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Characterization and Comparison of N-, O- and N+O-functionalized Polymer Surfaces for Efficient (HUVEC) Endothelial Cell Colonization

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Summary :

Surface modifications are often required to enhance cell adhesion and growth around implanted biomaterials. This study compares various functionalization processes in their ability to create high densities of oxygen- and/or nitrogen-containing functional groups, mostly on a polymeric biomaterial, polyethylene terephthalate (PET). Primary amine (NH_2)-rich surfaces were prepared by low-pressure plasma-polymerization (L-PPE:N), plasma modification (functionalized PET, "PETf"), chemical vapour deposition (Parylene diX AM), and grafting of polyallylamine (PAAm). Plasma polymerization was also used to obtain oxygen-rich (L-PPE:O) as well as hybrid (L-PPE:O,N) films, which were respectively compared to oxygen-rich tissue culture polystyrene (TCP) and hybrid (Primaria™) culture plates. Compositions and bond types were studied by X-ray photoelectron spectroscopy. Finally, the effect of each surface on cell adhesion and growth was assessed using human umbilical vein endothelial cells (HUVECs). Amine-containing surfaces manifested a wide

[NH_2] range, up to 8.9 %. Hybrid surfaces, Primaria™ and L-PPE:O,N, showed lower [NH_2] in spite of high [N], suggesting more varied and complex functionalities. Except for Parylene, all O- and NH_2-rich surfaces promoted HUVEC adhesion and growth similarly, despite differing chemical compositions.. Primaria™ showed the best cell behaviour, but L-PPE:O,N did not reproduce this apparent synergistic effect. To conclude, both N- and O-rich surfaces displayed good cell-colonization properties, particularly plasma polymers, while "hybrid" surfaces appear somewhat ambiguous and call for further investigation.

1 Introduction

A very extensive technical and scientific literature bears witness to the fact that commercial polymers, as-received from vendors, generally perform poorly in most applications involving their surface properties, including biomedical applications.[1,2] The main reason for this is their low surface energy, concomitant poor wettability and inability to form strong adhesive bonds. However, a sizeable and growing portion of that literature, some going as far back as the 1950s, teaches us that *modifying* the outermost surface region of a given polymer can greatly enhance its wettability, its ability to form strong bonds, even its biocompatibility, without adversely affecting the desirable bulk properties of the material.[1–4] Among the most powerful surface-modification techniques is the exposure of a polymer surface to "cold" electric discharge plasmas, either at atmospheric pressure (AP) or under partial vacuum, hereafter "low pressure" (LP).[2,4] Indeed, there now exists a vast array of commercialized AP and LP plasma processes for surface modification of polymers, so many that we shall restrict our discussion only to the ones that apply directly to biomedical applications. A good example is laboratory wares made of medical-grade polystyrene. Some quite recent patents,[5,6] as well as many older ones, describe how LP plasmas can modify cell-culture dishes to enhance cell

adhesion. In the following text we shall refer to "tissue culture polystyrene" (TCP), a surface with a high concentration of bonded oxygen, [O], and to Primaria™, the trade name of a product with both high [O] and nitrogen concentration, [N]; these products will both be discussed in more detail later.

In addition, there are many other biomedical applications of polymers that call for efficient cell colonization; the case of particular interest in the present authors' laboratories are synthetic vascular grafts (VGs), commonly made of polyethylene terephthalate (PET) or expanded polytetrafluoroethylene (ePTFE).[7,8] Small-diameter (< 6 mm) VGs tend to fail due to hyperplasia and thrombosis, which lead to occlusion of the blood vessel.[9–11] The likelihood of such events can be minimized by a full endothelialization of the graft luminal walls;[12] therefore, various surface treatments have been proposed in order to foster endothelial cell (EC) adhesion and proliferation, either directly since surface properties such as roughness, chemical composition and associated surface energy, and electrical charge are all known to affect EC behaviour,[3,13–15] or indirectly through immobilization of certain biomolecules on the surface.

In "*Plasma Methods for the Generation of Chemically Reactive Surfaces for Biomolecule Immobilization and Cell Colonization - A Review*",[16] Siow et al. described the state of knowledge up to about ten years ago. Important for the present context, the authors reviewed not only *surface modification* of polymers via cold plasma treatments, but also the even more powerful technique of *plasma polymerization*, whereby a thin organic coating with tailored composition and properties can be applied to virtually any desired solid surface, not only polymers. The authors particularly emphasized surfaces with O- and N-based functional groups. Indeed, primary amine groups (NH_2) are particularly well known to enhance cell adhesion,[16–19] likely due to their positive charge at physiological pH that allows them to attract negatively-charged biomolecules like adhesive glycoproteins (fibronectin, vitronectin), which adsorb on the surface and in turn enhance cell binding via integrin receptors at the cell

surface. Thus, a low-pressure plasma-polymerized primary amine-rich coating, "L-PPE:N", developed in our laboratory,[17] has proven very efficient in promoting the adhesion and proliferation of U937 monocytes,[20] of vascular smooth muscle cells (VSMCs)[21] and of endothelial cells (EC),[22] as well as for improving resistance of the latter to shear stress. Amine functional groups also widely serve to covalently graft other biologically active molecules.[23–25]

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In a similar manner, O-containing surface functionalities are also of great interest to support cell colonization, as illustrated by the example of TCP, often used as a positive control reference for cell attachment and growth. Even better performance has been reported for the "hybrid" commercial surface, Primaria™, that combines both N- and O-bearing functional groups.[26,27] This would suggest a complementarity or a synergistic effect of O- and N-based moieties, although there are contradictions in the literature regarding the chemical composition of Primaria™.[6,27–29] Therefore the present article will focus exclusively on O- and N-functionalized polymer surfaces (including their combination, as in Primaria™). Various processes enabling the creation of N- (or rather NH_2)-rich, O-rich or hybrid (O+N)-rich surfaces, were compared in regard to their chemical compositions and their efficacy for promoting endothelial cell (EC) colonization. Low-pressure plasma-polymerization ("L-PP") was carried out on polyethylene terephthalate (PET) film substrates to create thin coatings of plasma-polymerized ethylene with bonded N ("L-PPE:N"), bonded O ("L-PPE:O"), or a combination of the two ("L-PPE:O,N"). Other amine-rich surfaces were obtained by plasma-modifying PET (plasma treatment in NH_3, "PETf"), by chemical vapor deposition (Parylene diX AM),[30] or by covalent grafting of polyallylamine ("PAAm") by wet chemistry. Commercial cell culture surfaces (TCP and Primaria™) were investigated for comparison.

