Cytocompatibility of PET Films after DC Helium Plasma Treatments and Collagen Immobilization

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Films of poly(ethylene terephthalate) have been subject to a direct current helium discharge, the most efficient adsorption of collagen on the plasma-treated surfaces being recorded for longer treatment times. All treated samples sustained the adherence and the proliferation of the endothelial cells.

Keywords: poly(ethylene terephthalate), helium plasma, protein adsorption, endothelial cell

The chemical composition and physical surface morphology of polymers are key factors as far as their compatibility with biological systems is concerned [1, 2]. The physico-chemical properties of the surface influence the adsorption of proteins on a substrate, therefore triggering reactions to a foreign body, i.e., the biocompatibility of the material.

Poly(ethylene terephthalate) (PET) is widely used in various forms, such as films, fibers and engineering plastics, for a large variety of applications. PET exhibits good mechanical strength, toughness, fatigue resistance at elevated temperatures and a high crystalline melting temperature. Besides the desirable bulk properties of PET, good interfacial bonding is of great importance for the application of biomaterials. Interface properties, such as chemical bonding and structure, play an important role in the adsorption of proteins to polymers [3, 4].

Chemical reactions, as well as physical treatments, can be used in order to add chemical groups onto a polymer surface. It is demonstrated [5] that treatments on an He discharge induce both a functionalization and a cross-link of the polymeric film uppermost layers. The residual oxygen, which partially originates from the polymer, induces surface oxidation. The cross-linked content critically depends on the residence time and concentration of implicated species [5].

Collagen, the most abundant protein in the body, has been widely examined as a potential tissue-engineering scaffold [6-8]. The use of collagen for the obtaining of biomaterials offers such advantages as biocompatibility and low toxicity to most tissues, as well as controlled structural, physical, chemical and immunological properties. Moreover, collagen is readily isolated and purified in large quantities and can be processed into a variety of forms [9]. In its purified form, collagen forms a weakly cross-linked thermo gel, therefore, for tissue engineering applications, covalent intermolecular cross-links between collagen molecules in macromolecular fibrils using appropriate biocompatible molecules, is essential for developing stable materials with a high degree of mechanical integrity.

Cell affinity is the most significant factor to be concerned with when polymeric materials are utilized as cell scaffolds in tissue engineering [10, 11]. Cell affinity includes cell attachment and cell growth. The cell attachment belongs to the first phase of cell/materials interactions and the quality of this phase will influence the cell’s capacity to proliferate and to differentiate itself when in contact with the material [12].

The aim of this study was the evaluation of helium-plasma treatments influence on poly(ethylene terephthalate) (PET) films in order to obtain support surfaces for the immobilization of biologically active molecules and living cells. Even if radio frequency plasma is often used in this types of treatments, in this study one has obtained good results using direct current plasma. The plasma-functionalized PET films were then placed in a collagen-buffer solution and the obtained polymeric supports were used for EA.hy 926 endothelial cell line cultures.

Experimental part

Materials and methods
Plasma treatments
The experiments were performed in a direct-current plasma device. The plasma was produced in a glass vessel by a DC discharge sustained between a heated filament cathode and a metal foil negatively biased with respect to the foil. As background gas one used helium at a pressure p = 5 × 10^{-3} mbar. A PET film E (30μm thickness) was immersed into the plasma filling the gas vessel by diffusion, helium being the most efficient of the inert gases for cross-linking the outermost polymeric monolayers. This feature is due to its increased rate of diffusion and the large amount of energy available to transfer to the polymer surface via ion neutralization, Auger de-excitation and Penning ionization in the polymer [13].

After plasma treatment, the films were incubated in 3 mg/mL type I collagen/phosphate buffered solution (PBS, pH = 3.4) for 24 h at 24°C. The collagen immobilized films were rinsed with ethanol solution and then with de-ionized water in order to remove the free collagen.
Cell culture on collagen samples

Human endothelial cell line EA hy 926 (human aortic endothelial cells) were purchased from American Tissue Culture Collection. Endothelial cells were maintained in DMEM high glucose medium (Dulbecco’s Modified Eagle Medium), supplemented with 100 U/mL streptomycin, 100 U/mL penicillin, 50 μg/mL neomycin, 2 mM glutamine, 1 mM HEPES (GIBCO), and 10 % FBS (fetal bovine serum) (GIBCO). PET films were sterilized with 70 % ethanol and conditioned in the same culture medium, then inoculated with endothelial cells (5 x 10⁴ cells/mL). Cells were maintained in culture media at 37°C, in incubators, with 5 % CO₂ in air (v/v) and relative humidity over 95 %. Chemicals used for cell culture were obtained from Sigma Aldrich.

Cells were maintained in culture media on PET films for five days and then stained with hematoxylin and eosin.

Characterization methods

AFM images were recorded in air at room temperature, in the tapping mode using a Scanning Probe Microscope (Solver PRO-M, NT-MDT, Russia) with commercially available NSG10/Au Silicon cantilevers. The root-mean-square roughness, RMS, was calculated as the average value for the set of AFM frames of certain scales.

In order to calculate the content in original and PET treated samples, the compositional analysis of the studied samples was carried out by X-ray photoelectron spectroscopy (XPS) using a PHI-5000 VersaProbe photoelectron spectrometer (ULVAC-PHI, INC.) with a hemispherical energy analyzer (0.85 eV binding energy resolution for organic materials). A monochromatic Al Kα X-ray radiation (hv = 1486.7 eV) was used as excitation source. The standard take-off angle used for analysis was 45°, producing a maximum analysis depth in the range of 3-5 nm. Spectra were recorded from at least three different locations on each sample, with a 1 mm x 1 mm area of analysis. Low-resolution survey spectra were recorded in 0.5 eV steps with 117.4 eV analyzer pass energy. In addition, high-resolution carbon (1s) spectra were recorded in 0.1 eV steps with 58.7 eV analyzer pass energy.

