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How to Confer a Permanent Bio-Repelling and Bio-Adhesive Character to Biomedical Materials through Cold Plasmas

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Abstract: Plasma Enhanced–Chemical Vapor Deposition (PE-CVD) of polyethylene oxide-like (PEO)-like coatings represent a successful strategy to address cell-behavior on biomaterials. Indeed, one of the main drawbacks of organic and hydrophilic films, like PEO-like ones, often consists in their poor adhesion to the substrate, especially in biological fluids where the biomaterial is required to operate. In this paper, low pressure (LP) and aerosol-assisted atmospheric pressure (aerosol-assisted AP) PE-CVD of PEO-like coatings is compared. The stability of the two different classes of coatings was investigated, both in water and in the cell culture media, during cell culture experiments. The obtained results show that, when deposited at atmospheric pressure (AP), the adhesion of the PEO-like coatings to the substrate has to be granted by an intermediate gradient layer. This interlayer can match the properties of the substrate with that of the topmost coatings, and, in turn, can dramatically improve the coating's stability in complex biological fluids, like the cell culture medium. An accurate modulation of the experimental conditions, both at LP and AP, allowed control of the film chemical structure and surface properties, to permanently promote or discourage the cellular adhesion on the surfaces of biomaterials.

Keywords: PE-CVD; aerosol assisted plasma deposition; atmospheric pressure plasma; PEO; non-fouling

1. Introduction

Plasma processes are well known as a powerful tool to deposit polymer-like thin coatings on materials surfaces, whose properties tightly depend on plasma experimental conditions. The process is known as PE-CVD, and is one of the most common plasma polymerization techniques, at LP as well as at AP [1]. One intrinsic advantage of PE-CVD is that the surface chemistry of the deposited film, in principle, only depends on plasma conditions regardless of the type of the substrates [2,3]. In fact, after the initial deposition of the first atomic or molecular layer of material, the film growth is substrate-independent [2]. PE-CVD techniques are, nowadays, exploited in a wide range of applications in Materials Science and Technology, including many in the biomedical field [4].

Plasma deposited films are generally referred to as "plasma polymers" because of their structural analogy with such materials. However, while the atomic stoichiometry is often undefined, the retention of the starting monomer structure can be modulated to resemble the respective conventional polymer.

The first degree of freedom when designing a plasma polymer is thus the monomer choice [5]. When the complexity of the monomer increases, careful tuning of the plasma conditions is needed to allow tailoring of the retention of the structure of the monomer in the coating, and the density of surface functionalities to suit any specific application [6]. In this way, it is possible to tune PE-CVD processes to improve film stability over time or upon soaking in liquids [7].

For most plasma polymers, chemical composition, the degree of retention of the monomer functionalities, and the final properties are highly dependent on the energy used to ignite the plasma. For instance, it is well accepted that high power inputs and low monomer-flow rates lead to stronger monomer fragmentation in the plasma and higher crosslinking of the resulting deposited coating. While this generally improves the stability of the coating, in contrast, its chemical composition can significantly deviate from that of the monomer. Carefully balancing the precursor flow rate and power allow, in principle, to achieve both stability and the desired chemical properties. Another way to adjust the chemical structure of a PE-CVD coating is to mix different precursors in the same discharge, to confer chemical features that belong to the used monomers [8]. To improve the fragmentation and activation of less reactive precursors, a carrier gas could be necessary [9]. Selecting the proper plasma regimes and source, at low or at atmospheric pressure, is also important for the final characteristic of the coating.

Despite the possibility to finely tune the composition of the plasma deposited films, one of the most critical points of the process is the interaction of the film with the substrate. Even if the chemistry and the growth of the film are independent of the substrate, important features of the final coating, such as the depth profile, the aging or the mechanical stability, and others, strongly depend on the quality of the plasma and substrate interactions [5]. Films deposited by PE-CVD at LP and AP could exhibit poor adhesion to the substrate, delamination, and cracks when submitted to deformation, friction, or shear stress, such as those induced by cell adhesion in biomedical applications [10]. As plasma conditions are changed, processes need to be finely tailored to suit such specific applications and improve the interaction with substrates.

In this context, LP plasma polymerization processes should induce better adhesion between the polymeric coating and the substrate due to the initial activation of the surface, through ion bombardment [11]. On the other hand, only a few strategies have been explored, and not completely, to improve the stability of the substrate-coating interface in AP plasma when chemical or physical characteristics of the substrate, and the PE-CVD film, are different [5]. Several studies explored the potential of plasma-synthesized surfaces to control interactions of biological entities at the surface of biomedical materials. The design of plasma processes for biomaterial surfaces must be based on understanding the requirements of the bio-interface aimed in the application.

In recent years PEO materials emerged as potential candidates for many biomedical applications, due to their non-specific and non-fouling biological properties toward a broad range of biomolecules and cells. In literature, it is shown that PEO-based materials can prevent the adsorption of proteins, the adhesion of platelets, and thrombus formation both in vitro and in vivo [12]. The antifouling properties of PEO are predominantly explained with the presence of high concentrations of hydrophilic ether oxygen moieties $(CH_2\text{-}CH_2\text{-}O)_n$ [13,14]. These can bind four water molecules each in a water environment, so that the hydrated PEO surfaces do not look much different from the solution, from the energetic point of view. In this way, biomolecules and other biological entities do not "see" energetic advantages in the adhesion to the PEO surface, and remain in solution. Furthermore, the molecular weight of the PEO chains and surface density of $CH_2\text{-}CH_2\text{-}O$ moieties (known as PEO-character), are also considered crucial to determine the non-fouling behavior of PEO-coated surfaces [15–18]. Currently, conventional PEO polymers, approved by the Food and Drug Administration in the

US, are used for fabricating blood-contacting devices and the manufacture of hydrogels for drug delivery [14,19].

PE-CVD of PEO-like coatings could be used to tune cell adhesion on biomaterials by changing the PEO-character. Two strategies can be envisaged, at least: (1) deposition of organic coatings starting from a monomer with "cell-repulsive" moieties at optimized plasma parameters (i.e., input power, monomer flow rate, etc.); (2) co-deposition of organic coatings from two monomers, one with "cell-repulsive" motifs, and the other showing "cell-adhesive" ones, at a controlled relative ratio in the feed (Figure 1). In the second route, a monomer for PEO-like deposition (i.e., a glyme) is fed with a monomer characterized by other functional groups, like carboxyl (COOH) or amine (NH_2) groups, that are suitable for promoting non-specific cell adhesion [20]. The copolymerization of two monomers is a convenient and versatile method for adjusting the surface density of functional groups [21] and modulating the un-fouling character of the resulting surface.

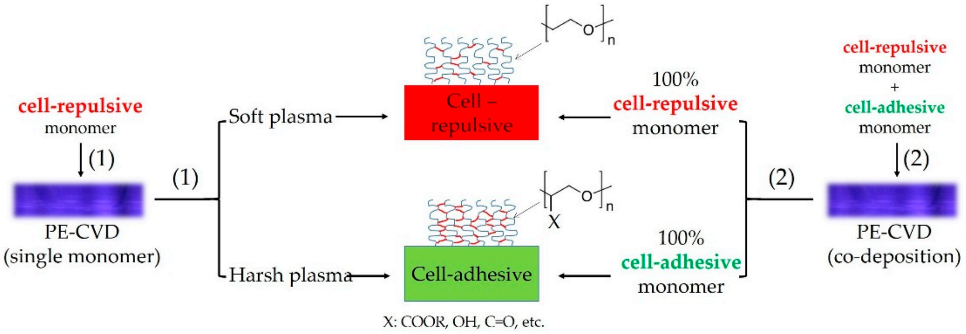

Figure 1. Scheme of the modulation of the cell-adhesive and cell-repulsive character following two strategies: (1) Single monomer PE-CVD deposition; (2) PE-CVD co-deposition. In strategy 1, starting from a "cell repulsive" monomer (i.e., glyme) it is possible to go from a cell-repulsive coating toward a cell-adhesive one by changing the experimental plasma conditions from "soft" (i.e., low input power, high pressure) to "harsh" (i.e., high input power, low pressure). Strategy 2 is generally applied in "soft" plasma deposition by mixing a "cell-repulsive" monomer with a "cell-adhesive" one (i.e., a monomer containing COOH, NH_2 functional groups). Red lines among film chains represent the inter-chain cross-linking.

While several reviews on AP or LP processes have been published in recent years [22,23], there is no detailed overview of differences, advantages, and disadvantages, in depositing PEO-like coatings using AP vs LP conditions, and this contribution is designed to address this gap.

In the present study, the deposition of polyethylene oxide (PEO)-like functional coatings was carried out in LP on inorganic substrates of biomedical interest like titanium, and in AP plasmas on plastics (like polycarbonate, (PC)), as an example of the future application in biomaterials manufacturing (i.e., blood stents) or in microfluidic. The effects of three major operating parameters (deposition potential, input power, and gas feed composition) on the surface properties of PEO-like coatings were investigated. Different characterization techniques, such as contact angle (CA) measurement, FT-IR spectroscopy, X-ray photoelectron spectroscopy (XPS), and optical microscopy were used to examine the changes in the hydrophilicity, surface chemistry, and topography of the PEO-like coatings. Finally, the cell adhesion and repulsive properties of the PEO-like coatings were investigated by in vitro cell culture tests.

