oxidation and formation of peroxide and hydroperoxide species [54]. These new species formed on the CO_2 plasma-pretreated PVDF surfaces (after air exposure) can serve as active sites for the subsequent adsorption/grafting of proteins (see below).

As the surface of all treated samples is enriched in oxygen-containing functionalities, one may safely assess that hydrophilicity of the surface films is improved by CO_2 plasma treatment, in accordance with contact angle measurement results. The CO_2 plasma technology provides superior surface treatment, to improve adhesion on a wide variety of materials. The combination of CO_2 with plasma discharges significantly increases the polar component of the free surface energy. The CO_2 plasma produces ablation of the outermost oxidized layer of the polymer surface and noticeably increases the adhesive bond strength, because of the different chemical and morphological surface modifications produced by the plasma. Formation of carboxyl groups is one of the main effects of PVDF film exposure to the CO_2 plasma [24, 55].

Figure 4 provides the high-resolution C 1s, N 1s, O 1s and F 1s XPS spectra for untreated and modified PVDF samples; also, variation of the corresponding areas—table 2—was considered to evidence differences between samples.

The PVDF C1s spectrum (figure 4(*a*)) can be curve-fitted with four peak components, from the chemically non-equivalent carbon atoms: two major peaks (noted C1 and C4) at 286.38 eV and 290.97 eV are related to CH_2 and CF_2 species, respectively, and two smaller peaks (C2 and C3) at 285.07 eV (attributed to C–C/C–H) and 288.9 eV (characteristic of the C–O bond) [56, 57].

The C2 and C3 peaks detected for the virgin PVDF sample, in addition to the expected peaks for the CH_2 and CF_2 species, suggest that the PVDF film exhibits low oxidation, possibly arising during sample storage. When comparing the spectrum of untreated PVDF with the spectra of CO_2, N_2 and N_2/H_2, plasma-treated and coated/grafted with protein A or triglycine modified samples, some important differences appear. The shape of the C1s peaks changes after treatments, indicating that new groups are implanted. Basically, the C 1s core-level spectra of the modified samples are similar (in broadness, with only small position shifts) with the spectrum of the reference sample, except for the reorganization of the C2 peak and the ratio of peak intensities. At a binding energy around 288.4 eV, the C3 peak could be attributed to a carbon surrounded by fluorine and nitrogen [58]. The most considerable changes were observed for the CO_2 plasma-treated and coated/grafted with protein A samples. A quick decrease in the percentage of the C4 peak (assigned to the CF_2 component; around 290.0 eV), accompanied by an increase in the percentage of C2 (typical of oxygenated or nitrogen species; between 286.24 and 286.44 eV) for each modified PVDF sample, comparatively with the reference sample, is evident. The XPS C 1s core-level spectra for protein-coated surfaces were deconvoluted in 4 or 5 peaks: 284.6 and 285.4 eV (C–C and C–H), 286.3 and 286.5 eV (C with amine), 288.1 eV (C with O, carbonyl or amide bond) and 298.3 eV (C with carboxylic acid) [59]. The surface coated with proteins displayed a significant increase in the C–N and amide group. Variation of O 1s and N 1s with respect to the C 1s signal ratios was correlated with the

Table 2. Area (at%) of C 1s peaks for untreated and modified PVDF samples.

	Area (at%)													
	Carbon					Nitrogen			Oxygen				Fluorine	
Sample/atom type	C1	C2	C3	C4	C5	N1	N2	N3	O1	O2	O3	O4	F1	F2
PVDF	38.3	5.3	16.5	39.9	—	—	—	—	19.2	80.8	—	—	57.6	42.4
PVDF/CO_2 plasma	57.1	25.9	2.9	14.1	—	87.7	12.3	—	88.5	11.5	—	—	4.6	95.4
PVDF/CO_2 plasma/coated protein A	52.7	28.7	8.2	10.3	—	93.1	6.9	—	49.6	46.3	4.1	—	57.6	42.4
PVDF/CO_2 plasma/grafted protein A	37.0	35.4	9.6	18.0	—	76.8	23.2	—	54.8	41.9	3.3	—	4.6	95.4
PVDF/CO_2 plasma/coated TG	43.4	33.1	2.8	2.2	18.5	94.1	5.9	—	80.9	10.4	8.7	—	100.0	—
PVDF/N_2 plasma	42.8	34.4	6.3	7.7	8.8	100.0	—	—	46.5	53.5	—	—	100.0	—
PVDF/N_2 plasma/coated protein A	55.2	6.8	—	38.0		67.0	33.0	—	80.4	19.6	—	—	100.0	—
PVDF/N_2 plasma/grafted protein A	57.9	28.2	4.0	2.5	7.4	85.3	2.4	12.3	93.0	7.0	—	—	100.0	—
PVDF/N_2 plasma/coated TG	35.1	30.6	16.0	18.4	—	94.7	5.3	—	81.5	18.5	—	—	100.0	—
PVDF/N_2 plasma grafted triglycine	72.2	22.2	0.9	3.1	1.6	89.6	3.3	7.1	22.3	59.6	11.1	7.0	17.4	82.6
PVDF/N_2/H_2 plasma	33.5	37.1	13.6	15.8	—	100.0	—	—	75.1	24.9	—	—	100.0	—
PVDF/N_2/H_2 plasma/coated protein A	50.0	32.2	9.9	7.9	—	100	—	—	54.7	43.9	1.4	—		
PVDF/N_2/H_2 plasma/grafted protein A	62.3	28.8	4.9	2.8	1.2	100.0	—	—	92.0	8.0	—	—	4.4	95.6
PVDF/N_2/H_2 plasma/coated TG	60.1	24.9	4.4	9.9	0.7	100.0	—	—	93.7	6.3	—	—	100.0	—
PVDF/N_2/H_2 plasma/grafted TG	46.8	35.1	11.4	5.4	1.3	100	—	—	75.2	17.4	7.4	—	6.9	93.1