2 Experimental Section

2.1 Materials and Chemicals

50 µm-thick PET film samples, obtained from Goodfellow (Huntington, England), were first cleaned by sonication in ethanol for 15 min before use. Tissue-culture polystyrene plates, TCP (Falcon^{R}) and Primaria™ (both from Corning Inc.), as well as Crystal Violet, were purchased from Fisher Scientific (Ottawa, Canada). Resazurin dye was obtained from Biotium. Ethylene (C_2H_4), ammonia (NH_3), nitrous oxide (N_2O) and oxygen diluted in argon (O_2/Ar) gases (purity > 99.9%) were purchased from Air Liquide (Montreal, Canada). Polyallylamine (PAAm, 15% w/v in water, M.W. = 15 000), 1,4-dioxane (anhydrous, 99.8%, M.W. = 88.11 g/mol), NaOH, KCl, 4-(trifluoromethyl)benzaldehyde (TFBA, 98%) and methanol (anhydrous, 99.8%) were all purchased from Sigma Aldrich (Oakville, Canada). Phosphate buffered saline (PBS) was obtained from Wisent Inc. (Saint-Jean-Baptiste, Canada), hydrochloric acid (HCl, 37.7% v/v) from VWR International Ltd. (Mont-Royal, Canada), and sterile water from Baxter (Mississauga, Canada).

2.2 Functionalized Surfaces

2.2.1 Plasma Polymerization

Plasma polymerization was carried out on PET film samples in a capacitively-coupled (13.56 MHz, ENI) radio-frequency (r.f.) glow discharge plasma reactor. The plasma was created in a cylindrical aluminium /steel vacuum chamber, approximately 20 cm in diameter and 20 cm high; this system was aleady illustrated earlier, so that there is no need for repetition.[17] A turbo-molecular pump, backed by a two-stage rotary vane pump was used to evacuate the

chamber to a base pressure of < 10^{-4} Pa. Flows of high-purity feed gas were then admitted into the chamber using electronic flow meter/controllers (Vacuum General Inc.) and a "shower head" gas distributor (10 cm in diameter). Plasma was generated under mild conditions, with applied r.f. power P = 10 W (resulting in a negative d.c. bias voltage, V_B = -40V), and at an operating pressure p = 80 Pa, maintained constant by a butterfly throttle valve, in combination with a capacitive pressure gauge (Baratron, MKS Instruments). Various reagent gas mixtures were used as follows: ethylene (C_2H_4) was combined with ammonia (NH_3), molecular oxygen diluted in argon (10% O_2 + 90% Ar), or nitrous oxide (N_2O), to respectively obtain low-pressure plasma-polymerized ("L-PP") thin (≈ 100 nm thick) coatings containing N ("L-PPE:N"), O ("L-PPE:O"), or both ("L-PPE:O,N"). The flow rate of C_2H_4, $F_{C_2H_4}$, was kept constant at 20 standard cubic centimeters per minute (sccm), while the other reagent gas flow was varied; the gas flow ratios R (e.g., $F_{NH_3}/F_{C_2H_4}$) were respectively R_{NH_3} = 0.75; R_{O_2} = 0.15; and R_{N_2O} = 0.6. Note that O_2 was diluted in argon to minimize the occurrence of competing ablation (etching) by atomic oxygen. The above-cited R values, based on our earlier work,[31–33] yielded coatings with maximum hetero-atom content and with low solubility in water or cell culture medium. N_2O-based coatings had not been investigated earlier, but this was done as part of the present work; for lack of space it has been decided not to present further details, but coatings corresponding to R_{N_2O} values up to 1.0 were found to be virtually insoluble.

2.2.2 Plasma Modification

Plasma modification of PET films ("PETf") was carried out in the same system and under similar conditions, but with exposure to NH_3 plasma alone, for a 10-min treatment duration.

2.2.3 Aminolysis

PAAm was grafted onto PET film samples by an aminolysis reaction, using the protocol adapted from Noel et al. (**Figure 1**).[34,35] PAAm was added to an alkaline solution of NaOH and KCl (1:1, 100 mM in Milli-Q water), then mixed with 1,4-dioxane (3.8 : 69.5 : 26.7% v/v). Prior to dioxane addition, pH was adjusted by progressively adding a few µL of HCl (12.3 M). PET films were then immersed in the resulting PAAm solution. The reaction was carried out for 24h at 70°C, after 20 min of sonication. Finally, the treated PET samples were successively rinsed in Milli-Q water, methanol, and again Milli-Q water.

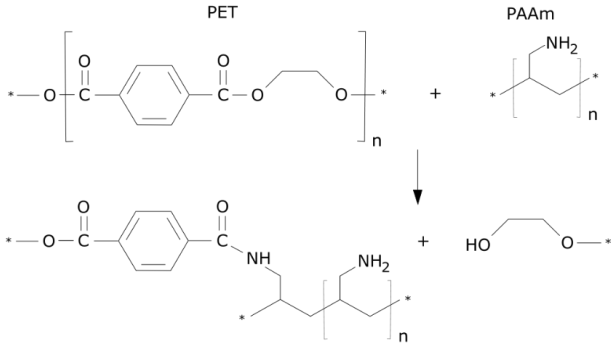

Figure 1. Aminolysis reaction between PET and PAAm

2.2.4 Chemical Vapor Deposition

Parylene diX AM samples roughly 100 nm in thickness (**Figure 2**) were prepared by chemical vapor deposition on biaxially oriented polypropylene (BOPP) substrates, with an intermediate "inert" Parylene C layer (500 nm thick); they were graciously prepared and sent by Drs. B. Elkin and C. Oehr (*Fraunhofer* Institute for Interfacial Engineering and *Biotechnology* IGB, Stuttgart, Germany). To avoid aging,[36] they were shipped under inert gas (Ar) and subsequently stored at -80°C. Parylene deposition was invented and patented by W.F. Gorham,[37] later described in detail by Lahann et al.,[30] so that details of the process need not be repeated here.