2. Materials and Methods

2.1. Materials

Alumina (Al_2O_3) plate used as a dielectric barrier (CoorsTek, 96% purity, 70 × 70 mm^2, 1.9 mm thick). He (99.999% purity, i.e., 5.0, Sol SpA Modugno, Bari, Italy). Ethylene (99.95% purity, i.e., 3.5, AirLiquide, Milano, Italy). Tetra ethylene glycol dimethyl ether (TEGDME) and Di-ethylene glycol dimethyl ether (DEGDME), ≥99% purity, Sigma Aldrich, St. Louis, MO, USA. AP plasma deposition processes were carried out on 1 cm × 1 cm PC square samples (0.125 mm thick, Goodfellow, London, UK); LP plasma depositions were carried out on Titanium. Titanium discs (14.5 mm in diameter/1.6 mm in thickness) were manufactured from commercially pure grade 4 titanium (ISO 5832-2, Medartis, Basel, Switzerland). The diameters were chosen to ensure that the discs would match the diameter of 24-wells plates. Titanium discs were first sonicated for 15 min with a 2% Hellmanex II alkaline cleaning solution (Helma^{TM}, Fisher Scientific, Landsmeer, NL, USA); after thorough rinsing with ultrapure water, they were sonicated twice for 15 min with absolute ethanol (Sigma Aldrich, St. Louis, MO, USA). PC samples were sonicated for 5 min in absolute ethanol and dried under an N_2 flow.

2.2. Plasma Deposition in LP and Aerosol-Assisted AP

The plasma deposition of cell-repulsive and cell adhesive coatings in LP regime was carried out in radiofrequency (RF), 13.56 MHz, glow discharges ignited in stainless steel (SS) reactor with asymmetric vertical parallel plate electrodes, as well described elsewhere [13,24]. The inter-electrode gap is 60 mm between the grounded electrode (180 mm diameter) and the RF-powered electrode (80 mm diameter). The smallest electrode is connected to the RF power supply through a matching network unit.

Different experimental conditions were investigated, to study the effect of the input power, and of the gas feed on the final chemical characteristics of the deposited films and, in turn, on their cell-repulsive attitude. Vapors of diethylene glycol dimethyl ether (DEGDME, $CH_3O(CH_2CH_2O)_2CH_3$, purity 99.98% Sigma Aldrich, St. Louis, MO, USA), also known as di-glyme, were used as a precursor with or without vapors of Vinyl acetate (VAc), $CH_3CO_2CH{=}CH_2$, ≥99% (Sigma Aldrich, St. Louis, MO, USA) to modulate cell adhesive propriety. Input powers were changed in the range 5–50 W; the VAc/DEGDME ratios were changed from 5/0 (i.e., 5 sccm VAc plus 0 sccm DEGDME) to 0/5 (i.e., 0 sccm VAc plus 5 sccm DEGDME). Samples were fixed with double-sided copper adhesive tape on a sample holder positioned on the ground electrode. Argon (Air Liquide, 99.95) was used as the carrier gas in the case of plasma depositions performed with only DEGDME to increase deposition rate [5]. A turbo-rotary pump system (Pfeiffer) was used to maintain the process pressure at 53 Pa, as monitored with a baratron (MKS Instruments, Andover, MA, USA). The deposition time was kept constant at 60 min.

The home-made experimental apparatus for aerosol-assisted AP plasma deposition is composed of a parallel plate dielectric barrier discharge (DBD) electrode system (4 mm inter-electrode gap), enclosed in an airtight Plexiglas chamber. Each electrode (50 × 50 mm^2 area) is covered with a Al_2O_3 dielectric plate. The plasma was generated with a high voltage alternating current (AC) power supply (SG2 STT Calvatron) working in the 15–50 kHz frequency (f) range. [25] Experiments were run at 16 kHz and 26 kHz at applied voltages (Va) of 6.5 kV_{p-p} and 8.5 kV_{p-p}. The applied voltage was measured with a High Voltage probe (Tektronix P6015A). The current (I_m) and the charge (Q) were determined indirectly by measuring (with a Tektronix P2200 probe), respectively, the voltage drop across a 50 Ω resistor and a 4.7 nF capacitor connected in series with the ground electrode [26]. Data were recorded with a digital oscilloscope (Tektronix TDS20143). The power dissipated in the discharge was calculated with the Manley method, by using voltage-charge (V-Q) Lissajous figure [27].

The DBD was fed by He, ethylene, or the aerosol of TEGMDE. Two different flows of He were used and controlled using mass flowmeters (MKS), to change the concentration of TEGDME precursor in the feed mixture. In particular, a fixed He flow rate of 3.15 slm (ϕHe-TEGDME aerosol) was utilized for the operation of a pneumatic atomizer (TSI, model 3076) and allowed introduction in the DBD 0.02 $g\cdot min^{-1}$ (i.e., 1.7 sccm) of TEGDME. A variable He flow rate (ϕHe-carrier) was used to transport the aerosol in the discharge (4.85 or 6.85 slm). The ethylene flow rate was kept fixed at 8 sccm. The feed mixture was let longitudinally in the inter-electrode gap through an inlet slit and pumped out (membrane pump) through a second slit on the opposite side. The pressure inside the Plexiglas chamber was monitored with an MKS baratron, kept constant at 101,325 Pa by regulating the pumping speed with a needle valve. Before each experiment, the Plexiglas chamber was flushed with He (4 slm) for 15 min. A three-step plasma deposition process has been designed to produce coatings exhibiting a vertical cross-linking gradient in their subsurface and to improve, in turn, their adhesion to the underlying substrate. As reported in Table 1, the three steps are: step 1, plasma deposition of a hydrocarbon polyethylene (PE)-like coating (ϕC_2H_4 8 sccm, ϕHe carrier 8 slm, applied voltage, 8.5 kV_{p-p}, frequency 16 or 26 kHz, deposition time, t_d, 60 s); step 2, plasma deposition of a highly cross-linked PEO-like coating obtained by mixing C_2H_4 and TEGDME as precursors (ϕC_2H_4 8 sccm, (ϕHe-TEGDME aerosol 3.15 slm, He carrier ϕHe 4.85 sccm, applied voltage 8.5 kV_{p-p}, frequency 16 or 26 kHz, deposition time 10 s); step 3, the uppermost PEO-like layers exhibiting different cell adhesive or repulsive properties were deposited using He/TEGDME feed mixtures by changing the flow of the carrier gas (ϕHe-carrier) from 4.85 slm to 6.85 slm (deposition time 300 s) to increase the total He flow rate from 8 to 10 slm, respectively. By reducing the ϕHe-carrier, with the aerosol flow rate kept constant at 3.15 slm (i.e., by increasing the ϕHe-TEGDME aerosol/ϕHe-carrier ratio), the concentration of the TEGDME aerosol in the feed mixture and the residence time of the gas feed in the discharge increase. Thus lower monomer fragmentation is expected and consequent high retention of the PEO-character could be achieved for the films deposited with ϕHe carrier of 4.85 slm [28]. These films from now on will be named PEOA and distinguished from the films deposited at a higher carrier flow rate (PEOB). All the PEO-like coatings of the (third step) were deposited at 16 and 26 kHz except for the PEOB coatings, which were deposited only at 26 kHz. The frequency remained the same for the whole duration of the multistep process. As an example, if the first step is carried out at 16 kHz, the same value is kept constant until the third step. The Va value of 8.5 kV_{p-p} was used in the first two processes to ensure high degree of cross-linking of the interlayers. The third step was performed both at 6.5 kV_{p-p} and at 8.5 kV_{p-p} to study the effect of the applied voltage on the chemical composition of the deposited coating. All conditions are listed in Table 1.

Table 1. Experimental conditions for the three-step deposition. PEOA and PEOB coatings were obtained following the three-step approach by changing respectively the flow rate of the carrier gas (ϕHe-carrier) from 4.85 to 6.85 slm, the applied voltage (Va), the frequency (f) and the deposition time (t_d). The other parameters like flow of ethylene (ϕC_2H_4) and He flow used in the atomizer to deliver 0.02 g·min^{-1} of TEGDME (ϕHe-TEGDME aerosol) were kept constant.

Deposition Step	Name	ϕC_2H_4 (sccm)	ϕHe-TEGDME (slm)	ϕHe-Carrier (slm)	Va (kV_{p-p})	f (kHz)	t_d (s)
Step 1	-	8	-	8	8.5	16; 26 ^{2}	60
Step 2	-	8	3.15	4.85	8.5	16; 26 ^{2}	10
Step 3	PEOA *	-	3.15	4.85	6.5; 8.5 ^{1}	16; 26 ^{2}	300
	PEOB *	-	3.15	6.85	6.5; 8.5 ^{1}	26	300

* Coatings deposited both as a single step or as the third step of a multi-step approach; ^{1} coating deposited at 6.5 or 8.5 kV_{p-p}; ^{2} coatings deposited at 16 or 26 kHz.