significant presence of chemical species on the coated/grafted surfaces. For the untreated sample surface, the O 1s core-level spectrum displays a broad peak that can be curve-fitted with two peak components, with binding energies at 532.27 and at 533.73 eV—figure 4. After CO_2 plasma treatment, these signals are shifted to slightly higher binding energies. From the obtained XPS results, some qualitative comparisons can be drawn: (a) change in the shape of the O 1s photoelectron peak and a relative intensity of curve-fitted peak components during CO_2 plasma treatment and coating/grafting protein process; (b) emerging of the O3 component in the spectra shown in figures 4(*c*), (*d*) and (*e*) (O 1s spectra) is a reasonable consequence of the grafting or coating process.

In the case of an untreated film (figure 4, N 1s spectrum), no N component was detected on the sample surface, which is consistent with the absence of nitrogen in the PVDF structure. For all CO_2 plasma-treated samples, an asymmetric signal, with a main peak (N1, at 400.0 eV), attributed to the amino tail groups, and a second one (N2, at 401 or 402 eV), due to the positively charged amine, were detected. Peak N1 represents 76.8–94.1% (table 2) of the whole N 1s signal and suggests that the free NH_2 groups are the major form of nitrogen on the modified surface. The presence of this signal reinforces the validity of the C 1s signal deconvolution, where a C3 component appears after proteins' coating or grafting process.

High-resolution scans of the XPS spectra of F 1s levels for the untreated PVDF film show two peaks at 686.85 eV (57.6%), assigned to the F–C–F groups (F1), and at 688.48 eV (42.4%), due to the C–F groups (F2). The presence of these signals indicates the existence of more than one fluorine compound on the surface of the untreated polymer and suggests a significant conversion (57.6%) of the F–C–F to C–F groups. After plasma treatment, F2 is dramatically reduced. This behaviour, as well as the fluorine concentration, still significant in the modified samples (table 1), reinforces the idea that the loss of fluorine during all kinds of plasma and grafting treatments is accompanied by a drastic decrease in the F–C–F groups, which is compensated by an increase in the C–F groups.

XPS spectra show that the treatment in CO_2 plasma leads not only to the oxidation of PVDF (7.5%), but also to the formation of CF and terminal CF_3 groups. Taking into account that the decrease in the F/C ratio (from 0.80 to 0.108) exceeds the increase in the O/C ratio (from 0.045 to 0.094), one can conclude that the CO_2 plasma treatment leads to the effective formation of double bonds and weak cross-linkings. As illustrated in table 2, the peak area of F1 decreased more than 12 times after CO_2 plasma activation.

The XPS results agree with ATR-FTIR spectra (not shown). The CO_2 plasma treatment led to new absorption peaks with maxima at 1881 cm^{-1} and 1725 cm^{-1}, which can be attributed to COF carboxylic acid fluoride and to –CF=CF– double bonds, respectively. Characteristic bands were observed at 3200 cm^{-1} (OH str.), 1710 cm^{-1} (C=O str.) and 800 cm^{-1} (COOH out-of-plane def.) in the ATR-FTIR spectrum [60].

The AFM image—figure 5—of the PVDF-treated CO_2 plasma (*b*) proves surface modification. Also, surface roughness increased from 0.084 to 0.135 μm.

It can be concluded that the acidic surface was created by the CO_2 plasma treatment, surface roughness being increased. Plasma treatments produce pits on the surface, ablation of the oxidized surface layer and functionalization by implantation of oxygen functionalities, mainly carboxyl groups [25, 55, 61, 62].