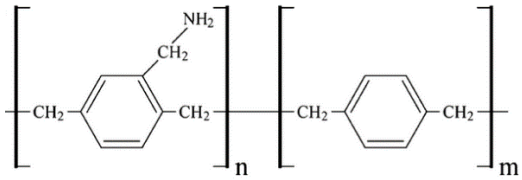

Figure 2. Chemical structure of Parylene diX AM (poly-aminomethyl-[2,2]-paracyclophane)

2.2.5 Commercial cell culture plates

TCP (Falcon™) and Primaria™, both from Corning Inc., were also included in this study. For analytical characterization, both Petri dishes and 24-well plates were used, samples being obtained by cutting the products into ≈ 0.5 cm^2 pieces that could fit into the XPS instrument. For purposes of cell culture, 24-well plates of both products were used, because Primaria™ was unavailable in 48-well plate format.

2.3 Surface-analytical Studies

2.3.1 X-Ray Photoelectron Spectroscopy

Surface chemical compositions of all samples were determined by X-ray Photoelectron Spectroscopy (XPS) using a VG ESCALAB 3MkII instrument with non-monochromatic Mg Kα radiation (hν = 1253.6 eV).[38] Overall compositions were determined on hand of survey spectra, while the binding states of the hetero-atoms, oxygen (O) and nitrogen (N), were determined from high-resolution C1s spectra, as elaborated further below.

The emitted photoelectrons were captured at various angles with respect to the surface-normal (known as *Angle-Resolved* XPS, AR-XPS), namely 0°, 45, 70 and 80° (grazing angle), in order to analyze the extreme outermost surface region of plasma-modified samples (PETf, TCP, Primaria™), the region accessible to the proteins and cells. The effect of surface roughness (in the nm rms range) on the detected photoelectrons at grazing angles was considered to be insignificant, therefore neglected. In the case of plasma polymer coatings,

the composition is known to be constant throughout the ca. 100 nm thickness.[39] Surface charging during XPS was corrected by referencing all peaks to the carbon (aliphatic C-C, C-H) C1s peak at binding energy BE = 285.0 eV, and elemental compositions were obtained using Avantage v4.12 (Thermo Electron Corporation) by integrating the area under a specific peak after a Shirley-type background was subtracted, and by using sensitivity factors from the Wagner table.[40]

Bond types were identified and quantified by analyzing the high-resolution (HR) C1s sub-peaks, using CasaXPS v2.3.16 (CasaSoftware Ltd., Teignmouth, England). The identified components and their relative binding energies and full width at half maximum (F.W.H.M.), along with the constraints associated in the deconvolution of C1s peaks, are presented in **Table 1**. Note that peak A includes all carbon-carbon bonds, including unsaturated ones, because their binding energies are very close; F.W.H.M. for F is not cited because this peak is inherently broad. The constraints on the C-O position and width have been slightly increased in order to avoid overlapping with the C-N peak.

Table 1. Sub-peaks used for the deconvolution of C1s peaks

Peak	Bond	Energy E_b (eV)	F.W.H.M. (eV)
A	**C-C/C=C**	285.0 ± 0.2	1.5 – 1.7
B	**C-N**	286.0 ± 0.2	1.5 – 1.7
C	**C-O**	286.5 ± 0.1	1.5 – 1.6
D	**C=O/N-C=O**	288.0 ± 0.2	1.5 – 1.7
E	**COOR/H**	289.3 ± 0.2	1.5 – 1.7
F	**Shake-up**	291.8 ± 0.2	-

In the case of N-containing surfaces, XPS analyses were also conducted after chemical derivatization with 4-(trifluoromethyl)benzaldehyde (TFBA), in order to quantify primary amine concentrations [$-NH_2$]:[41] samples were exposed to TFBA vapor in a closed glass vessel for 3h at 45°C, thereby selectively covalently linking TFBA to primary amines ($–NH_2$) via imine bonds. The treated samples were then analyzed by XPS, and [NH_2] content was

deduced from their fluorine concentration, [F], using equation (1),[17] where subscripts 'u' and 'd' respectively refer to underivatized and derivatized samples:

$$[\mathrm{NH_2}]_\mathrm{u} = \frac{[\mathrm{F}]_\mathrm{d}[\mathrm{N}]_\mathrm{u}}{3[\mathrm{N}]_\mathrm{d}} \times 100 \qquad (1)$$

In the authors' very extensive experience with XPS analyses, they have never had cause to suspect significant radiation damage (e.g. fluorine loss) by incident X-ray photons. However, we should point out here that Klages and coworkers[42] have recently cautioned users of this technique that it might overestimate the true [NH_2] value, because TFBA also reacts with imine groups. We shall return to this point later.

2.3.2 Orange II Dye

An alternate method for measuring the concentration of primary amine groups is based on their interaction with Orange II (*4-(2-Hydroxy-1-naphthylazo)benzenesulfonic acid sodium salt*, **Figure 3**),[34] a colorimetric method. Orange II molecules, negatively charged at the SO_3^- group, are made to bond with the positively-charged NH_2^+ groups in an acidic (HCl, pH 3) solution at 40°C for 30 min, enough time to assure complete reaction; the reverse reaction occurs in a basic solution of NaOH at pH 12. One can therefore quantify the desorbed dye by colorimetry at λ = 484 nm after having performed a calibration experiment.