2.3. Chemical and Physical and Biological Characterization of Materials

XPS measurements were performed with a Theta Probe Thermo VG Scientific instrument (monochromatic Al Kα source). Survey (0–1100 eV) and high-resolution spectra (C1s, O1s) were recorded. Samples were neutralized for electrostatic charging with a flood gun (Mod.822−06 FG, 1 eV). Angle-resolved (AR) analyses were performed at different take-off angles ranging from 28° (lowest sampling depth) to 78° (highest sampling depth). Where not specified a take-off angle of 52° was used. The take-off angles reported are calculated concerning the sample surface. The hydrocarbon component of the C1s spectrum was used as a reference for the Binding Energy (BE) centered at 285.0 eV; C1s spectra were best-fitted into four peak components: I (285.0 ± 0.2 eV, C-H/C), II (286.5 ± 0.2 eV, C-OH/OC), III (287.9 ± 0.2 eV, C=O/O-C-O), IV (289.2 ± 0.2 eV, COOH/C). A full width at half maximum (FWHM) value of 1.5 ± 0.2 eV was used for all components. The relative importance of the II component (PEO-character) was related to the non-fouling character of the coatings, as reported in the literature [29,30].

Fourier Transform Infrared Spectroscopy (FT-IR) was used to characterize the bulk of the coatings deposited onto silicon slices. FT-IR spectra (32 scans per analysis, 4 cm^{-1} resolution) were obtained in transmission mode with a Vertex 70 V Bruker spectrometer. The spectrometer was evacuated to less than 150 Pa for 10 min before each acquisition. Spectra were elaborated with a Bruker OPUS software and normalized by keeping the peak of CH_x stretching (2940–2810 cm^{-1}) as reference.

Static water contact angle (WCA) was measured with a Ramé–Hart manual goniometer (model A−100). Static WCA was obtained using 3 μL drops of double-distilled (DD) water. An average value was calculated (3 measurements for each sample, 3 samples per experimental condition).

The thickness of the coatings was measured with a AlphaStep® D-120 Stylus Profiler, to calculate their deposition rate.

The biological experiments performed to test the cytocompatibility of the produced surfaces were done independently with two different types of fibroblast-like cells: Human dermal fibroblasts as a model of primary cells and Saos-2 osteoblast cell lines a model of immortal cell lines widely used for in vitro experiments in the tissue engineering field. Commercial normal human dermal fibroblasts (NHDF adult, Promocell, Germany) were used for the experiments on coatings deposited in LPregime. NHDF fibroblasts were cultured in a medium containing 72% Dulbecco's modified Eagle medium (Sigma Aldrich, St. Louis, MO, USA), 18% Medium M199 (Sigma Aldrich, St. Louis, MO, USA), 9% fetal calf serum (Sigma Aldrich, St. Louis, MO, USA), and 1% Penicillin−Streptomycin (Sigma Aldrich, St. Louis, MO, USA). They were maintained at 37 °C in a saturated humid atmosphere (95% air 5% CO_2). Cells were sub-cultured by detachment with Trypsin/Ethylene diamine tetraacetic acid (Trypsin/EDTA, Sigma Aldrich, St. Louis, MO, USA) at approximately 90% confluence. Fibroblasts from the 2nd to 6th passage were used for all experiments. To study adhesion and growth, cells were fixed in 4% formaldehyde/Phosphate Buffer Solution (formaldehyde/PBS, Thermo Fisher Scientific, Waltham, MA, USA), at RT for 20 min, permeated with PBS containing 0.1% Triton X-100 and incubated with Alexa Fluor488 phalloidin (Thermo Fisher Scientific, Waltham, MA, USA) at room temperature and for 20–30 min. Vinculin was detected with a monoclonal antibody raised in mouse (Sigma Aldrich, St. Louis, MO, USA). For vinculin staining, a secondary antibody against mouse IgG conjugated with Alexa Fluor546 (Thermo Fisher Scientific, Waltham, MA, USA) was used. The osteoblastoma Saos-2 cell line (ICLC, Genova, Italy) was used on coatings deposited in aerosol-assisted AP plasma. Cells were routinely grown in Dulbecco's Modified Eagle Medium (DMEM, Sigma Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS), 50 IU/mL penicillin, 50 IU/mL streptomycin, and 200 mM glutamine (Sigma Aldrich, St. Louis, MO, USA). They were maintained at 37 °C, in a saturated humid atmosphere (95% air, 5% CO_2). For experiments on plasma deposited samples, cells were detached with a trypsin/EDTA solution (Sigma Aldrich, St. Louis, MO, USA), re-suspended in the correct medium, and seeded at a concentration of 1×10^5 cells/mL on native and coated substrates (squares of PC of 1cm × 1cm) placed in 24 multiwell plates. Each experiment was performed, at least, on 3 samples per each condition. To study their adhesion to the surfaces, cells were stained with Coomassie Blue. After 24 h of culture, Saos-2 cells seeded on differently coated substrates were fixed with a 4% Paraformaldehyde/PBS solution (15 min), and stained 3 min with a 0.2% Coomassie Brilliant Blue R250 (Sigma Aldrich, St. Louis, MO, USA) solution (50% methanol, 10% acetic acid and water). After staining, cells adhering to different substrates were observed at different magnifications with an inverted phase-contrast microscopy (Leica DM ILI); digital images were acquired through a Charge Coupled Device (CCD) camera (Leica DC100).

3. Results

3.1. LP Plasma Deposition of Differently Cell-Repulsive Coatings

This part of the research aims to demonstrate how, by working at low pressure, it is possible to finely modulate cell-adhesion by using both strategies, 1 (i.e., changing input power) and 2 (i.e., mixing monomers with different typologies of cell-adhesive and cell-repulsive motifs), explained earlier in

Figure 1. VAc was mixed with DEGDME in order to modulate the cell-repulsive character of the resulting PEO-like coatings as a function of the relative amount of ether and ester groups in the final structure of deposited coatings.

As shown in Figure 2, when different VAc/DEGDME ratios are used to feed the deposition process (i.e., strategy 2 of Figure 1), the chemical composition of the bulk of the deposited coating changes in terms of a decrease in the contribution centered at 1100 cm^{-1} (C−O−C stretching), and a gradual increase in the contribution at 1723 cm^{-1} (C=O stretching), as the VAc content in the feed increases. Low input power and high-pressure values (i.e., soft plasma conditions) were chosen to guarantee careful control over process conditions. The low energy and soft ion bombardment could, in principle, enable the breaking of labile or double bonds which are characterized by a higher reactivity, without causing significant dissociation of the rest of the molecule [5]. In this case, the chemical structure of the deposited polymer closely resembles that of polymer analogs obtained by conventional radical polymerization processes. The co-presence of ether and carbonyl groups in the coatings confirm that, as expected by the experimental conditions used, a high monomer structure retention is obtained in the coating.

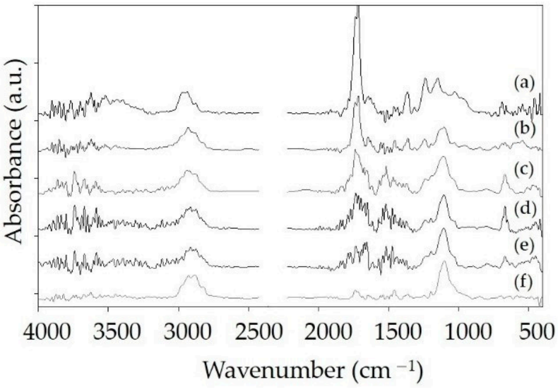

Figure 2. Fourier-transform infrared spectroscopy (FTIR) spectra acquired for plasma polymers obtained at 5 W and 53 Pa for 60 min from a feed composed by mixtures of VAc/DEGDME at different ratios (i.e., feed total flow 5 sccm): (**a**) 5/0; (**b**) 4.5/0.5; (**c**) 4/1; (**d**) 3 /2; (**e**) 1/4; (**f**) 0/5.

All coatings deposited with a flow of DEGDME higher than 0.5 sccm appear sticky and poorly adhering to the underlying substrate. This effect could be due to a combination of milder conditions used (i.e., low input power value) for the process, and the condensation of the scarcely volatile DEGDME monomer in the reactor during the process at high flow rates. Moreover, the high deposition rate of VAc, and the absence of carrier gas, can further contribute in synthesizing a poorly cross-linked coating containing long hydrogel-like chains [15].

To guarantee a correct balance between the retention of the functional groups and a certain cross-linking degree (i.e., non-sticking polymers), the flow of DEGDME was kept constant at 0.5 sccm, while that of the VAc that polymerizes easily was fixed at 4.5 sccm. Thus, from now on, when we speak about coatings obtained by VAc/DEGDME mixtures, we will refer only to those obtained with a VAc/DEGDME ratio of 4.5/0.5. Coatings obtained from DEGDME only indeed were deposited with 0.5 sccm of DEGDME in mixture with Ar [5] to confer the correct coating cross-linking and, at the same time, keep the ether functionalities in the coating.