3.2. Protein covering of the surfaces

Two techniques can be used to simplify the process with strong adhesion of the activated layer to substrates for covalently binding the proteins: plasma modification of polymers (if the substrates are polymers) and plasma deposition of polymer-like materials onto any solid surface, by adding polymer precursors into the plasma [63]. If proteins are immobilized on surfaces with non-specific and uncontrolled weak interactions, they are susceptible to detach quickly with time, within a few hours or days, which may result in a loose contact between

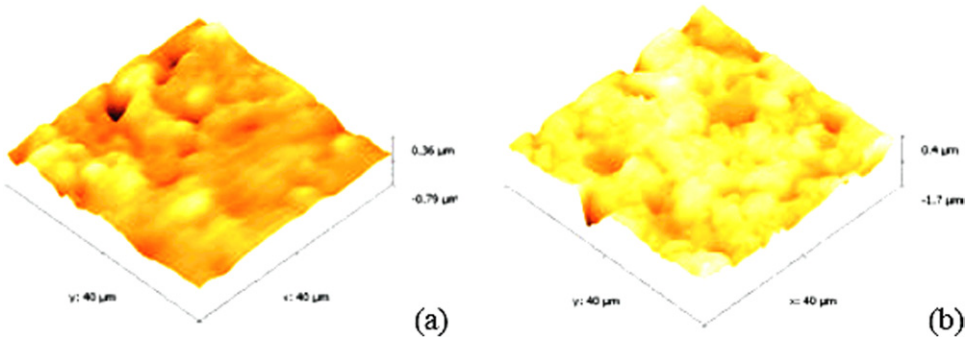


Figure 5. AFM images of the samples: PVDF untreated (*a*) and pretreated with CO_2 plasma (*b*).

implants and the biological tissues, or in an uncontrollable decrease in biosensors' sensitivity.

Comparing our present data with that previously obtained [25, 30] and also with literature results, we can conclude that the above-mentioned three kinds of active surfaces obtained under optimal conditions of plasma treatment, underwent covering with proteins, namely acidic surfaces (obtained by CO_2 plasma activation) [61], basic surfaces (obtained by N_2/H_2 plasma activation [64]) and amphoteric surfaces (obtained by N_2 plasma activation [65, 66]), in all cases after exposure to air for the same period of time of about 1 min.

Protein coating was achieved by both physisorption and grafting, according to the procedures described above.

Covering/grafting with proteins leads to an increased film weight, up to an average of 4.8–5.6 $\mu g\,cm^{-2}$ for adsorbed TG; 2.8–3.1 $\mu g\,cm^{-2}$ in the case of grafted TG; 2.5–3.2 $\mu g\,cm^{-2}$ for adsorbed protein A and 1.3–2.8 $\mu g\,cm^{-2}$ for grafted protein A.

Comparing the samples treated in different plasmas, it appears that, in all cases, the contact angle with water decreases with respect to the untreated PVDF—figure 6. It is obvious that, in all cases, the PVDF surface becomes more hydrophilic. The larger decrease observed for N_2/H_2 plasma—figure 6(*a*)—could be explained by the different kinds of implanted functional groups and also by their possible increased number. Favia *et al* found that N_2 or NH_3 plasma treatments give rise to N-containing functionalities [64], such as amine ($–NH_2$), imine (–CH=NH), nitrile (–C≡N) on polymer surfaces, as well as to oxygen-containing groups, such as amide ($–CONH_2$), due to post-discharge atmospheric oxidation. The phenomenon is consistent with the fact that the water contact angle measurement is an even more surface-sensitive technique than XPS. Water contact angle measurements have a surface sensitivity of 1 nm or less in comparison with a probing depth of about 7.5 nm for the C 1s photoelectrons, at a take-off angle of 75° in an organic matrix. Generally, covering with proteins does not significantly change the surface wettability of the PVDF films treated in N_2/H_2 plasma, except for the case when the sample is grafted with TG; for this one, the contact angle with water has a higher value, evidencing that this surface becomes more hydrophobic than the other ones.

The XPS survey spectra of the plasma-treated and protein-covered samples (figure 3) also present some major differences compared with that of the untreated PVDF film as follows.

