JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 11 (2000) 327–331

Growth of human endothelial cells on plasma-treated polyethyleneterephthalate surfaces

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Different methods have been proposed to reduce the surface thrombogenicity of small caliber vascular grafts, using plasma treatments of polymer surfaces in order to improve the adhesion and the proliferation of human endothelial cells (HEC). Plasma modified polyethyleneterephthalate (PET) substrates were employed to grow HEC, isolated from the umbilical vein. A combination of X-ray photoelectron spectroscopy (XPS) and Sessile contact angle (SCA) measurements allowed the study of the surface modifications produced soon after nitrogen and hydrogen plasma treatments with respect to an untreated PET substrate, used as reference. It was possible to select a number of PET substrates while actually performing the HEC seeding experiments. The HEC proliferation was evaluated by light microscope image analyzes.

1. Introduction

The ideal blood contacting surface of a vascular prosthesis would be an endothelial lining, because endothelium is the natural non-thrombogenic lining of blood vessels. It has been observed that human beings have a different capability compared to animals, as human endothelium is not automatically generated in the inner surface of a prosthesis and this problem is more significant for small caliber grafts (diameter < 6 mm) used in the reconstruction of peripheral arteries [1].

On the other hand, in the literature [2, 3] it can be found that the use of medium- and large-diameter vascular grafts are generally employed in the surgical field as a well established operative technique. The possibility of obtaining the optimal interaction between the artificial vascular surface and endothelial cells can lead to a complete coverage of the prosthesis surface, projecting *in vitro* the blood vessels intima.

A proper modification of the chemical and morphological surface substrate plays an important role in determining different biological answers and time-lasting effects. By means of proper treatments, optimal polymer surface conditions can be realized in order to give rise to a homogeneous and uniform endothelial cell coverage of the artificial surface prosthesis, overcoming the thrombogenic problems linked to the lack of success of small vascular grafts.

Previous papers [4–6] have shown that the adhesion of human endothelial cells (HEC) on polytetrafluoroethylene (PTFE) and polyethyleneterephthalate (PET) polymers is always reduced or even impossible, when their surfaces have a hydrophobic nature.

Plasma treatments are a well known procedure employed to implant various functional groups containing OH, CO, COOH and nitrogen on the surface, in order to increase (or decrease) their adhesion, wettability and hardness [7–12].

It is reported [7, 13] that the presence of nitrogen containing groups on the polymeric surface, and particularly primary aminic groups, improve the growth and adhesion of endothelial cells. Our focus point is therefore to generate polyethyleneterephthalate (PET) substrates, materials used in the fabrication of Dacron vascular grafts, with improved surface chemical condition by means of the implantation of nitrogen functional groups with a proper chemical surface modification method.

In particular, in the present work a plasma surface treatment has been considered with different N_2 and H_2 flux ratios at two power sources, 80 and 40 Watt from the reactor. The choice of only7 two power source values has been suggested by previous experiences of one the authors on the use of the reactor employed in this work. The plasma glow discharge method was systematically used to modify commercial PET surfaces. The induced surface chemical modifications were studied by X-ray photoelectron spectroscopy (XPS) and Sessile contact angle (SCA) measurements, while the human endothelial cells (HEC) growth was evaluated by light microscopy. Further cell image elaborations allowed us to determine

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some discriminating coverage set of parameters, after four days of cell incubation.

2. Experimental procedures

Commercial PET films were cut into disks 15 mm in diameter, rinsed in deionized water, and submitted to a plasma surface modification.

2.1. Plasma treatment

The plasma glow discharge treatment was performed in a reactive ion etching system reactor (Leybold R.I.E. 600). Nitrogen and hydrogen were the reactive gases in the plasma and the total flux was always 50 s.c.c.m., to keep the reactor chamber pressure at constant 266 Pa. The duration of plasma treatment was five minutes in no-heating conditions of the substrate plate for all samples. Table I lists the relative percentages of hydrogen and nitrogen for the two set of samples obtained at different powers 80 W and 40 W (a and b respectively,). The reproducibility of the plasma process was provided by analysing the surface chemical state generated with a certain treatment by XPS three times. A reference untreated PET sample (uPET) was used to compare the chemical behavior of plasma treated PET substrates.

After plasma treatment, to avoid aging effects and consequently the rearrangement of the surface functional groups through interaction with the atmosphere, care was taken to reduce to a few minutes the treated substrates transfer time for surface analyzes and HEC cultures.