In Figure 3 are reported the FTIR spectra acquired on coatings deposited at different input power values from only DEGDME, only VAc, and from a VAc/DEGDME mixture at 4.5/0.5 ratio. The deposition from DEGDME allows obtaining a coating with an enhanced stretching peak of the ether groups centered at 1100 cm^{-1}, usually reported for conventional PEO polymers. The absence of a shift to higher wavenumbers for coatings deposited at low power (red spectrum of Figure 3a) demonstrates that such coatings are characterized by a not amorphous state of the macromolecular segments that is, on the contrary, generally typical for plasma polymers. This finding allows assessing that, working at low power values, the experimental conditions reported in this paper enables the deposition of plasma polymers very similar to that of conventional PEO. The FT-IR spectrum reveals also the stretching band of the CH_2 groups (2940–2810 cm^{-1}) and that of carbonyl groups (1723 cm^{-1}). By changing the input power from 5 to 50 W (strategy 1 of Figure 1) it is possible to see the decrease in the COC ether contribution at 1100 cm^{-1}, and the increase in the carbonyl band at 1723 cm^{-1}. This result could be due to inter-chain crosslinking or to the fragmentation of polymer chains (i.e., the prevalence of short chains).

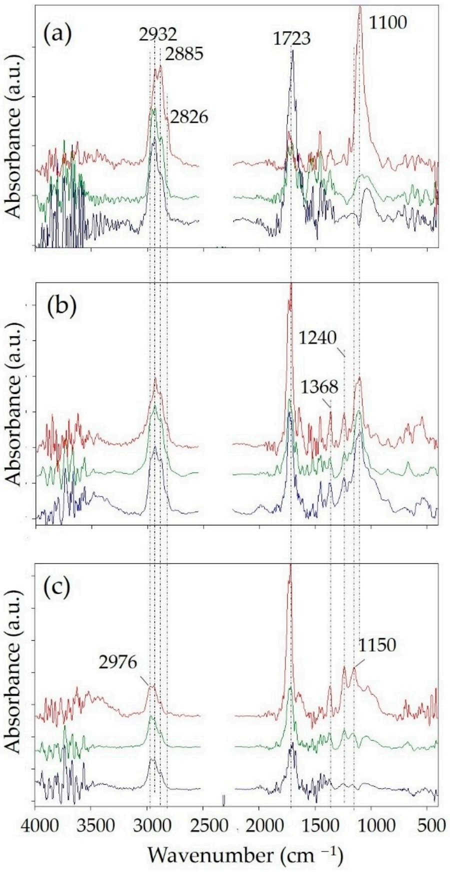

Figure 3. FT-IR spectra acquired on coatings deposited from different gas feeds: (**a**) DEGDME (53 Pa, 60 s); (**b**) mixture of VAc/DEGMDE (4.5/0.5 ratio, 53 Pa, 60 s; (**c**) VAc (53 Pa, 60 s). The spectra were acquired for coatings deposited at 5 W (red), 15 W (green), and 50 W (blue).

When VAc is used as the monomer, the twisting and wagging of CH_3 groups at 1240 cm^{-1} and 1368 cm$^{-1,}$ respectively; appear in the FT-IR spectra of Figure 3; moreover, the C=O stretching band (1723 cm^{-1}), that is the characteristic fingerprint of the VAc structure, decreases as the input power increases from 5 to 50 W. Coatings deposited from the blend of the two monomers (Figure 3b) show the characteristic bands of both monomers, especially at low input power. The C=O stretching peak at 1723 cm^{-1} decreases as the input power increases from 5 to 15 W, then it increases again at 50 W.

Besides the bulk, the surface composition of the coatings is very important for applications, for it is the arena where the interactions between biological entities and material occur and could be engineered to pre-determined effects such as non-fouling or cell-adhesion properties.

In Figure 4 the XPS C1s spectra of coatings deposited from DEGDME, VAc, and a mixture of them, at different powers values are reported. The chemical composition of the coatings in terms of PEO-character tightly depends on the DEGDME content in the feed, and the power input delivered to the discharge (Table S1). The PEO-character decreases with the increase in power from 70% to 30% in coatings deposited in discharges fed with only DEGDME (Figure 4a–c), and decreases at least 20%

from a pure DEGDME discharge to a process fed with only VAc, at any power delivered to the plasma (compare Figure 4a,g, Figure 4b,h, Figure 4c,i).

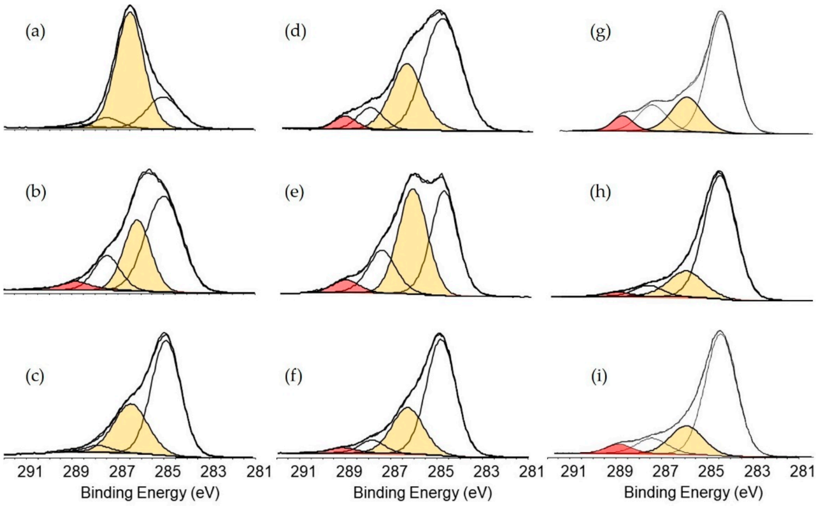

Figure 4. XPS C1s spectra acquired on plasma polymers deposited at 5 (**a**,**d**,**g**), 15 (**b**,**e**,**h**) and 50 W (**c**,**f**,**i**) with different feed composition at the following experimental conditions: (**a**–**c**) 0.5 sccm DEGDME, 5 sccm Ar, 53 Pa, 60 min; (**d**–**f**) 4.5 sccm VAc, 0.5 sccm DEGDME, 5 sccm Ar, 53 Pa, 60 min; (**g**–**i**) 4.5 sccm VAc, 0.5 sccm DEGDME, 5 sccm Ar, 53 Pa, 60 min. The intensity of C1s peaks (ordinate axis) is expressed as arbitrary units (a.u.).

The coatings deposited from only DEGDME at 50 W are less stable in water than those coatings deposited at 15 W and 5 W (i.e., the PEO-character of DEGDME coatings deposited at 50 W decreases 20% after soaking, Table S1). The water soaking does not allow the complete dissolution of the deposited films as attested by the absence of Ti signal in the acquired low resolution (survey) XPS spectra before and after water immersion (Figure S1). A similar behavior, although not as drastic, is observed also for coatings deposited from VAc/DEGDME mixtures and VAc (i.e., changes of PEO-character up to 10% after water soaking, Table S1). Only variations of oxygen on carbon ratio (O/C) composition were revealed, no titanium from the underlying substrate was detected, so the modifications after soaking in water could be ascribed to leaching of weakly bound fragments rather than to delamination or dissolution of the PE-CVD layer. Coatings deposited from a VAc/DEGDME are less stable in the water when deposited at 5 W, as attested by the increasing of the O/C ratio and of the PEO-character (see Table S1), probably for the low cross-linking degree of the coatings deposited at low power, and presence of polar groups that could contribute to the dissolution of weakly bound fragments. The more stable VAc/DEGDME coatings are the ones deposited at 15 W.

The results of cell growth after 1 and 5 days on the most stable coatings on titanium substrates are shown in Figure 5. Coatings deposited from DEGDME at 5 and 15 W of power (i.e., DEGDME_5W and DEGDME_15W), from VAc at 5 W and 15 W (i.e., VAc_5W and VAc_15W) and from VAc/DEGDME 4.5/0.5 mixture at 15 W (i.e., VAc/DEGDME_15W) have been investigated. It is possible to observe that cells cover almost completely, to confluence growth, all substrates after 5 days except those with a PEO-character of around 70% (DEGME_5W). On these surfaces, of clear non-fouling character, only a few adhering cells can be observed after 1 day of growth. Very few cells are present also after 5 days, clustered together due to their minimal adhesion to the non-fouling surface. It is very interesting to compare how cells behave on VAc/DEGDME and DEGDME coatings, deposited at the same power of 15 W, after 1 day of growth. The latter discourages cell adhesion more than the former although the PEO-character is higher in the VAc/DEGDME_15W coating. This behavior attests to a possible contribution of C-OH cell adhesive alcohol moieties to the II component, where the non-fouling

CH_2CH_2O moieties also contribute, as they can positively contribute to promote cell adhesion and amplify the cell adhesive effect of the ester groups in the IV component.