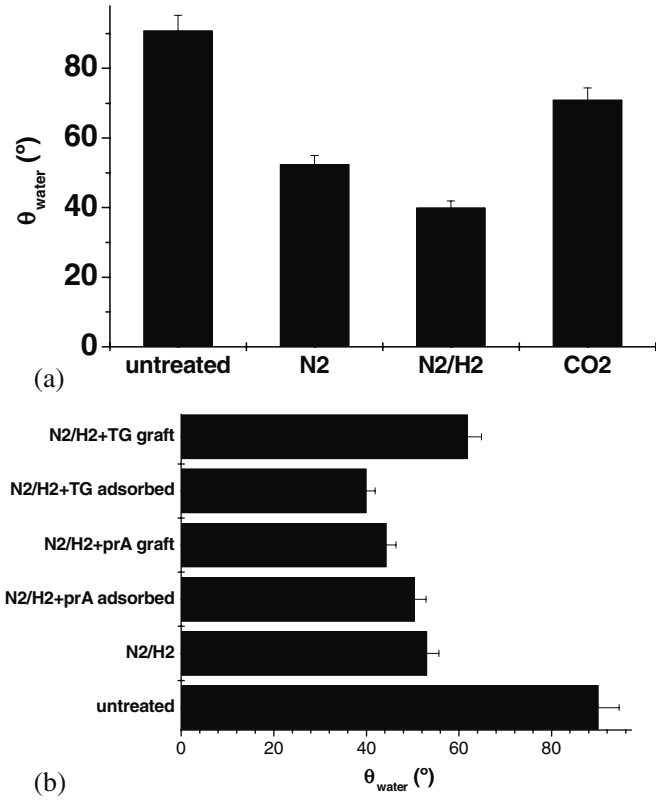


Figure 6. Contact angle of water on the PVDF surface plasma treated with different gases (*a*) and after coating/grafting with proteins (*b*).

(a) The O 1s and N 1s peaks, centred at about 534 eV and 400 eV, respectively, are well-defined and prominent only in the XPS survey spectra of the modified samples, because of the presence of the oxygen- and nitrogen-containing species on the PVDF surface, meaning that an interaction between the proteins used and the polymer surface may have taken place. (b) The C 1s characteristic peaks and their corresponding content (%) (table 2) are higher for all modified samples than for the untreated one. The highest value for carbon content (%) was registered for the PVDF/N_2/H_2 plasma/grafted protein A sample. (c) After each modification step, the F/C atomic ratio subsequently decreased, while the O/C atomic ratio increased. (d) The appearance of N 1s peak is accompanied by a decrease in the F/C atomic ratio (not shown) and an increase in the O/C ratio, after protein covering or grafting, as indicated

by the presence of amino groups on the modified PVDF surface (table 1). (e) Analysis of atomic composition (table 1) revealed a very low concentration of fluorine and a significant concentration for oxygen and nitrogen, indicating a very good anchoring of protein A onto the PVDF surface.

A significant increase in N content may be observed after deposition/grafting of proteins, the highest one being observed when using protein A—table 1. This observation is consistent with the highest value (N/F = 0.5) obtained in the case of the CO_2 plasma-treated sample coated with protein A. The drastic defluorination of the modified PVDF samples and the emergence of new O and N peaks, characteristic of multiple nitrogen or oxygen functions, testify to the presence of proteins on the modified samples' surface. As the surface of all treated samples is enriched in nitrogen- or in oxygen-containing functions, it may be safely assessed that the hydrophilicity of films' surface is improved by plasma treatment.

As expected for plasma treatments, new peaks (noted as C2, C3, C5) emerged in each C 1s spectrum (figure 4) between 285.86 and 290.06 eV (for all treated samples, except the N_2 plasma-treated and coated with protein A polymer sample), as a consequence of PVDF surface oxidation.

The appearance of C2, C3 or C5 peaks indicates the formation of oxygen–carbon (O–C–O, C=O, O=C–O) and nitrogen–carbon bonds (N–C=O, C–NH_2) between the polymer and proteins—table 2.

These new characteristic signals are typical of the C–N, C–N–F, O–C–O and O=C–O components. A large increase in C1 concentration, from about 38.3% (untreated PVDF) to 57.9% (the N_2 plasma-treated and grafted with protein A PVDF sample), and to 72.2% (N_2 plasma-treated and grafted with triglycine) (table 2) is observed, this enrichment being attributed to the two grafted proteins, which contain aliphatic moieties. The XPS data of table 2 show that the content of C1 is slightly increased (42.8%) in the case of the N_2 plasma-treated PVDF sample, and it is only insignificantly (35.1%) or dramatically (6.8%) decreasing for PVDF treated with N_2 plasma and coated with triglycine and protein A. It can be seen that the variation of the C1, C2, C3 and C5 (when present) low binding energy is simultaneously compensated by the significantly reduced intensity of the high-binding-energy C4 component, attributable to the CF_2 groups. As indicated in the literature, even a mild plasma pretreatment of fluorine-based polymers induces a substantial damage of the polymer surface, as a result of defluorination [22]. Consequently, chain mobility and surface permeability of the PVDF polymer are significantly increased, thus possibly or substantially facilitating grafting of the polar segments from proteins.

For all treated samples, in addition to the N1 and N2 main peaks, two other peaks, which can be assigned to the nitrogen involved in oxidized environments (probably between the terminal amino group and oxygen group near the surface), are found.

Peak N1 represents 76.8–94.1% of the whole N 1s signal and suggests that free NH_2 groups are the major form of nitrogen on the modified surface—table 2. The presence of this signal reinforces the validity of signal N 1s deconvolution, where a N3 component appears after proteins' coating or grafting.

A large increase in the nitrogen percentage and a significant increase in the oxygen content are registered for all protein-modified surfaces, reflecting that the two proteins are anchored to the PVDF surface. A comparative analysis shows that protein A is grafted or coated more efficiently than triglycine on the acidic (CO_2 plasma-treated) and basic (N_2/H_2 plasma-treated) PVDF surface.