2.2. XPS analysis

XPS measurements were performed by means of an ESCALAB 210 spectrometer (VG Scientific) using an A1 $K\alpha_{1,2}$ non-monochromatic X-ray source ($E = 1486.6\,eV$), and a five channeltron hemispherical analyzer working at constant pass energy (P.E.). The take-off angle employed for all the measurements was 90° and large area analyzes were executed in order to obtain average experimental surface information. Wide energy range spectra were acquired at 50 P.E., while detailed scans (20 eV P.E.) were made of C 1 s, O 1 s and N 1 s and then compared to the uPET surface. The atomic relative percentages have been extracted by correcting the experimental areas with theoretical sensitivity factors (Slater-Hartree-Fock approximation), after a Shirley background subtraction procedure.

TABLE I Plasma treatment conditions for PET substrates: suffix ''a'' denotes samples submitted to 80 W power source, while the ''b'' suffix stands for the 40 W power source

Samples	$\%H_2$	$\%N_2$
0a	100	0
1a, 1b	20	80
2a, 2b	30	70
3a, 3b	50	50
4a, 4b	70	30
5a, 5b	80	20
6a	0	100

2.3. Contact angle measurements

The SCA measurements were determined using a 100 NRL goniometer, by placing a drop of water on the substrate surface and recording the angle between the horizontal plane and the tangent to the drop at the point of contact with the substrate.

2.4. Cell culture

Endothelial cells were isolated from human umbilical cord vein according to the method of Jaffe *et al*. [14] and cultured in 80 cm^2 culture flasks (Nunc), precoated with 1.5% gelatin (Difco) in a complete culture medium composed of Medium 199 with Hepes buffer, 2 mM L-glutamine, 100 U/ml penicillin-streptomicin, 1 μg/ml gentamicin, 2.5 μg/ml fungizone and supplemented with 20% foetal calf serum, 50 μg/ml endothelial growth factor, prepared in our laboratory from bovine brain and 100 μg/ml porcine heparin (Sigma). All reagents were supplied by Mascia Brunelli (Milan, Italy). Cultures were maintained in a 37 °C incubator with 5% of CO_2 in a humidified atmosphere.

Plasma treated PET disks were located in a 24-multiwells plate (Corning) and cells were seeded at a density of 3.0×10^4 cells/cm^2.

After four days, of incubation samples were fixed with 4% formaldehyde for 10 min at room temperature, stained with blue toluidine and dehydrated through a grade series of ethanol. The samples were examined by light microscopy (Axiovert 100, Carl Zeiss) and the images obtained were elaborated through an interactive image analyzer. At least 15 microscopic fields per sample were randomly acquired in the central and peripheral regions with a × 20 magnification objective lens.

The image analysis was performed with a software program (NIH Image 1.60, Bethesda, MD) and after a threshold contrast with respect to the substrate the morphological cell features were evidenced. The main parameters considered were the cell surface density (cell per unit area in cm^2) and the percentage cell-covered area with respect to the whole.

2.5. Statistical analysis

Results of the image analysis are expressed as mean ± standard deviation for each group of plasma treated samples. After the assessment of significant differences by one-way variance analysis (ANOVA method), differences among groups were established by T-student test analysis by a two population comparison. Statistical significance was considered at a probability $P < 0.05$.

3. Results

Since there is no exact correlation in the literature between the choice of the discharge gas fluxes and the density and typology of implanted functional groups, in the present work a gas mixture of nitrogen and hydrogen were selected for plasma treatment and in different flux ratios, at two power sources, 80 W and 40 W, keeping constant all other parameters.

It was reported [15] that nitrogen plasma treatments on PTFE substrates produce a modification of carbon

containing groups, and then a chemical modification of the original surface composition.

XPS analyzes (Fig. 1) performed on freshly treated substrates have shown: (1) in any gas mixture, flux condition and at both power sources nitrogen containing groups were recorded; (2) highly evident modifications in the C 1 s peak shape were found in the 80 W treated substrates with respect to the reference untreated uPET. In particular, Fig. 1 reports the behavior of C 1 s, O 1 s, N 1 s for samples 3a, 3b and uPET. Relative XPS atomic percentages of the main components of the surface (C, O, N) are reported in Table II and showed higher nitrogen containing groups when an 80 W supply is employed. A further nitrogen increase in the discharge has not been considered as an element to improve the implant efficiency of the desired groups. It is proof of the fact that the use of nitrogen alone in the discharge causes a sudden decrease in the nitrogen containing groups, underling the importance of hydrogen in the plasma to create active centers of anchoring to nitrogen groups and causing the reduction of the nitrogen groups in aminic groups. The hydrogen-only treated substrate shows, in fact, the evidence of an ionic bombardment of its original surface.