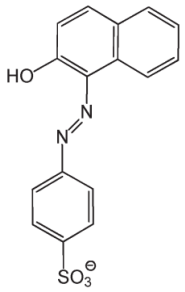

Figure 3. Orange II molecule

2.4 Cell Culture

Human umbilical vein endothelial cells (HUVECs, Lonza, Mississauga, Canada) were cultured on the various substrates in *Endothelial Cell Growth Medium-2* (ECGM-2 bullet kit; Lonza, Canada), which was changed every 2-3 days. Given HUVEC sensitivity to the number of passages,[43] cells were used at passage 3 and 4 only. First, substrate samples were cut into disks of 1 cm diameter and placed face-up on the flat bottoms of 48-well plates (TCP Falcon™); they were then sterilized by immersion in 70% ethanol for 5 min and rinsed in sterile water. After drying, cloning cylinders were placed on each sample so as to limit the area (internal area $\approx 0.5\ cm^2$) and to maintain the film substrates at the bottoms of their respective wells. Cells in a 200 µL suspension at a concentration of 75 000 cells/mL (that is, 15 000 cells/well) were seeded onto the sample surfaces, and incubated at 37°C in air with 5% CO_2. After 4h incubation, the cloning cylinders were removed and the samples were rinsed with PBS to remove non-adherent cells; the plates were then placed back into the incubator. As mentioned before, HUVECs were also seeded on both TCP and Primaria™, for comparison.

2.4.1 Cell-adhesion and -growth

Cell adhesion and growth was evaluated after 24h and 4 days respectively, using the Alamar Blue method for measuring cellular activity by fluorescence.[44] At each time-point, samples were rinsed in PBS, immersed in Alamar Blue solution (10% v/v in in complete medium, 500 µL/well) and incubated for 4 more hours (37°C, 5 % CO_2). The solution was then transferred into a 96-well plate, and the fluorescence signal, proportional to metabolic activity, [44] was measured using a spectrophotometer at excitation and emission wavelengths of 560 and 590 nm, respectively. After transferring the Alamar Blue solution, the samples were immersed in PBS; they were then stained by Crystal Violet stain and rinsed 3 times in Milli-Q water for direct observation of cells under optical microscopy. This last step was carried out to confirm the correlation between metabolic activity and the number of adherent

cells. Each set of conditions was investigated in 4 different wells and repeated a minimum of 3 times.

2.5 Statistical Analysis

Results are expressed as *mean* ± *standard deviation*. For cellular assays, a statistical analysis was performed using one-way analysis of variance (ANOVA) with Tukey's post-hoc analysis in the case of multiple comparisons. Independent two-sample t-test with equal variance was carried out in the case of two-group comparisons. A p-value < 0.05 was considered significant for all tests.

3 Results

3.1 Surface Characterization Experiments

3.1.1 AR-XPS

AR-XPS was used to analyze plasma-initiated surface modifications (PETf, TCP, Primaria™) since these techniques may lead to a surface gradient, while the composition values *at the surface* (or in a very shallow surface-near layer) are those of importance in regard to cell adhesion / culture. **Figure 4**(a,b) shows respective plots for TCP and Primaria™ corresponding to hetero-atom concentrations (in (a): [O]; in (b): both [O] and [N]) versus depth, d (in nm), of the shallow layer probed; the escape depth of X-ray photoelectrons for each hetero-atom was calculated for the particular substrate material, polystyrene, using Cumpson's tables.[45] The electron emission angles with respect to the surface-normal were, with increasing d values: 80°, 70°, 45°, and 0°, respectively. The [O] and [N] values tend towards asymptotic maximum concentrations, those for the 70° and 80° grazing angles being essentially the same as one approaches the surface, d = 0 nm. This Figure illustrates that XPS

analyses *normal* to the sample surface (d ~ 10 nm) lead to serious underestimations of true surface concentrations for the case of *plasma-modified* polymers. Regrettably, not all authors specifically mention the emission angles they used when citing XPS measurements.

In the following we shall only be referring to concentrations measured for 70 or 80° grazing emission angles in the case of all plasma-modified samples. In the case of thin coatings, plasma-deposited or other, the normal (0°) emission angle was used, because the coatings were essentially homogeneous throughout their thickness of ca. 100 nm, as already mentioned above.

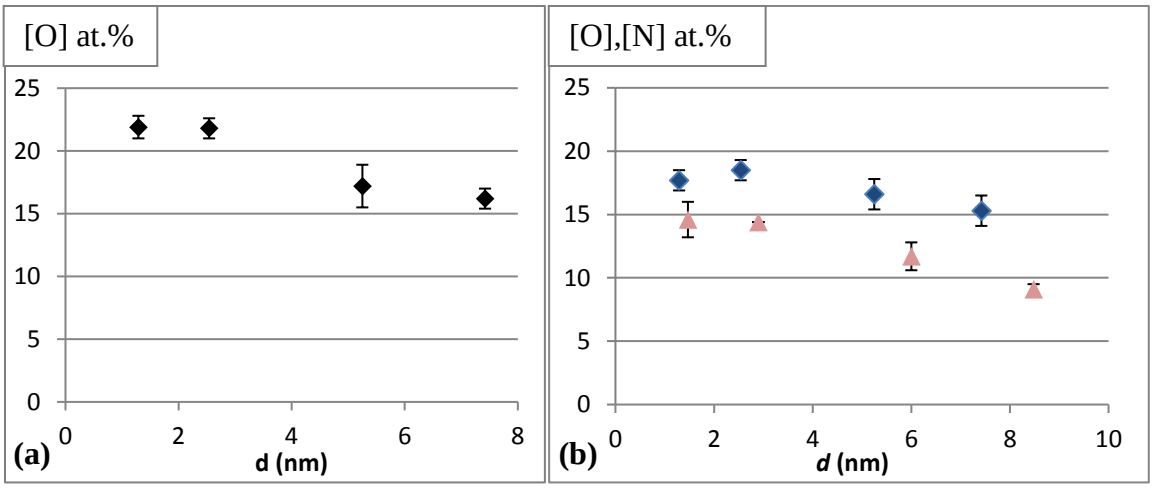

Figure 4. Plots of (a) [O]; and (b) [O] and [N], versus escape depth of X-ray photoelectrons from polystyrene, *d*, for (a) TCP and (b) Primaria™, respectively. Symbols: diamonds = [O]; triangles = [N].