Peak N 1s is very prominent in the XPS high-resolution spectra of the N_2/H_2-modified samples (while the control PVDF sample spectrum does not show such a characteristic peak). A single strong symmetrical N 1s peak, centred at about 400 eV for the N_2/H_2 plasma-treated sample and at 397.33–397.48 eV for the protein-coated or grafted samples, suggests that the nitrogen-containing groups (neutral amino tail) are successfully anchored on the PVDF chain *via* direct grafting or protein monolayer deposition. As shown in table 2, grafting of triglycine (3.6% N) is more successful than that of protein A (1.9%).

In oxygen core-level spectra, an additional very broad minor component peak at about 3.5 eV in binding energy is detected in the O core-level spectrum of the plasma-treated samples and of the coated or grafted protein surfaces (not shown), which is a reasonable consequence of the grafting or coating process. Change in the shape of the O 1s photoelectron peak and the relative intensity of the curve-fitted peak components occurred after plasma treatment and after coating or grafting with protein.

The occurrence of the O3 component in the spectra is a reasonable consequence of the grafting or coating process. The signal is asymmetrical and has two peaks (except for the N_2 plasma-treated and grafted with triglycine PVDF sample that presents four signals) from chemically inequivalent oxygen atoms. By deconvolution of the two peaks at about 532.5 eV and 534.5 eV, respectively (table 2), the intensity increases at 532.5 eV and decreases at 534.5 eV, indicating that surface chemistry changes after the plasma treatment and coating/grafting with proteins. It is well known that the plasma treatment is a complex process and its exact mechanism is not precisely established. However, it is generally agreed today that a plasma treatment induces surface oxidation and consequently enhances the content of polar species responsible for the increase in hydrophilicity.

Two types of oxygen species are evidenced for the untreated PVDF film, the N_2/H_2 plasma-treated sample, the N_2/H_2 plasma-treated and grafted with protein A sample, while the N_2/H_2 plasma-treated and coated with triglycine samples exhibit three kinds of oxygen atoms: O–C=O, C–O, O–C–O. These results are very satisfactory and are in good agreement with reported data for similar systems.

After plasma treatment and protein coating or grafting, F2 is reduced dramatically (in the case of protein A) or completely (for the TG protein). This behaviour and also the fluorine concentration, still significant in the modified samples (tables 1 and 2), reinforce the idea that loss of fluorine takes place during plasma and grafting treatments and that, in some cases, coating with proteins is not uniform. The loss of fluorine is related to the plasma treatment, this effect being more pronounced for the PVDF samples activated with the CO_2 and N_2/H_2 plasmas,

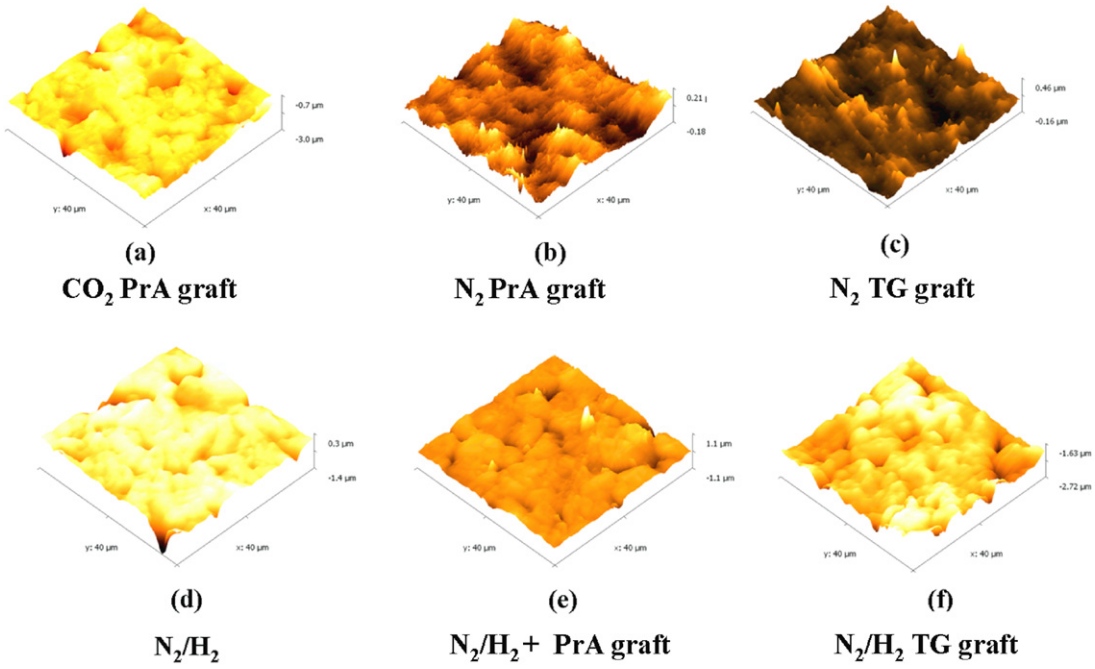


Figure 7. 3D AFM images of the PVDF surfaces after plasma activation and deposition of protein A and TG ($40.2 \times 40.2\,\mu m^2$).

comparatively with the effect observed for the PVDF sample treated with the N_2 plasma.