Standard hematoxylin and eosin staining were performed in order to observe the cells morphology and sample colonization after five days of culture on collagen-immobilized PET samples. Representative fields were photographed at 10 x magnification using a Sony DSC-S75 Digital Camera and a Nikon inverted microscope.

Results and discussions

Significant changes in the surface topography induced by collagen immobilization on plasma treated PET films were evidenced, as well, by performing tapping-mode AFM experiments.

The images of the native PET film (fig. 2a) reveal a flat and homogeneous surface, with Sq of 1.43 nm. In addition, the cross-section profile taken along the solid line from 2D height image and the height histogram, shown in figures 2b and 2c, allowed the calculation of the average height, the value of 4.5 nm being obtained. The aspect of the statistical distribution of z-values within the image confirmed that the film surface was uniform.
Following collagen immobilization onto plasma treated PET surfaces, the AFM images highlight the distribution of collagen molecules, evidencing an irregular sphere-like appearance, due to the occasional overlapping of some collagen molecules to each other (fig. 3 and 4).

Figure 3 displays the AFM illustrations of the collagen immobilized on 3 min plasma treated PET surface. The height images of the treated PET film (fig. 3a) reveal a surface with Sq of 2.09 nm. In addition, the cross-section profile taken along the solid line from 2D height image and the height histogram, exhibited in figure 3b and 3c, allowed the calculation of the average height, the value of 6.5 nm being obtained. The aspect of the statistical distribution of z-values within the image confirmed that the film surface was uniform.

The AFM measurements for collagen immobilized on 10 minutes plasma treated PET film revealed a surface structure with small grains, showing the tendency to cluster, as well (fig. 4a). The height images of the treated PET film (fig. 4a) reveal a surface with Sq of 9.97 nm. In addition, the cross-section profile taken along the solid line from 2D height image and the height histogram, exhibited in figure 4b and 4c, allowed the calculation of the average height, the value of 30 nm being obtained. The aspect of the statistical distribution of z-values within the image confirmed that the film surface was not uniform.

Surface chemical modifications induced by plasma treatment and collagen immobilization were determined by XPS measurements. Figure 5 shows the wide scan spectra of the untreated and plasma treated PET surfaces, these ones mainly containing C1s, O1s and N1s peaks. The elemental compositions and atomic ratios were summarized in table 1, the untreated PET film has an O/C ratio equal to 0.26, while after plasma treatment and collagen immobilization the O/C atomic ratio for 3 min plasma and 10 min plasma are increased to 0.30 and 0.31. The slight increase in O/C atomic ratio suggests that new oxygen-containing polar groups are formed on the PET films surface after treatment. The XPS measurements indicate, as well, that the surface treatments lead to an increase in the N/C atomic ratio from 0.14 for plasma 3 min to 0.25 for plasma 10 min. Therefore, nitrogen-containing functional groups are formed on the PET surfaces after plasma treatment and collagen immobilization. Adsorption of collagen led to the appearance of a N1s peak, which is increasing with plasma exposure time.

The C1s high resolution spectra of untreated PET film is shown in figure 6. The peaks may be identified at 284, 285.5, and 288.1 eV, respectively, which can be assigned to C–C/C=H, C–O and O=O–C=O, respectively [18]. The spectra of treated films (fig. 7 and 8) reveal, as well, peaks for C–C/C=H, C–O and/or C–N, O=O–C=O and/or N–C=O and an additional peak at 287.1 eV, which may be attributed to N-
C=O groups [14]. The changes in content of each chemical component are given in Table 2, which shows that the C–C component decreases significantly after plasma treatment, and, at the same time, most of the C–O oxygen containing polar groups, O=C–O and N–CO–N are increasing in the surface of the plasma treated polymer surfaces. These results indicate that some of the C–C/C–H bonds present in polymer surface are broken by the plasma treatment, the broken C–C/C–H bonds recombining with the oxygen and the nitrogen atoms [15, 16].

Photomicrographs of haematoxylin and eosin stained samples (Fig. 9) showed a homogenous cell distribution in all cases. All tested samples sustained adherence and proliferation of the endothelial cells. After 5 days in the culture media, on collagen samples the endothelial cells grew as a monolayer and presented a typical polygonal shape, with large nuclei.

**Conclusions**

In the present study, PET films activated by a DC helium plasma treatment and placed in a collagen-buffer solution were thoroughly investigated. The changes in the surface topography induced by collagen immobilization on plasma treated PET films were evidenced by using tapping-mode
AFM experiments. The roughness values and the average height, obtained from the AFM images interpretation of different regions of the treated polymer surface, demonstrate an increase of the roughness with the increasing of plasma treatment time.

XPS measurements revealed the appearance of a N1s peak, which is increasing with plasma exposure time and demonstrates the adsorption of collagen through the formation of nitrogen-containing functional groups on the PET surfaces after plasma treatment and collagen immobilization. The higher value of the nitrogen concentration is obtained for 10 min plasma treatments followed by collagen immobilization.

By using cytotoxicity and cytocompatibility tests, the biocompatibility of the modified PET surfaces was assessed by studying the behaviour of human aortic endothelial cells. All tested samples sustained the endothelial cells development. The cytocompatibility tests revealed an increase in cell growth as a monolayer and presented a typical polygonal shape, having large nuclei.

This study is, therefore, reporting that the proposed configuration of DC plasma device represents an appropriate method to be used for collagen immobilization and for producing biocompatible PET surfaces.

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