3.1.2 Surface composition of modified surfaces (XPS, including derivatized samples)

(A) Nitrogen- (Amine-)containing Materials

Table 2 presents [O], [N] and [NH_2] for all modified surfaces. It should be noted right away that amine-rich samples have a well-documented shortcoming in common, namely that they age quite rapidly in air: for the case of Parylene diX AM, St-Georges-Robillard et al.[36] showed that these groups oxidized to amides in air according to 1st order reaction kinetics, the characteristic time (time constant) being about 9 days. Therefore, all present samples were either kept under inert gas or vacuum (Parylene was stored at -80°C), or they were prepared at

most 24 hours before being used in cell-culture experiments. The analytical results presented in **Table 2** (top portion) all pertain to freshly prepared materials, but even here non-zero [O] values can be noted: in the case of the "thick" NH_2-rich coatings, Parylene diX AM and L-PPE:N, [O] (≤ 5 at.%) can be attributed to highly reactive surface-near groups, primary amines in the former and predominantly trapped free radicals in the latter case, both of which oxidize rapidly when the pristine coating is exposed to atmospheric air.[16,17,32,36] For PETf and PAAm, of course, the high observed [O] values (> 22 at.%) can be explained by the PET structure. The various surface modifications techniques varied in terms of amine selectivity ([NH_2]/[N] in %), amine efficiency ([NH_2]/[C] in %), and N efficiency ([N]/[C] in %). While [N] values of the four different surfaces are seen to have varied between ca. 4 and 17 at.%, [NH_2] values differed only between ca. 3.5 and 8.9 at.%: for both Parylene and PAAm practically all of the bonded nitrogen occurred in the form of NH_2 groups, but amines, imines, nitriles and other N-bearing functionalities were also present in PETf and L-PPE:N[17,46,47]. For Parylene diX AM, the coating structure (see **Figure 2**) predicts the measured result: [N] ≡ [NH_2] = 6 at.%.

Table 2. Summary of XPS surface-near compositions of all eight materials examined in this research (note: [NH_2] values originate from derivatization experiments)

Composition (at.%)	[C]	[N]	[O]	[NH_2]	[N]/[C]	[O]/[C]	[NH_2]/[C]
Parylene diX AM	93.5 ± 0.4	5.3 ± 0.1	1.1 ± 0.4	**5.9 ± 0.6**	5.7 ± 0.1	1.2 ± 0.4	6.3 ± 0.7
L-PPE:N	80.2 ± 2.9	15.3 ± 1.5	4.5 ± 2.0	**6.7 ± 1.0**	19.1 ± 2.4	5.6 ± 2.7	8.4 ± 1.2
PETf*	60.6 ± 2.0	16.8 ± 1.6	22.7 ± 0.7	**8.9 ± 1.2**	27.8 ± 3.4	37.5 ± 2.3	14.8 ± 2.5
PAAm (pH=12.5)	72.9 ± 2.5	3.7 ± 0.9	23.4 ± 1.7	**3.5 ± 1.1**	5.1 ± 1.4	32.2 ± 3.5	4.8 ± 1.6
TCP*	79.0 ± 1.6	-	21.0 ± 1.6	-	-	26.6 ± 2.6	-
L-PPE:O	65.5 ± 1.0	-	34.5 ± 1.0	-	-	52.6 ± 1.8	-
Primaria™	67.7 ± 0.6	14.6 ± 1.4	17.7 ± 0.8	< 1.0	21.6 ± 2.3	26.1 ± 1.0	< 1.0
L-PPE:O,N	82.8 ± 0.7	5.5 ± 0.6	11.6 ± 0.5	1.5 ± 0.2	6.6 ± 0.7	14.0 ± 0.6	1.9 ± 0.2

The outcomes of [NH_2] determinations based on XPS / TFBA derivatization (section 2.3.1.) were compared to those based on Orange II dye (section 2.3.2.). **Figure 5** shows a plot of the

number of Orange II molecules reacted, versus the thickness *D* (in nm) of L-PPE:N coatings prepared at increasing deposition durations. The near-linear rise of [Orange II] with increasing *D* suggests that the Orange II dye molecules do not react only with amine (NH_x) groups in a very shallow (~ nm) surface-near layer of the L-PPE:N coatings, but rather diffuse deep into the L-PPE:N coating. Indeed, Fernandez et al.[48] have clearly shown that large organic molecules (in their case, the antibiotic Cyprofloxacin, 331.3415 g/mol) could diffuse into the free volume of L-PPE:O, for subsequent slow release to the outer surface. Therefore, it is reasonable to expect that Orange II, with slightly larger molecular weight, 350.3243 g/mol, is also able to diffuse into similar hydrophilic coating, L-PPE:N. The minor deviation from linearity noted in **Figure 5** may be due to kinetic or steric effects with rising *D* values. Thus, Orange II is clearly not well suited to determine amine concentrations near the surface of a polymeric biomaterial.

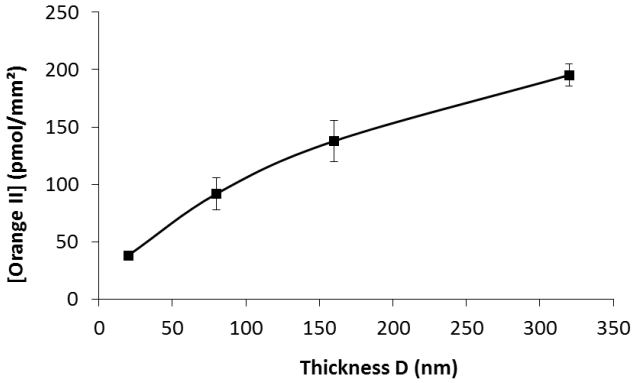

Figure 5. Concentration of Orange II molecules (in pmol/mm^2) presumed to have reacted with amine groups of L-PPE:N coatings of different thicknesses, *D*.