The change in the shape of the F 1s high-resolution spectra during plasma treatment and grafting/coating with proteins clearly shows the characteristics of the fluorine-rich species of the untreated PVDF sample surface, with a lower peak at 686.85 eV (57.6% of the whole N 1s signal) and a higher peak at 688.48 eV (42.4% of the whole N 1s signal). It is obvious that the F 1s peak is slightly shifted to a higher binding energy position after plasma treatment and coating/grafting, this positive shift indicating the decrease in electron density around the F atoms, and that the chemical state of fluorine changes after applied modifications.

On the basis of the analysed XPS data, the following can be concluded. (a) The carbon content decreases with respect to that of the reference in all cases, probably because surface composition is determined mainly by proteins. (b) There are no major differences between the surfaces covered by adsorption of proteins and the grafted ones; probably, the difference mainly refers to the stability of the deposited layer. When using TG, the nitrogen content is higher for the case in which the protein is grafted. (c) The oxygen content is also increased with respect to the reference in all cases and especially after covering with proteins, and the variation seems to be similar to that found in the nitrogen content. After plasma treatment, all surfaces became more hydrophilic, owing to the grafting of polar nitrogen or oxygen-containing functions. (d) N_2 plasma led to the grafting of a wide variety of polar species, leading to an amphoteric surface. (e) In the case of proteins' adsorption/grafting, both the acidic and/or the basic groups of proteins will interact with the complementary basic or acidic active sites, created on the surfaces by N_2 and N_2/H_2 or CO_2 plasma treatment, respectively. (f) The oxygen and nitrogen contents increase considerably after plasma treatment and coating/grafting with proteins of the PVDF reference sample, the highest values being registered for the N_2/H_2 plasma-treated sample and for PVDF N_2/H_2 plasma-pretreated/grafted with triglycine. This result suggests that, in this case, triglycine was more successfully grafted onto the PVDF surface than protein A, while in the other two cases of CO_2 and N_2 plasma activation, protein A was better grafted. (g) In every case, a strong surface defluorination was observed. The significantly reduced intensity of the F 1s signal and the related data presented in table 2 for all treated samples revealed a massive defluorination of the surface, in all cases, as a result of the electron attack generated by plasma action and surface coating with proteins. Considering that the fluorine peak was not totally suppressed after plasma treatment, a non-homogeneous grafting or coating of the PVDF surface with proteins occurred. (h) The most obvious change in all C 1s spectra of the modified samples is observed in the number and type of carbon atoms. The untreated PVDF exhibits two types of characteristic carbon atoms, while the treated samples have at least four signals—table 2. As expected for plasma-treated surfaces, some oxidized components of C 1s appear in the 285.85–293.30 eV range (e.g. C2, C3 and C5), in addition to the two main peak components assigned to the PVDF chains (e.g. C1 and C4). (i) Covalent grafting or physical deposition of a protein layer results in the introduction of amino groups on the PVDF surface. The signal at about 288.5 eV, attributed to N–C=O groups, overlaps the O–C=O signals, whereas the peak at 286.3 eV is characteristic exclusively of the C–NH_2 groups. This last mentioned peak is therefore a proof of protein's presence on the PVDF surface. (j) The intensity and content of the C4 peak is further decreased comparatively with that of the non-treated PVDF film, which suggests that the plasma treatment induces a loss of fluorine at the surface. This defluorination is accompanied by an enhancement of the newly formed multiple nitrogen or oxygen functions.