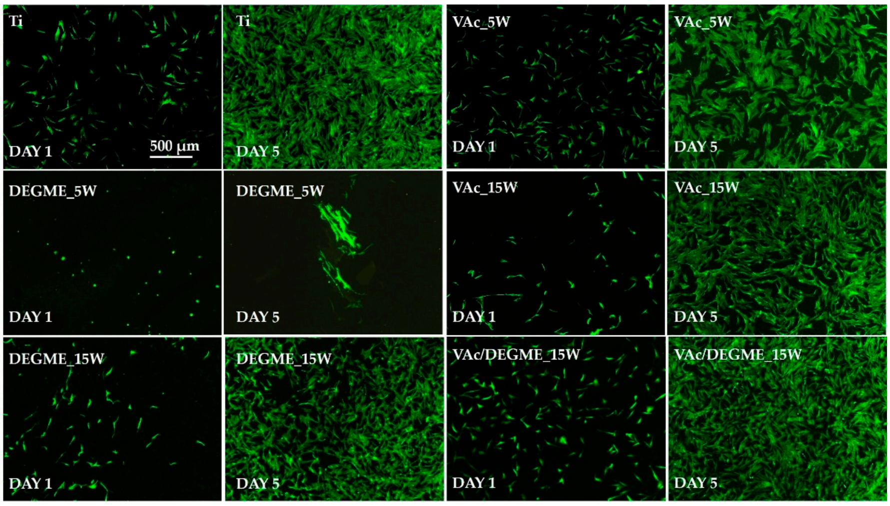

Figure 5. Images of actin cytoskeleton of NHDF cells grown on different samples for 1 (DAY 1) and 5 days (DAY 5) of cell culture. Samples are untreated Titanium (Ti); Ti PE-CVD coated with DEGDME at 5 W (DEGDME_5W; 5 W, 53 Pa, 5 sccm Ar, 0.5 sccm DEGDME, 60 min) and at 15 W (DEGDME_15W; 15 W, 53 Pa, 5 sccm Ar, 0.5 sccm DEGDME, 60 min); Ti PE-CVD coated with VAc at 5 W (VAc_5W; 5 W, 53 Pa, 5 sccm VAc, 60 min) and 15 W (VAc_15W; 15 W, 53 Pa, 5 sccm VAc, 60 min); PE-CVD coated with VAc/DEGDME mixture at 15 W (VAc/DEGDME_15W; 15 W, 53 Pa, 4.5 sccm VAc, 0.5 sccm DEGDME, 60 min).

The differences in cell behavior between the few on the PEO un-fouling surfaces and those adhering to the other surfaces are not related to the cell number and their growth rate. It is possible to observe, from high magnification images, as in Figure 6, an evident difference in morphology of the NHDF cells. The few cells adhering to the DEGME_5W surface are not only smaller, but they show no stress actin fibers and no focal adhesions, as shown from the vinculin staining, concerning those on the other surfaces. These further confirm the un-fouling character of the DEGME_5W surface.

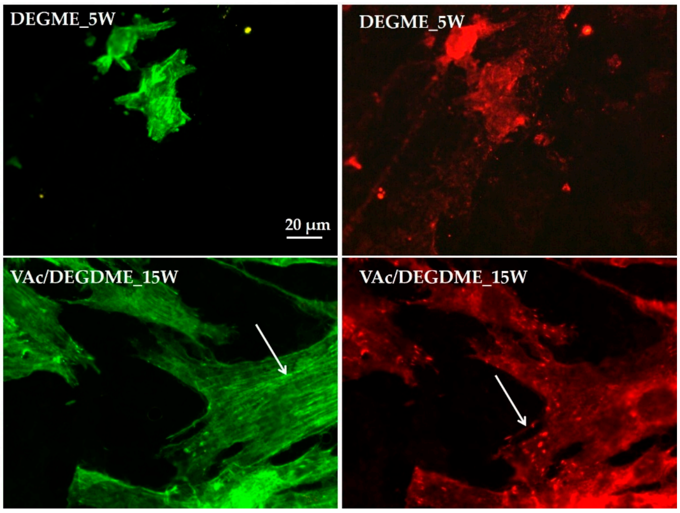

Figure 6. Images of the actin cytoskeleton of NHDF cells grown on the DEGME_5W and Vac/DEGME_15W surfaces. Actin is shown in green, vinculin in red. The presence of stress fibers (actin) and of focal adhesions (vinculin) is highlighted by the white arrows.

3.2. Aerosol-Assisted AP Plasma Deposition of Organic Coatings Starting from a Monomer with "Cell-Repulsive" Motifs

Plasma deposition of cell repulsive coatings has been also carried out in aerosol-assisted AP plasma using a DBD reactor, fed with a mixture He and TEGDME. PC substrates have been processed with a single or a multiple-step approach to investigate and improve the stability of the coatings during water soaking and cell adhesion experiments.

As described earlier, PEOA coatings were deposited at ϕHe-carrier of 4.85 slm, PEOB at a higher ϕHe-carrier flow rate of 6.85 slm in a single step process. In both cases, TEGDME was let in the DBD with 3.15 slm of ϕHe-TEGDME aerosol. Excitation frequency and applied voltage were kept constant at 26 kHz and 8.5 kV_{p-p}.

Optical images acquired on bare and PEOA coated (5 min) PC before and after 3 days of water, soaking is reported in Figure 7. The images show coatings characterized by a certain roughness, and no relevant morphologic changes after water soaking up to 3 days (Figure 7c,d), attesting to acceptable stability of the PEOA coatings in water.

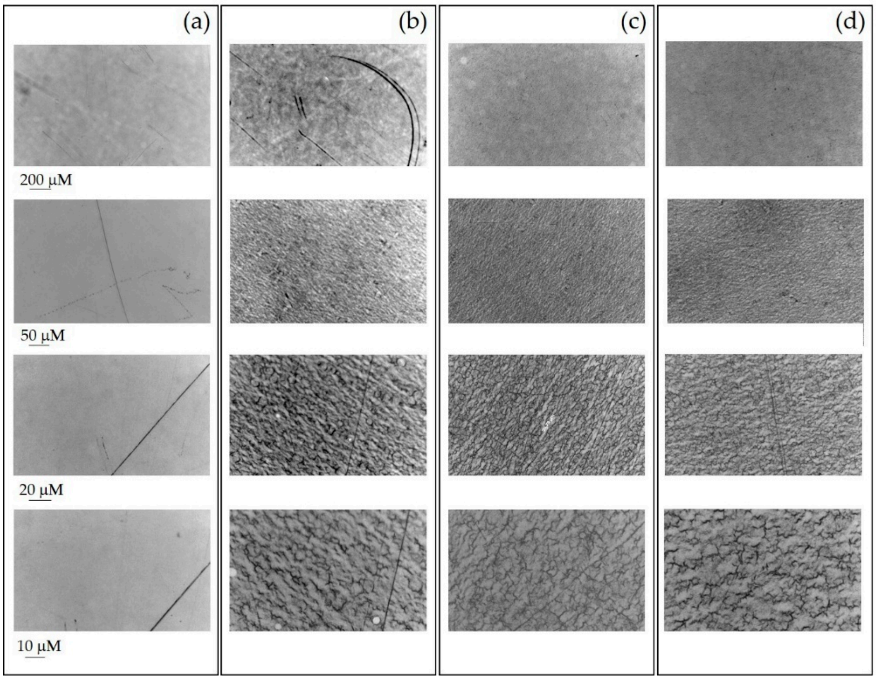

Figure 7. Optical microscopy pictures acquired on untreated PC (**a**) and PEOA coated one (3.15 slm He/TEGDME aerosol flow, 4.85 slm He carrier flow, 8.5 kV_{p-p}, f 26 kHz, 5 min) before (**b**) and after 5min (**c**) and 3 days (**d**) of soaking in double-distilled water. Pictures in the same rectangle report images acquired at different magnitudes.

The C1s best fitting data of Figure 8 show a slight decrease in the PEO-character from 70% to 67% for the PEOA coatings as the soaking time increases, while a constant value of 55% was measured for the PEOB coatings. The XPS results are in good agreement with the morphological characterizations reported in Figure 7, both indicating good stability of the coatings in water media, as well as a stable adhesion to the polymer substrate underneath.

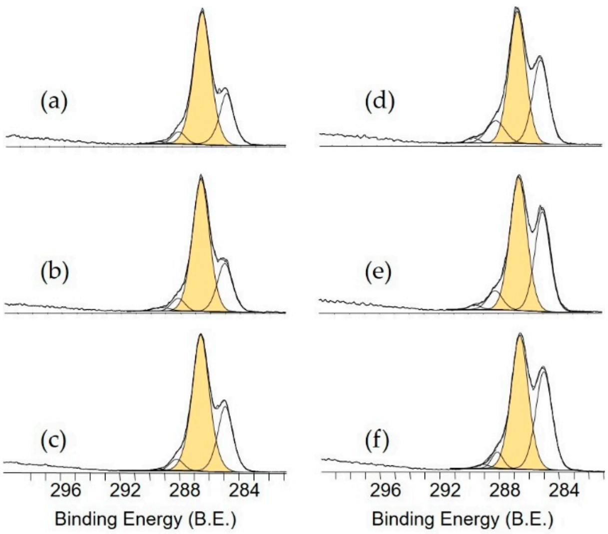

Figure 8. C1s best fitting data for PEOA (**a**–**c**) and PEOB (**d**–**f**) single-layer coatings as-deposited (**a**,**d**), after 5 min (**b**,**e**) and after 3 days (**c**,**f**) in double-distilled water. The percentage contribution II (PEO-character) remain almost unchanged for PEOA (around 70% PEO-character) and PEOB (55% PEO-character) even after 3 days of water soaking.