Table II reports the N/C and N/O ratios for the 80 W plasma treated samples, and within the error in atomic percentage calculation (< 10%) it is helpful to consider only the second decimal number. The nitrogen-only treatments have a decrease of 48.8% with respect to the high efficiency treatment. Similar consideration can be run for the N/O ratio.

Another very important indication of plasma treatment is the surface contact angle trend versus the nitrogen amount in the glow discharge. In our samples the measured contact angles were always beneath the uPET contact angle value (63°) and the graph slope (Fig. 2) decreases when the nitrogen amount is increased for both power sources employed.

The seven samples plasma treated at 80 W, and previously chemically analyzed, were employed to verify the HEC behavior after their seeding on the polymeric surface.

The uPET sample, the tissue culture polystyrene-gelatin-treated (TCPS) and a commercial Thermanox PET (Nunc) were used as reference samples. In Fig. 3 the HEC density of treated PET substrates after four days' of incubation is represented. The surface cell density on plasma treated samples is always higher than the uPET reference sample and very similar to TCPS. The exception to this trend is represented by sample 5a, whose surface density is well below that of TCPS. The same thing applies to Thermanox. Therefore plasma treatments with both nitrogen and hydrogen result in even more effective cell growth than Thermanox. Fig. 4 shows the percentage of cell-covered area. All the treated PET substrates show a higher surface-covered area than uPET and samples 2a, 3a and 4a are comparable with TCPS. The Thermanox also has a surface-covered area below TCPS.

4. Discussion

References [4, 5] indicate that PET material is hydrophobic in nature and prevents cells from adhesion and growth onto the substrate results. It was our intention to realize PET surfaces suitable for endothelial growth by means of plasma treatments. In particular, N_2 and H_2 were employed to enhance the nitrogen containing