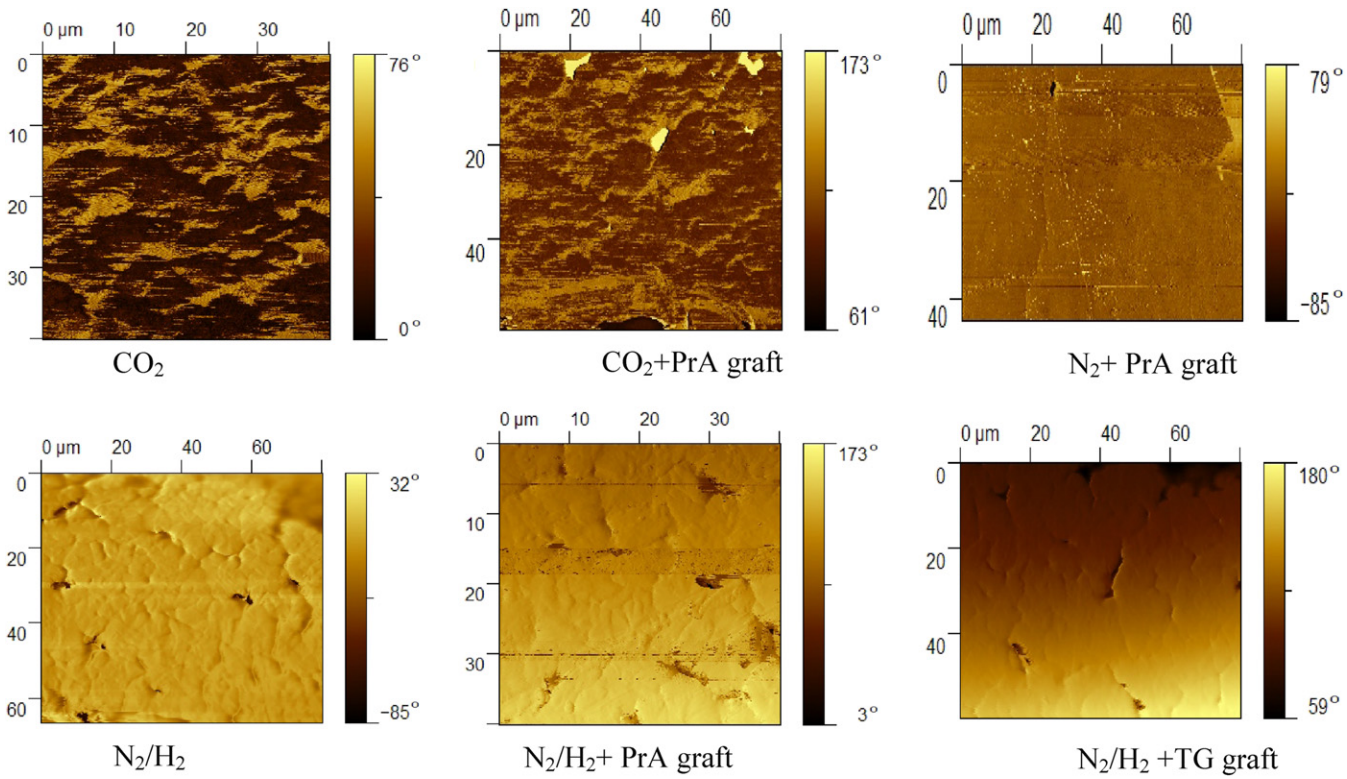


Figure 8. Phase images for PVDF after plasma activation and grafting with protein A or TG.

Table 3. Average roughness of the MW plasma-treated surfaces, and covered and grafted with proteins A and TG.

Sample	Roughness, nm for $10 \times 10\,\mu m^2$
Reference	84
CO_2	135
CO_2+PrA adsorbed	153
CO_2+TG adsorbed	172
CO_2+ PrA graft	143
CO_2+ TG graft	185
N_2	215
N_2+PrA adsorbed	125
N_2+TG adsorbed	147
N_2 + PrA graft	49.3
N_2+ TG graft	61.6
N_2/H_2	150
N_2/H_2+PrA adsorbed	60.4
N_2/H_2+TG adsorbed	165
N_2/H_2+ PrA graft	130
N_2/H_2+ TG graft	118

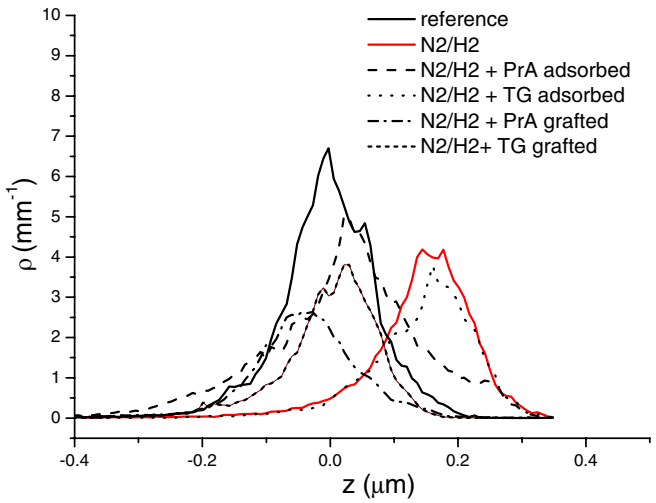
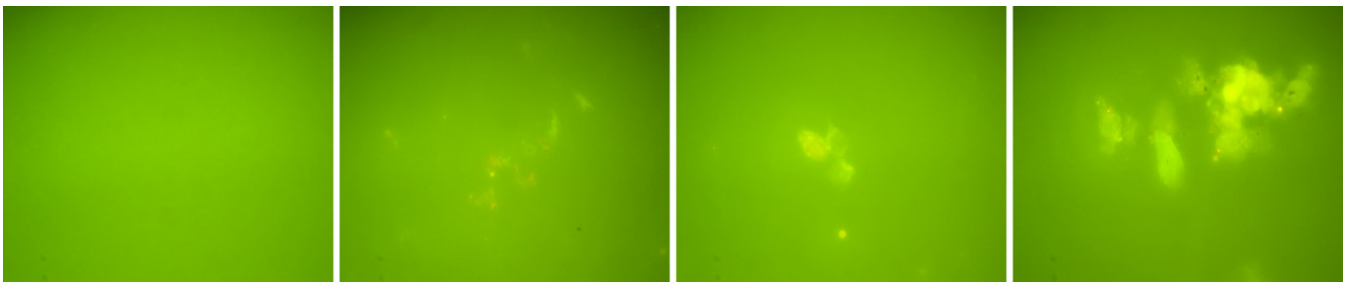


Figure 9. Histograms of the samples treated in different plasmas and covered/grafted with proteins.