Despite criticism by Klages and coworkers that imines also participate in the reaction[42], the method based on TFBA derivatization followed by XPS analyses appears far more reliable here: even though TFBA, too, reacts with NH_2 groups deep inside the bulk of the film, the small escape depth of photoelectrons ($\leq$ 10 nm) assures that [NH_2] is determined only by the near-surface region.

In the Discussion section, we shall comment on the extent to which the supposedly highly bioactive NH_2 groups contributed to EC adhesion and proliferation, a key assumption underlying this research at its outset.

(B) Oxygen-containing Materials

Figure 6 shows high-resolution (HR) C1s XPS spectra for TCP (Falcon®) and L-PPE:O, two materials with O-containing surface moieties. The total amounts of bound oxygen, [O], and the distributions of bonding types (C-O, C=O, COOR/H) differed greatly among the two. **Table 3** summarizes those values.

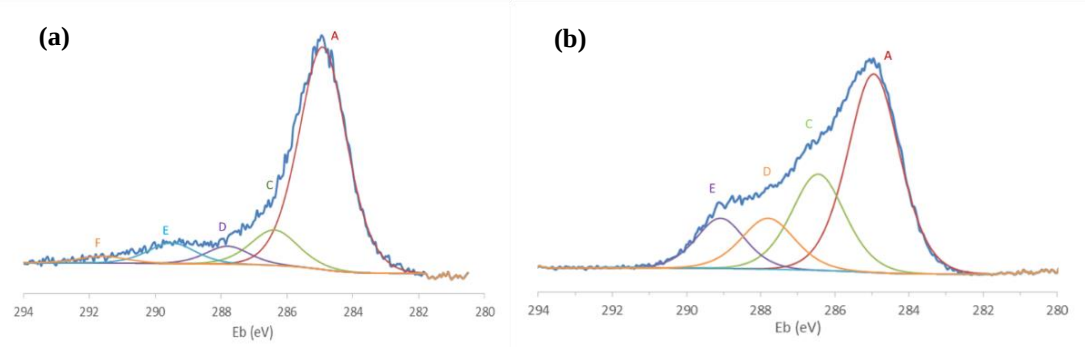

Figure 6. High-resolution XPS C1s spectra of (a) TCP (emission angle 70°); and (b) L-PPE:O

Table 3. Oxygen-containing surface compositions (in atomic %)

	C-C/C=C	C-O	C=O	COOR/H	Shake-up
TCP (Falcon^R)	72.3 ± 1.9	13.3 ± 2.6	5.9 ± 1.0	6.3 ± 0.7	2.2 ± 0.5
L-PPE:O	50.5 ± 1.4	24.9 ± 0.9	12.7 ± 0.9	11.7 ± 1.0	-

Some noteworthy features that can be pointed out are the following: for the case of TCP, plasma-functionalized polystyrene (PS), the shake-up peak very near the surface is greatly reduced in intensity compared with pristine PS due to the creation of the oxygenated moieties.

L-PPE:O contained almost twice as much bound oxygen as TCP, with a similar distribution of moieties (but obviously far fewer C-C/C=C bonds and no sign of a shakeup signal).

(C) Hybrid (N- and O-containing) materials

Figure 7 compares high resolution C1s spectra of Primaria™ and L-PPE:O,N, which both contain N- and O-based moieties; let us mention at the outset that two types of Primaria™ samples were examined, namely Petri dishes and 24-well plates, which were found to yield quite different [O] and [N] values, almost twice more [N] and 1.5 more [O] for the latter (data not shown). These differences among products bearing the same trademark (Primaria™) may explain that various compositions for Primaria™ can be found in the literature.[6,27–29,49] Hereafter, we shall exclusively refer to the more highly functionalized 24-well plates.

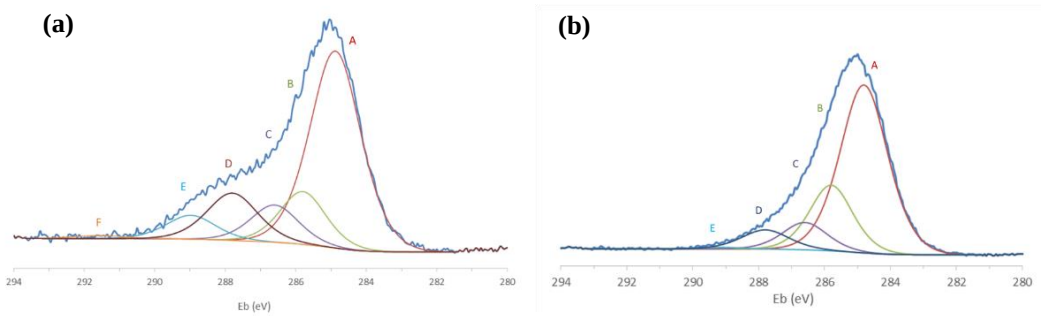

Figure 7. High-resolution XPS C1s spectra of (a) Primaria™ (emission angle 70°); and (b) L-PPE:O,N

Table 4 compares the respective compositions, obtained using the sub-peak characteristics presented in **Table 1**.

Table 4. Hybrid surface compositions (at. %)

	C-C/C=C	C-N	C-O	C=O/ N-C=O	COOR/H	Shake-up
Primaria™	52.3 ± 2.1	17.4 ± 2.2	9.2 ± 0.4	12.9 ± 1.5	7.4 ± 0.9	0.8 ± 0.1
L-PPE:O,N	62.3 ± 3.5	21.3 ± 2.2	8.9 ± 1.2	6.4 ± 0.7	1.0 ± 0.5	-

The total [O] and [N] values were higher for Primaria™ (cf. **Table 2**), but this might be compensated for the case of L-PPE:O,N by using R_{N_2O} > 0.6, which raises both to some extent (data not shown). As mentioned above, the surface chemistry of Primaria™ is not well defined in the literature, compositions varying quite widely among different research groups. Here, it is seen to be highly functionalized, for example with 7% carboxyl or ester groups, but these values differ for Petri dishes and multi-well plates, as already pointed out. L-PPE:O,N has far fewer C=O bonds, especially carboxyl and ester groups, and of course no aromatic functionalities (no shake-up peak). While L-PPE:O,N was found to have ca. 1.6 at.% of [NH_2], the corresponding value for Primaria™ was < 1 at.%. We shall return to all of these issues later.