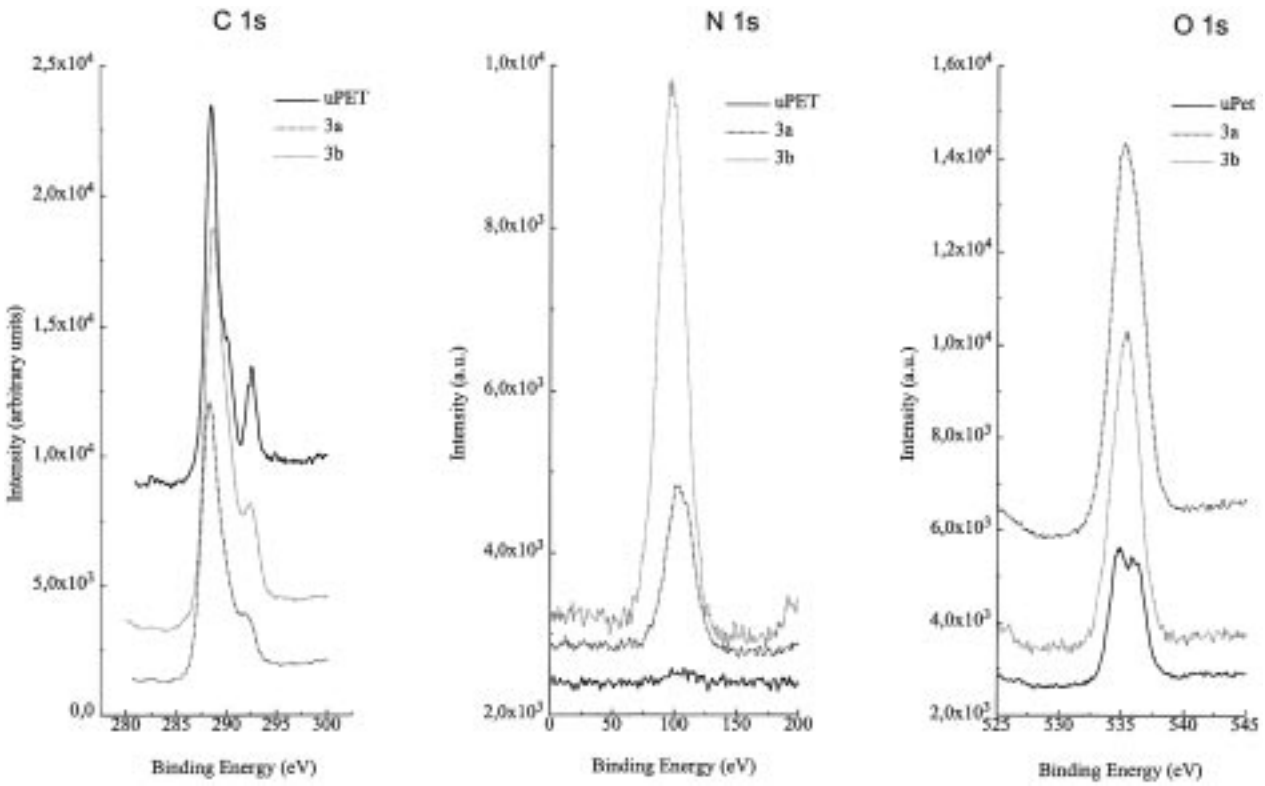

Figure 1 C 1 s, N 1 s and O 1 s XPS spectra not charged corrected for samples uPET (—), 3a (- - -) and 3b (· · ·).

TABLE II XPS quantitative evaluation of atomic relative percentages of C, O and N in differently treated samples

Samples	C 1 s%	O 1 s%	N 1 s%	N/C	N/O
uPET	71.6	28.4	0		
0a	76.2	23.8	0		
1a	66.4	26.0	7.6	0.114	0.292
2a	68.6	23.0	8.4	0.112	0.365
3a	71.4	21.4	7.2	0.101	0.336
4a	71.0	21.2	7.8	0.109	0.368
5a	71.9	20.8	7.3	0.101	0.351
6a	62.5	29.6	5.1	0.082	0.172
1b	64.7	28.2	7.1	0.109	0.252
2b	66.1	27.2	6.6	0.099	0.243
3b	67.0	25.0	8.0	0.119	0.320
4b	70.4	22.6	6.9	0.098	0.305
5b	70.8	22.5	6.7	0.094	0.298

groups, which appeared to be a possible cause for specific interactions with cells, proteins, biomolecules, tissues and biological fluids. In fact, $-NH_2$ groups are thought to become positively charged ($-NH_3^+$) at physiological pH, and to enhance surface interactions of polymers with cells and biomolecules carrying a net negative charge [13]. Covalent bonding with specific groups can also occur.

The constant parameters were fixed according to previous papers and experience on the reactor, while the best choice of power source was made comparing the results from a chemical point of view obtained at 40 and 80 W. The biological answer was assessed by image elaboration of the data set on 80 W PET treated samples and the results for treated samples were always better with respect to uPET and Thermanox. They were also similar to the positive control TCPS.

The good biological answer of the Th reference sample is also justified by the fact that XPS analysis indicated the presence of nitrogen on the surface, but the results for substrates treated in our laboratory are even better. XPS analyses gave a comparable N/C efficiency for all treated samples at 80 W. The sample with a high nitrogen percentage also has the best cell surface coverage parameter. The contact angle measurements confirm this observation and are in agreement with what can be found for other kinds of substrates [13]. Several parameters influence cell adhesion and growth: the local chemical conditions at the surface during plasma treatment, the polymer chemical composition and, of course, the parameters chosen for plasma glow discharge. A previous study [16] indicated that cell adhesion becomes maximal on the surface which had a certain range of contact angle against water and decreased with the higher or lower wettability with respect to that angle. These are the real conditions that affect the typology of the implanted groups. Unfortunately it was not possible to resolve the different components on the N 1 s peak as it appears in all treated samples as a unique wide envelope. It can be helpful to employ other techniques associated with XPS analyses such as the derivatization method to discern every single nitrogen component and to discuss the possible phenomena at the surface during the plasma treatment according to the variable parameter in the glow discharge.