The presence of a new surface layer was confirmed by AFM images of the surface. Coating with proteins leads to new surface characteristics—figures 7 and 8.

The AFM images (figure 7) show rough surfaces, whatever the nature of the treatment, while the phase images (figure 8) induce the idea of more smooth surfaces, especially with N_2 or N_2/H_2 plasma activation and adsorption/grafting of proteins.

Although the absolute roughness cannot be estimated, the root mean square roughness of the surface was determined systematically, both before and after treatments (table 3). Plasma activation induces a roughness from 83 nm to the highest value in the case of N_2 plasma treatment (215 nm). Protein deposition and grafting onto CO_2 plasma-treated surfaces lead to a relatively higher roughness than that of the plasma-treated surface (a few tens of nm more). While roughness of the protein–acidic surface is slightly increasing, that of the protein grafted or adsorbed on the amphoteric surface is decreasing (table 3). Almost all surfaces are smoother, as already shown by phase images. These results should lead to the conclusion of a high protein deposition or grafting with amino surfaces. On the AFM histogram corresponding to an area of $40 \times 40\,\mu m^2$, a Gaussian



(a) (b) (c) (d)

Figure 10. Immunofluorescence test results for virgin PVDF (*a*), N_2/H_2 plasma-treated PVDF with adsorbed TG (*b*), grafted with protein A (*c*) and grafted with TG (*d*).

distribution by height was observed in all cases—figure 9—which becomes narrower after plasma activation and larger by coating with proteins, the height of the curves being decreased for the samples grafted with proteins with respect to the reference samples and to the surface coated with adsorbed proteins. This should mean that, by these treatments, surfaces with different characteristics are obtained.

The immunofluorescence test incontestably proved the successful preparation of the PVDF surfaces for microorganism detection.

For the virgin or plasma-treated PVDF surface, no fluorescence zones were observed—figure 10(*a*). When treated in N_2/H_2 plasma and coated with adsorbed proteins, some fluorescence regions are observed (figures 10(*b*) and (*c*)), especially with adsorbed TG. Grafting of proteins induces larger fluorescence regions; the largest fluorescence zones are observed for the PVDF activated by the N_2/H_2 plasma and grafted with TG, which demonstrates the best coupling of the fluorescent antibody tracer with the TG protein grafted on the PVDF surface—figure 10(*d*). Similar results are obtained for the surfaces activated by either CO_2 or N_2 plasma but, in these cases, the larger fluorescence zones appear when using protein A in surface treatment, because, in these situations, covering of the surface with this protein was the most efficient—see the XPS results.

The results agree well with those obtained by XPS; the higher the amount or the stronger the binding of proteins, the larger the immunofluorescence zones observed.

4. Conclusions

CO_2 plasma treatment of PVDF leads to physico-chemical surface modifications, mainly by implantation of acidic groups onto the surface, due to the interactions between the polymer surface and the reactive species present in the plasma phase (including CO_2 species in different energy states, metastable, ions, atoms and radicals), which induces a functionalization characterized by the presence of oxygenated groups on the surface.

Two new methods are developed for polymer-surface functionalization by subsequent immobilization of protein A and triglycine through coating/grafting on a PVDF surface, followed by microwave plasma treatment with various gases, such as CO_2, N_2 and N_2/H_2.

Using XPS spectroscopy and ATR-FTIR, the formation of COF, COOH and O=C– groups after CO_2 plasma treatment, and amide and amine after other kinds of plasma activation and protein coating/grafting was detected and characterized. Water contact angle measurements show a gradual decrease in contact angles after proteins' coating/grafting, indicating an increased hydrophilicity in these two steps of modification, as also proved by AFM. It has been established that TG was better attached on a surface activated with N_2/H_2 plasma, while protein A on CO_2, N_2 plasma-activated surfaces.

The proteins immobilized on the PVDF surface exhibited the expected activity in coupling of the fluorescent antibody tracer, as assessed by an immunofluorescence test.

The purpose of these coatings is to create biosensors for antibody immobilization and detection. Plasma treatment of PVDF in a microwave plasma, followed by coating/grafting with different proteins, 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.

The procedure proposed in this paper is likely to have good potential in finding new biosensors (finally, the piezoelectric ones) that may play an important clinical role.

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