Differently from the data reported above, after water soaking, when PEOA and PEOB single-layer coatings were used in cell growth experiments, clear delamination of the coating from the substrates was observed after 4–5 h of culture in DMEM at 37 °C, as clearly visible in Figure 9. This phenomenon occurs whatever the chemical composition of the PEO-like coating is investigated.

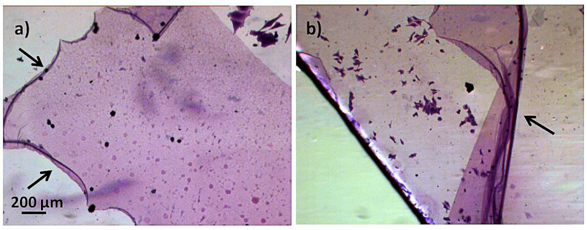

Figure 9. Delamination of PEOA (**a**) and PEOB (**b**) from the PC substrate (black arrows) under cell-growth conditions. PEOA (3.15 slm He/TEGDME aerosol flow, 4.85 slm He carrier flow, 8.5 kV_{p-p}, f 26 kHz, 5 min); PEOB (3.15 slm He/TEGDME aerosol flow, 6.85 slm He carrier flow, 8.5 kV_{p-p}, f 26 kHz, 5 min).

To improve the interface stability of PEO-like coatings deposited in AP plasma conditions in cell-culture experiments, a multi-step deposition process was carried out to obtain a vertical chemical gradient from the bottom, at the substrate and film interface, to the top side of the film, at the film and air or liquid interface.

The LP plasma deposition of coatings characterized by a vertical chemical gradient has been recently reported [31], to improve the adhesion of the coatings to a substrate. We show the AP PE-CVD synthesis of a vertical gradient through 3-layer deposition approach, starting from a process fed with only C_2H_4, followed by deposition with a mixture of C_2H_4 and TEGDME, and topped with deposition of only TEGDME. The three processes are run in continuo in the DBD reactor. We believe that this approach can be used in many cases to improve the adhesion between a coating and a substrate or to gradually increase the content of certain functionalities going towards its topmost layer, as shown by other authors with LP PE-CVD processes [32].

Figure 10 describes how thickness and WCA develop for two different PEOA coatings deposited at two different frequencies (see Table 1) on PC substrates with similar trends. After step 1, a cross-linked polyethylene-like (PE-like) hydrocarbon layer is deposited from the C_2H_4/He feeds, resulting in a 60 nm thick hydrophobic coating (WCA: 81 ± 2°). Step 2 from TEGDME, C_2H_4, and He resulting in a 20 nm thick coating characterized by WCA of 68 ± 2°. Step 3, finally, leads to the deposition of the PEOA film 180 nm thick showing a WCA value of 53 ± 3°. The WCA values are the same at the two frequencies used.

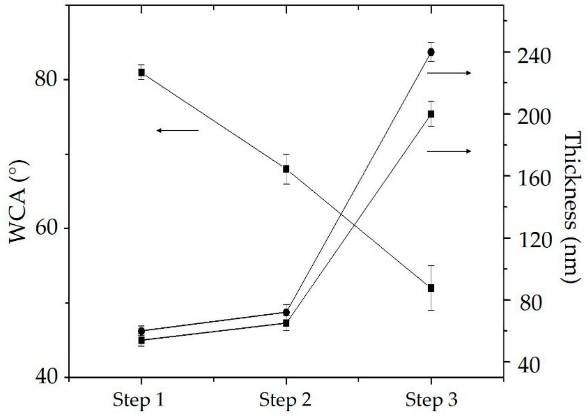

Figure 10. Evolution of WCA values and thickness of the vertical gradient film after the three-step AP PE-CVD deposition process carried out using an applied voltage of 8.5 kV_{p-p}, and frequency of both 26 and 16 kHz. The topmost layer deposited in step 3 corresponds to the PEOA film (step 3: 4.85 slm He-carrier, 3.15 slm He-TEGDMEaerosol, 8.5 kV_{p-p}, 300 s; see Table 1).

The AP deposition of PEO-like films has been performed by implementing the three steps described above. The effect of the frequency and the applied voltage was evaluated on the uppermost PEO-like layer, using in step 3 a carrier flow rate (ϕHe-carrier) of 4.85 slm (PEOB) coatings or 6.85 slm (PEOA coatings). Through different ϕHe-TEGDME aerosol/ϕHe-carrier ratios, therefore, different PEO-characters for PEOA and PEOB were obtained. The C1s best fittings of PEOA and PEOB coatings deposited at different frequencies and applied voltage values are reported in Figure 11.

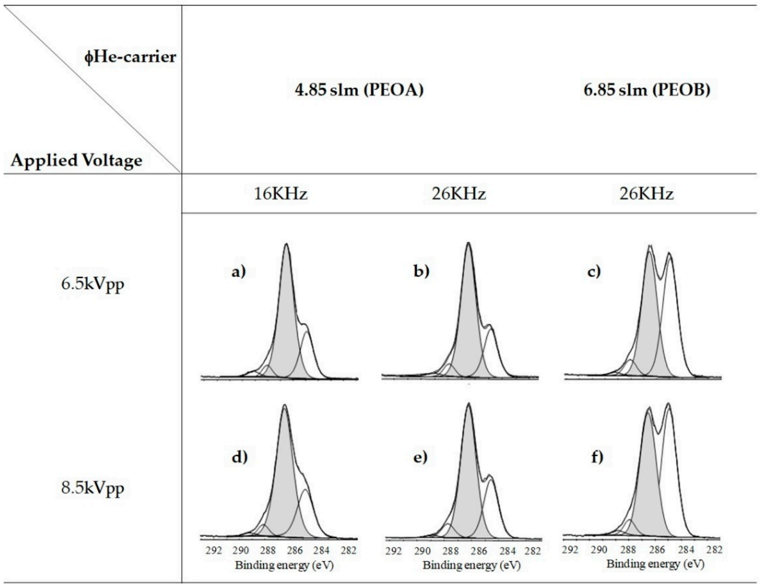

Figure 11. Best fitting of C1s spectra acquired on PEOA (**a**,**b**,**d**,**f**) and PEOB (**c**,**f**) coatings deposited at 16 kHz (**a**,**d**) and 26 kHz (**b**,**c**,**e**,**f**) by changing the applied voltage from 6.5 kV_{p-p} (**a**–**c**) to 8.5 kV_{p-p} (**d**–**f**). The ϕHe-TEGDME aerosol is fixed to 3.15 slm.

As observed earlier (Figure 8), the PEO-character of the PEO-like layer can be modulated by changing the relative flow of TEGDME aerosol in the discharge with respect to the He carrier gas [28]. This is observed in Figure 11 and is also shown for the uppermost PEO-like coatings deposited at the end of the three steps approach [28]. When deposited with ϕHe-carrier of 4.85 slm the coating exhibits a good monomer structure retention, with 70% of PEO-character (II component of Figure 11a,b,d,e) whatever other experimental conditions used. Instead, a higher ϕHe-carrier (6.85 slm) led to deposit PEO-like film with about 50% of PEO-character (Figure 11c,f). The effect of ϕHe-TEGDME aerosol/ϕHe-carrier ratio influenced the chemistry of the coating much more than the electrical parameters like excitation frequency and applied voltages, in the ranges investigated. Slight differences are reported, in fact, for coatings deposited at two different peak-to-peak voltages (6.5 and 8.5 kV_{p-p}) or frequency values (16 and 26 kHz) by keeping the other experimental parameters constant. This corresponds also to very slight wettability (WCA) differences in the coatings, that have WCA of about 50° whatever the plasma condition used (Table S2). The deposition rate of these coatings is around 30 $nm\cdot min^{-1}$ (Table S2).

AR-XPS analyses for PEOA samples indicated a progressive increase in the O/C ratio with the decrease in the sampling depth (Figure 12). The topmost layer shows a chemical composition and a C1s spectrum (Figure 12) very similar to PEO-like coating deposited in the single-step process reported in Figure 8.

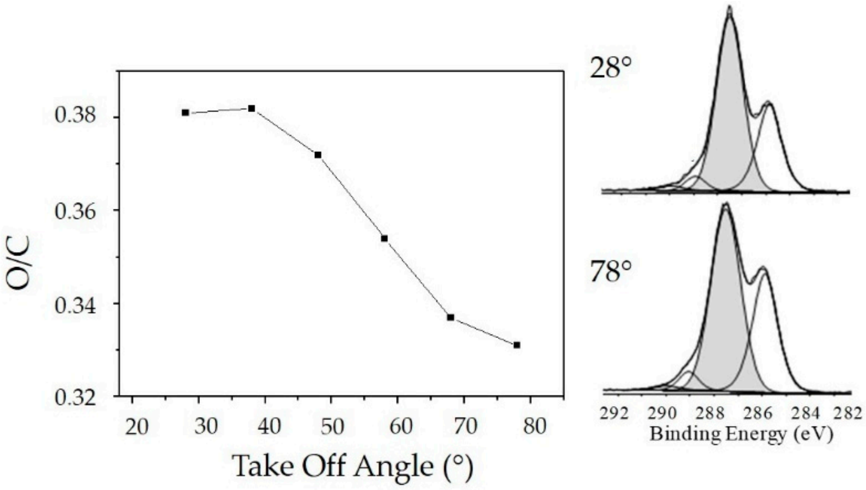

Figure 12. O/C ratio for PEOA (Step3: 4.85 slm ϕHe-carrier, 3.15 slm ϕHe-TEGMDE aerosol, 8.5 kV_{p-p}, 27 kHz, 300 s) measured in angle-resolved (AR) mode and C1s best-fitted spectra from the lowest (TOA 28°) to the highest sampling depth (TOA 78°). The II C1s contribution attributed to the PEO-character changes from 69% (TOA 28°) to 55% (TOA 78°) at deeper sampling depth.