In the present work it was possible to select a range of N_2 and H_2 flux ratios (with 30%–70% of nitrogen in the discharge) where there is a good surface wettability, meaning good adhesion, and where the biological answer (surface cell density and percentage surface cell coverage area) to human endothelial cell growth is also good. Therefore the plasma technique was revealed to be very useful in modifying the polymeric PET surface, introducing specific functional groups, without using

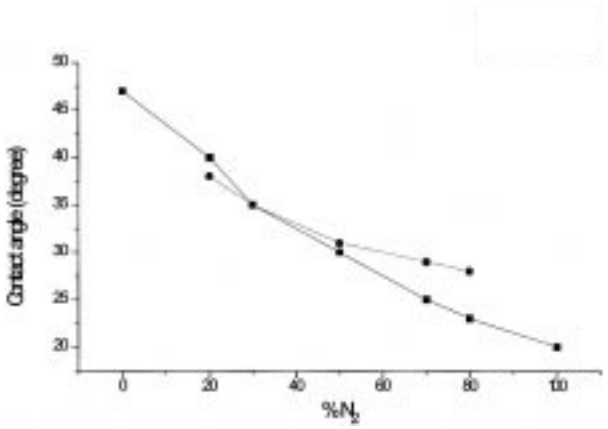

Figure 2 Experimental SCA measurements on plasma-treated PET samples, versus the percentage of nitrogen in the plasma discharge, at 80 W (■) and 40 W (●).

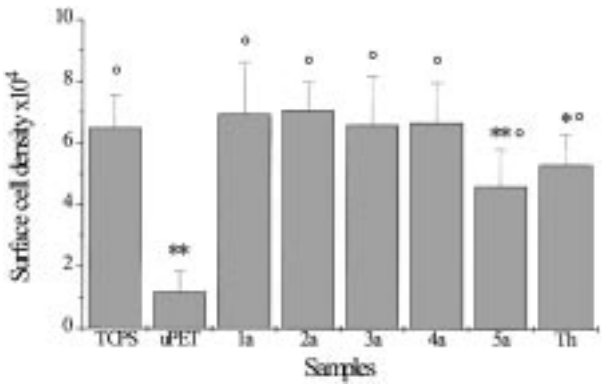

Figure 3 HEC density on plasma–treated surfaces (samples 1a, 2a, 3a, 4a and 5a) with respect to the uPET, Thermanox and TCPS after four days' incubation. Difference from TCPS: $^{*}P<0.01$; $^{**}P<0.001$. Difference from uPET: $^{\circ}P<0.001$.

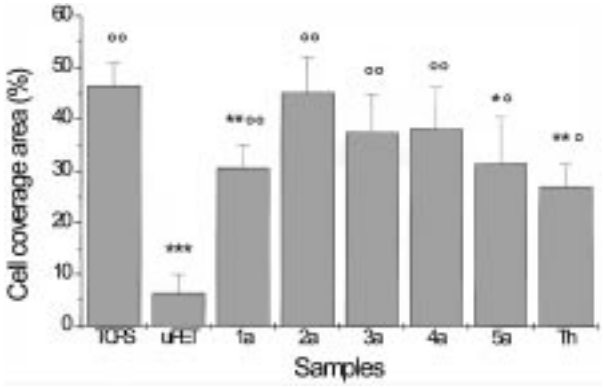

Figure 4 Percentage HEC coverage area on plasma-treated surfaces (samples 1a, 2a, 3a, 4a and 5a) with respect to the uPET, Thermanox and TCPS, after four days' incubation. Difference from TCPS: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Difference from uPET: $^{\circ}P < 0.01$; $^{\circ\circ}P < 0.001$.

proteinic coatings of the extracellular matrix (collagen, albumin, gelatin, fibronectin, laminin). For enhancing cell attachment and adhesion on artificial substrate materials proteinic coatings are, however, very commonly used [17–19].

5. Conclusions

The present study shows evidence that plasma treatments with nitrogen and hydrogen are a suitable method to improve the PET biocompatibility to HEC. In fact, the biological answer is good in treated samples with respect to the uPET and to the commercial Thermanox and similar to TCPS, without any need to add proteins to the extracellular matrix to promote cell adhesion and proliferation. The reproducibility and control of the chemical process induced by plasma and its advantages have overcome the need for sterilization of the surface as the plasma process by itself does not introduce foreign particles on the surface. The agreement of cell growth data with XPS functional groups identification and SCA measurements at 80 W plasma treatments confirmed the hypothesis that plasma surface activated nitrogen-containing groups are the reading-key of HEC biocomatibility with PET polymeric surfaces.

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Received 27 October 1997
and accepted 14 January 1999