3.2 Cell Culture Experiments

Figure 8 presents the results of HUVEC culture experiments performed on pristine PET, and on all of the eight bioactive (functionalized) surfaces studied here, the corresponding surface-analytical results having been summarized in **Table 2**. As explained in section 2.4., the term "cellular activity" refers to fluorescence measurements using the Alamar Blue method, and data are plotted with reference to 48-well TCP plates after 24h adhesion as control (≡ 100%).

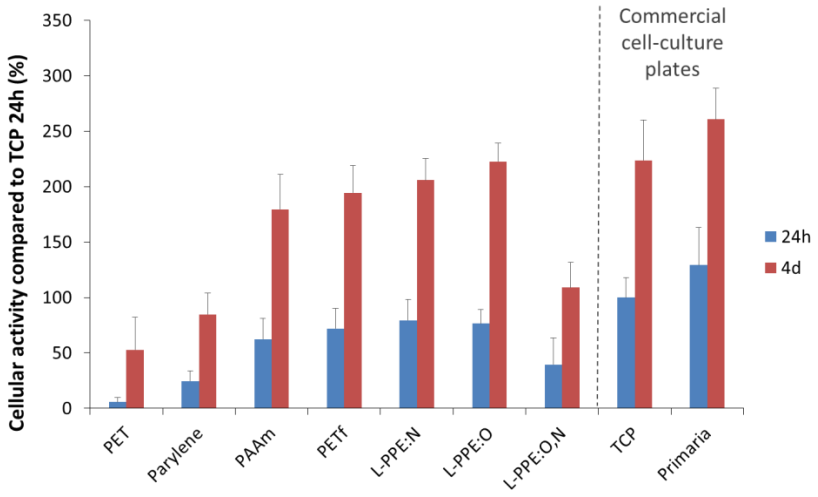

Figure 8 Endothelial cell (HUVEC) culture results for the various bioactive materials examined (blue bars: after 24h; red bars: after 4d). All results are normalized with respect to those obtained with TCP plates after 24h (≡ 100%).

First, regarding the N-functionalized materials L-PPE:N, PETf and PAAm, these all manifested quite similar HUVEC adhesion and proliferation results, within statistical limits, all vastly superior to pristine PET. Considering the different compositions, especially in terms of [NH_2] values, these outcomes are quite unexpected. On the contrary, Parylene diX AM surprisingly proved to be much less efficient than those other N-functionalized counterparts (but still higher than PET), despite its high [NH_2] content. L-PPE:O performed at least as well as its N-bearing counterpart, suggesting a comparable efficacy of O- and N-bearing moieties regarding HUVEC adhesion and growth. This is, however, not seen to be the case for L-PPE:O,N, in spite of confirmed superiority of Primaria™ over TCP: that plasma polymer coating performed comparably with Parylene diX AM, perhaps slightly better, but contrary to Primaria™ it showed no evidence of possible synergism between N- and O- functionalities.

The last two data sets on the right of **Figure 8** refer to commercial 24-well TCP and Primaria™ plates, because the latter were unavailable in 48-well format. The total active surface was evidently greater in these latter two cases than in the other seven, all of which

pertain to 48-well plates, as explained in section 2.4; therefore, the data for Primaria™ were plotted with reference to 24-well TCP plates.

Clearly, the use of these plasma-functionalized commercial cell-culture polystyrene surfaces in this study is strictly to provide a basis for comparison; in other words, they have little or no relevance to the task of enhancing endothelial cell adhesion on the inner surfaces of synthetic blood vessels, the driving motivation of this work pointed out in the Introduction. In agreement with data presented in **Figure 8**, the oxygen plasma-functionalized TCP is well documented for its very good general cell-proliferation properties, and therefore frequently used as a positive control for cell culture experiments. As such, it has here confirmed the generally very satisfactory properties for HUVEC colonization and culture, with the exceptions of Parylene diX AM and L-PPE:O,N. The hybrid (N- and O-) plasma treated Primaria™ was found to display even slightly (but statistically significant, $p<0.05$) better performance than TCP, suggesting that the combination of O- and N-containing moieties might constitute the new "gold standard" for cell culture dishes. This would agree with earlier-published observations by several groups.[26,50,51]

4 Discussion and Conclusions

The primary purpose of this research was to identify the most efficient surface modification technique for the lumen surfaces of synthetic blood vessels (Dacron™ PET, or expanded Teflon™), to enable colonization by a complete monolayer of endothelial cells. For simplicity, we have in the present work used flat, smooth PET surfaces seeded with HUVECs.