In vitro, biological experiments were carried out on PEOA and PEOB coatings deposited on PC as reported in Figures 13 and 14. These cell adhesion tests allow us to assess both the stability in complex water media (i.e., cell culture medium) and the biological responses to the coated PC with the three steps strategy. The cell repulsive films deposited in LP PE-CVD (PEO_LP, 0.5 sccm DEGDME, 5 sccm Ar, 53 Pa, 5 W, 60 min) with a PEO-character of 70% have been used as a reference.

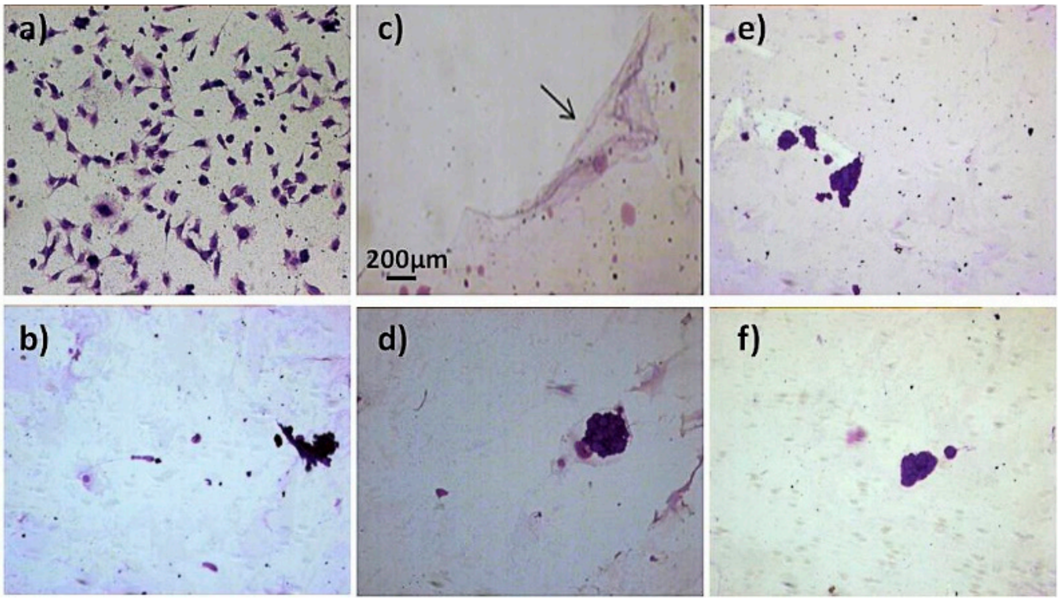

Figure 13. Cells fixed after 24 h of culture and colored with Comassie blue growing on different substrates: (**a**) Native PC; (**b**) PEO-like with 70% PEO-character, deposited at LP (PEO_LP, 5 W, 0.5 sccm DEGDME, 5 sccm Ar, 53 Pa, 60 min); (**c**) PEOA with 71% PEO-character deposited at AP (16 kHz, 6.5 kV_{p-p}, the arrow indicates the detachment of the film); (**d**) PEOA with 69% PEO-character deposited at AP (16 kHz, 8.5 kV_{p-p}); (**e**) PEOA coatings with 70% PEO-character deposited at AP (26 kHz, 6.5 kV_{p-p}); (**f**) PEOA coatings with 66% PEO-character deposited at AP (26 kHz, 8.5 kV_{p-p}).

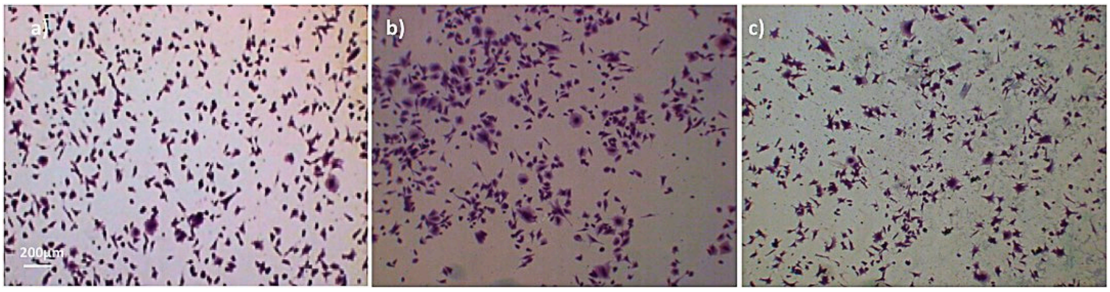

Figure 14. Osteoblast cells were grown after 24 h on different substrates: (**a**) PC native, (**b**) PEOB deposited at 26 kHz and 6.5 kV_{p-p} (**c**) PEOB deposited at 26 kHz and 8.5 kV_{p-p}.

As shown in Figure 13, cell culture data indicate that when the mildest AP PE-CVD conditions are used (i.e., lowest f and Va), the detachment of the coating from the underlying substrate is observed (Figure 13c). This effect can be, most likely, due to insufficient cross-linking of the PE-like primer coating (step 1 of the process). In fact, in this case, to avoid electrical instabilities of the discharge, the frequency remained the same during the three steps, and this could have led to a PE-like coating (step 1), and not enough cross-linked for optimizing the best adhesion of the coatings to be deposited in the next steps. When the process is carried out in higher fragmentation conditions of the feed (high Va or high f values), the resulting PE-like film, more cross-linked, remains attached to the substrate underneath and grants better adhesion of the layer to be deposited on top. An evaluation of the cell-adhesive and repelling character was carried out (Figure 13d–f). A clear non-fouling effect was tailored on these stable surfaces, due to their high PEO-character (~70%). Very few cells were found adhering to these coatings, all characterized by the globular morphology, assumed to minimize cell-substrate interactions. On the contrary, osteoblasts appear numerous and spread on native PC (Figure 13a) and PEOB coatings (Figure 14c).

The cells' behavior on PEO-like coatings deposited at AP PE-CVD in low monomer fragmentation conditions (i.e., low ϕHe-carrier, PEOA) could thus definitively be assumed comparable with those deposited in low fragmentation conditions at LP (PEO_LP, Figure 13b).

After 24 h of culture (Figure 14), many osteoblast cells could be found spread across PEOB surfaces (PEO-character lower than 70%) obtained at a higher carrier flow rate (ϕHe-carrier: 6.85 slm) indicating cell adhesion affinity similar to the control (not treated PC). Additionally, in this case, the strategy of three deposition steps in continuo resulted in successfully obtaining a three layer gradient coating stable in cell culture conditions. The results in terms of cell adhesion clearly show that no difference can be found in cell behavior on PEO-like coatings obtained with LP and AP plasma depositions, provided that at least 70% of PEO-character is obtained.

4. Discussion

Traditionally, studies on plasma polymerization of PEO-like coatings were focused on LP PE-CVD because they provide excellent control over the gas chemistry, and allow use of high energetic reactive species like positive ions bombarding the surface of substrates [11,15]. At present, though, following the development of processes and equipment for AP PE-CVD, many research groups are developing non-thermal equilibrium (cold) plasma processes for plasma deposition of PEO-like coatings in AP conditions [16]. Engineering coatings for biomedical applications implies, more stringently than for other applications, that controlled composition and adequate stability of the coating on the substrate are granted for the whole time of application in biological media like, for example, cell culture media.

Delamination of the coating, changes in chemical composition, and uncontrolled cell adhesion can occur if the process is not properly optimized, and the final function of the plasma modified surface could be compromised.

The plasma is characterized by different active species with energies up to tens of electron volts (eV), which provide sufficient energy to break most of the chemical bonds of the organic precursor monomer employed in this technology. The plasma polymeric films obtained are usually highly cross-linked, thermally stable, pinhole-free, highly conformal, and strongly bound to most substrates [11,33]. LP and AP plasma deposition processes, though, have different features, that need to be addressed for properly optimizing the properties of the treated surfaces. As an example, LP deposition of soft organic coatings on inorganic substrata could produce unstable films due to slight ion bombardment of the underlying substrate, while in the case of using thick plastic substrates in AP we could promote some electrical instabilities that are reflected in the coating composition if the substrate is included in the electric circuit [1].

In this paper, we have shown two different approaches to deposit PEO-like coatings with chemical characteristics able to discourage (non-fouling) or promote (cell-adhesive) cell adhesion.

Monomers with high boiling points have been used to produce, in LP, cell-repulsive or cell-adhesive coatings that were stable both in water and in cell culture medium during cell culture experiments. Following strategy 1 of Figure 1, PEO-like coatings from DEGDME vapors were deposited at different input power values of the process, resulting in a PEO-character that decreases as the input power increases.

To add and modulate cell-adhesive character in the coatings, DEDGME vapors were mixed with VAc ones following strategy 2 of Figure 1: in this way, COOH groups were included during the growth of PEO-like coatings. The use of VAc as the monomer is nearly absent in literature; indeed, it offers the advantage of a double bond in its structure, that allows easier polymerization through the conventional mechanism, and ester/carboxyl groups (-COOR (H)) useful to promote cell-adhesion [20]. In Figure 2, it is demonstrated that the chemical composition of coatings deposited in LP PE-CVD can be tuned by using VAc/DEGDME feed mixture with DEGDME flow lower than 0.5 sccm, low input power, without Ar as a buffer gas. In this way stable coatings are produced, that resist to water immersion, useful for biomedical applications. Sticky oily coatings may result when high boiling point monomers are used in high fraction (i.e., ϕDEGDME > 0.5 sccm) if the experimental conditions are not properly selected and controlled (i.e., low input power and absence of a carrier gas), due to low fragmentation and condensation of the monomer and oligomer molecules on the substrates. Although non-sticky coatings were deposited at LP with 0.5 sccm of DEGDME in the gas mixture, these coatings, when deposited at the lowest input power values investigated (i.e., 5 W) needed for low fragmentation and high retention of the structure of the monomer, poor stability in water was recorded, as reported in Table S1, thus attesting once again the need of more energetic conditions for a tight bond to the substrate and more cross-linking. However, increasing the input power from 5 to 50 W, by following strategy 1 of Figure 1, decreases the COC and increases the carbonyl contribution (Figure 3a) in coatings deposited from only DEGDME. The rearrangement of ethers and their conversion into carbonyl groups probably occur due to the high density of radicals produced at high input power values needed for low fragmentation and high retention of the structure of the monomer, poor stability in water was recorded, as reported in Table S1, thus attesting once again the need of more energetic conditions for a tight bond to the substrate and more cross-linking. At present, many research groups are developing cold AP plasma processes and reactors to conduct plasma polymerization processes of PEO-like coatings [16,34]. Many different types of AP plasma reactors are being developed, e.g., DBD reactors with various electrode configurations (e.g., classical parallel-plate geometries, surface DBD, etc.) plasma jets, and torches. These are suitable candidates for replacing traditional coating techniques, due to their simple scalability, and limited need of vacuum pumps. The control over the plasma polymerized film properties has improved greatly in the last few years; nevertheless, often hydrophilic films on hydrophobic surfaces can undergo delamination in water media when deposited in AP plasma conditions [34]. Moreover, a higher monomer structure retention is generally expected in AP plasmas with respect to LP ones, particularly when the monomer is injected in form of an aerosol. The deposition of PEO-like coatings, in aerosol, assisted DBD, produces rough coatings in our experimental conditions, probably due to the contribution of aerosol droplets to the polymerization or deposition of the monomer vapors that can lead to the formation of aggregates. [35,36]. Stallard et al. [37] deposited PEO-like coatings on Si wafer surface using TEGDME and diethylene glycol vinyl ether monomers in a non-equilibrium AP plasma jet and reported the formation of particulate in the process as a result of a high degree of polymerization. Further experiments are necessary to assess the mechanism of formation of these micro-nano-structures, as that is far from the aim of this paper.

The results reported in Figure 11 clearly show that the PEO-like coatings deposited at lower carrier flow (higher TEGDME concentration in the discharge) had higher percentages of PEO-like CH_2CH_2O moieties, whatever the applied voltages investigated, similar to those obtained in LP conditions. By contrast, at a higher carrier gas flow rate, the precursor molecules underwent greater fragmentation, likely due to the higher density of He active species, which provide sufficient energy to break most chemical bonds in monomer, resulting in increased cross-linking and lower PEO-character of the deposited coating. These results are similar to those obtained when high input power is used in LP processes. It has been demonstrated by XPS that the PEOA and PEOB coatings with different PEO-character are stable after 3 days of double distilled water soaking when deposited in a single step process but, during cell culture experiments, they delaminate to a certain extent. Since the detachment was constantly observed after 4–5 h of cell-culture and no detachment was observed during the water stability tests described previously, we attribute this effect to the pulling force exerted by the adhering cells on the coatings during the early time of growth. This effect was already observed in [38] on conventional PEO polymers. Considering that similar behavior has been observed for our non-fouling coatings (i.e., PEOA of Figure 9) where almost no cells were observed on them, the delamination of the coatings could be likely due to components of the cell culture medium (i.e., ions, proteins) that may change the interfacial liquid and coating interactions and promote its detachment from the underlying substrate. Similar behavior was observed for conventional PEO films deposited by spin coating [39,40]. In literature, it is reported that PEO-like films swell in PBS of approximately 20%, as revealed by in situ liquid-cell ellipsometry measurements at a time scale of several minutes [41]. Owing to the repetition of CH_2CH_2O ether groups, it is known that PEO forms complexes with various metal cations in organic solvents [42]. Complex formation between conventional PEO and such metal cations in water have been reported in the literature [40], demonstrating that PEO coatings show unique swelling behavior in aqueous salt systems. As noted earlier, since the chemical nature of oxygen in PEO is different concerning other hydrocarbon polymers, like polyvinyl alcohol, the interaction with metal cations present in the cell culture medium results in different swelling behavior. These findings on conventional PEO could explain also the behavior observed on plasma deposited PEO-like coatings [40].

The multi-step approach proposed in this paper for AP plasma processes was revealed to be a successful strategy to improve the resistance of the coating to delamination during in vitro cell-adhesion tests. AR-XPS analysis (Figure 12) performed on three-layer PEOA coatings shows that also in the last layer (~200 nm thick, Figure 10) there is a gradual enrichment of the O-containing moieties toward the topmost layer. The topmost layer shows a composition very similar to the PEOA deposited in a single step and to that deposited at LP. It is important to notice that the last deposition step, step 3, was carried out in-continuous, without interrupting the discharge, after closing the on-off valve of C_2H_4 flow to the reactor. This process produces a vertical chemical gradient in the last layer due to the progressive consumption of C_2H_4 after the closure of the on-off valve that connects the plasma chamber to the C_2H_4 line. In fact, it is supposed that 5 min of discharge, is not enough for purging or consuming unreacted ethylene and its active species produced in the previous steps. Thus, probably ethylene contributes actively in a co-deposition with TEGDME in the last step going toward a topmost layer richer of COC motifs when C_2H_4 is completely consumed. Almost all AP coatings resulting from the multilayer approach are stable in water and during cell culture experiments, except PEOA deposited at 16 kHz and 6.5 kV_{p-p} that, similarly to AP coatings deposited in a single step approach, delaminates during cell culture experiments (Figure 13c). The improvement in film adhesion is due to a hydrophobic interlayer, like the substrate below, well attached to it, thanks to a plasma polymerization process that involves ethylene which is a highly reactive precursor. If soft conditions are used (i.e., low frequency and low peak-to-peak voltage) this strategy fails, probably due to a reduced crosslinking in the film.

5. Conclusions

By comparing the results of the chemical and biological tests of the coatings presented in this paper, it is possible to assess that the attitude of plasma deposited PEO-like coatings, to discourage cell adhesion, tightly depends on their PEO-character, both in the case of LP and AP PE-CVD films. Aerosol assisted AP PE-CVD of non-fouling PEO-like coatings is one order of magnitude faster than the process run at LP (few minutes' vs one-hour). LP PE-CVD, though, allows depositing coatings with better adhesion to the substrate concerning AP processes. To overcome this limit, a multistep AP PE-CVD approach has been proposed in this paper, which led to a dramatic improvement of the stability of PEO-like coating in cell culture experiments. These findings show once again that a correct activation of the underlying substrate, a tightly bound film, and substrate interface, as well as a certain cross-linking degree of the coatings, grant satisfactory film and substrate adhesion also in complex media like cell-culture ones. These characteristics need to match with the correct retention degree of the monomer structure in the coating through a careful optimization of any LP or AP plasma process used. The use of two monomers in soft plasma conditions at LP (i.e., low input power, low carrier gas flow) guarantees the retention of the chemical structure of the monomers, allowing the co-presence of domains with a certain moiety (i.e., ether groups) and domains characterized by another (i.e., ester groups). In soft fragmentation conditions, though, the use of monomers with very low volatility (high boiling point) could compromise this effect. The mixture of two monomers in AP conditions can improve the stability of the coatings in complex media by inducing proper cross-linking, as demonstrated in this paper for coatings deposited by mixtures of ethylene and TEGDME (steps 1 and 2) before depositing the uppermost PEO-like layer.

The results obtained demonstrate, once again, that properly optimized plasma processes are valid alternatives to conventional coating methods to produce non-fouling and cell adhesive surfaces. When PE-CVD is oriented toward the biomedical field, additional attention is to be paid to control mechanical stresses and material and liquid interactions that generally occurs at the interface with biological entities, that drive, in the end, the success of the surface in the final application.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3417/10/24/9101/s1, Figure S1: representative survey spectra, Table S1: XPS chemical composition of PEO-like coatings deposited at LP, Table S2: C1s components, WCA and deposition rate for PEOA and PEOB coatings deposited in AP.

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