We have characterized plasma-modified surfaces (TCP, Primaria™, PETf), along with thin coatings prepared by chemical vapor deposition (CVD: Parylene diX AM), by covalent grafting by wet chemistry (PAAm, by aminolysis), or by plasma polymerization (L-PPE:N,

L-PPE:O, L-PPE:O,N), in regard to their surface compositions (section 3.1.) and their response to HUVEC colonization (coverage and time-dependence, section 3.2.). At the outset, there was reason to believe that surfaces presenting the highest density of reactive primary amines, [NH_2], would show particular promise for HUVECs in regard to the above-stated objective, because numerous authors, including these laboratories, have found amines to be effective with various cell-types.[16,18,20,36,52] As already pointed out above, Klages and coworkers[42] have taken issue with [NH_2] values based on derivatization with TFBA, on the grounds that imines (and possibly others) also contribute; while acknowledging the high quality of those authors' work, we respectfully submit that our (and others') argumentation is valid, at least to the degree of precision required here. Regrettably, due to spatial constraints, we cannot now present further supporting evidence. A presumed and often-proposed reason for the role of (NH_2) groups is their reaction in aqueous medium at physiological pH, near 7.4[53], which attracts negatively charged proteins present in the surrounding fluids:

$$R\text{---}NH_2 + H_2O \qquad R\text{--}NH_3^+ + OH^- \qquad (2)$$

Because the cells depend on specific adhesive proteins for anchorage and extracellular instructions, the composition of the adsorbed protein layer is a key factor for cell adhesion. The polymer surface chemistry does not only influence the type and amount of adsorbed adhesive proteins, but also their possible denaturation, all of which are known to influence cell binding activity. Thus, Steele et al.[26] showed that N-containing polymers stimulate cell attachment through two alternative pathways, either through adsorbed vitronectin (Vn) or adsorbed fibronectin (Fn; both these proteins could adsorb without significant denaturation onto the amine and amide plasma polymers of that study), while cell adhesion on O-containing plasma polymers was mainly through adsorbed Vn.

For the case of U937 monocyte adhesion, our group observed the existence of a "critical" concentration, $[NH_2]_{crit}$ = 4.2 at.%, below which no U937 adhesion at all was observed, including on commercial cell-culture plates[20]. In this present study, except for Parylene, all

NH_2-bearing surfaces examined (PAAm, PETf, L-PPE:N) performed indistinguishably well with endothelial cells, in spite of [NH_2] values varying between 3.5 and 8.9 at.% (see **Figure 8** and **Table 2**). Since overall [N] values differed even more widely, other N-bearing functional groups appeared to have little or no systematic effect: for example, PAAm, for which [N] = [NH_2] = 3.5 at.%, performed as well as L-PPE:N samples that present a large variety of N-bearing moeities.

The relatively poor cell results obtained with Parylene diX AM were surprising, since these surfaces present a high density of primary amines ([NH_2] ≈ 5.9 at.%). St-Georges-Robillard *et al.*[36] already pointed out that Parylene behaved "anomalously" compared with L-PPE:N, for reasons that remain unexplained. This topic is worthy of further investigation. For example, we have so far not investigated its wettability (surface energy), which may influence the extent of protein denaturation. A difference in protonation of amines would also affect protein adsorption and cell colonization.

Considering what has just been explained above, it was somewhat surprising that surfaces bearing only O-functionalities (L-PPE:O) performed at least as well as their N-functionalized counterparts (see **Figure 8**). However, several authors have shown that surfaces containing carboxylic acid (R – COOH) groups also promote cell adhesion, probably because they react in aqueous media to form R – COO^-, which can attract positively-charged biomolecules.

$$R\text{ --- }COOH \rightleftharpoons R\text{ --- }COO^- + H^+ \qquad (3)$$

In the case of hybrid surfaces, of course, one might expect both reactions (2) and (3) to participate to some extent. Several authors[26,51] have underlined the superior bio-response of Primaria™ in cell culture experiments, an observation we appear to reconfirm here, see **Figure 8**.

In their very elegant study using growth media selectively depleted of specific proteins, Steele et al. explained the difference between Primaria and TCP with the amount of fibronectin (Fn) adsorbed, which was much higher on Primaria, while the amount of adsorbed vitronectin (Vn, also involved in cell adhesion) was similar[26]. Their results also suggest that Fn and Vn bind to different binding sites. The mechanism influencing the amount adsorbed is complex, but involves the competition between serum proteins for "protein binding sites", as higher Fn adsorption onto Primaria™ versus TCP was not found when incubating Fn in serum-free solutions. Since then, the use of micro-patterned surfaces[54] has also shown the importance of protein localisation in cell binding efficacy, through focal adhesion complexes.

Regarding the somewhat disappointing performance of L-PPE:O,N compared with Primaria™, this may well be due to its very low COOR content, already pointed out above. This may be construed as yet more evidence that carboxylic or ester groups are important in promoting HUVEC colonization. In retrospect, as pointed out further above, we might have preferred to select R_{N_2O} = 1.0 instead of 0.6 to obtain increased COOH content, since both these conditions led to near-insoluble L-PPE:O,N coatings, but the latter displayed much greater surface roughness after prolonged immersion in water. For lack of space, we have decided not to further elaborate on this point.

In section 3.1.2, we had already quite amply discussed the common shortcoming of all amine-rich surfaces or coatings, their inevitable oxidation when exposed to atmospheric oxygen. This, of course, leads to the conversion of primary amines to amides[36] unless the materials are rigorously shielded from even small amounts of O_2 gas. In view of the comparably beneficial performance of L-PPE:O noted in **Figure 8**, a coating which evidently is far less sensitive towards atmospheric O_2, it may well be the preferred choice of culture surface for future applications. However these surfaces are also prone to aging through the diffusion of functional groups into the bulk and/or loss of low-molecular-weight oxygen-containing fragments[16]. Moreover, there is at least one aspect inherent to the NH_2-bearing surfaces and

coatings that renders them important and irreplaceable, namely covalent grafting of other bio-active molecules containing COOH groups, such as chondroitin sulfate (CS) or other glycoaminoglycans[21,23,55,56], via carbodiimide chemistry.Thus, CS has been shown to prevent platelet adhesion and possible thrombosis, as explained elsewhere.[24,56] It will be the object of further elaboration at a later date.

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Graphical Abstract

Various functionalized polymeric (PET) surfaces were compared in regard to their ability to enhance endothelial cell adhesion and proliferation. Most nitrogen-rich surfaces displayed excellent results, thanks to primary amine groups, and oxygen-bearing samples showed a similar efficiency. Hybrid surfaces, containing both O- and N-bearing moieties, presented a great potential to induce even better endothelialization, but still need further improvement.

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Characterization and Comparison of N-, O- and N+O-functionalized Polymer Surfaces for Efficient (HUVEC) Endothelial Cell Colonization