Cell Adhesion and Proliferation on Plasma-Treated and Poly(ethylene glycol)-Grafted Polyethylene

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Abstract
Polyethylene (PE) was modified by an Ar plasma. The plasma-activated PE surface was grafted with poly(ethylene glycol) (PEG, molecular weight 300 and 20 000). The depth profiles of the oxygen in the modified PE samples were determined using Rutherford Backscattering Spectroscopy (RBS). The changes in the surface wettability were examined by goniometry, and Atomic Force Microscopy (AFM) was used to determine the surface roughness and morphology. The modified PE samples were seeded with rat vascular smooth muscle cells (VSMCs) and their adhesion and proliferation was studied. The plasma treatment and the subsequent PEG grafting leads to dramatic changes in the PE surface morphology, roughness and wettability. The PEG grafting of the plasma-treated PE does not increase VSMC adhesion but it results in dramatic increase of VSMC proliferation.

Keywords
Polyethylene, plasma discharge, PEG grafting, cell adhesion and proliferation

1. Introduction
The performance of polymeric materials relies largely on the properties of their surfaces. Most polymers have hydrophobic, chemically inert surface which may present a serious problem in their applications requiring adhesion, coating, etc. [1–3].

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For many medical and biological applications (tissue engineering, cell culture substrates and cell bio-chips) the interaction of biological cells with the substrate is of crucial importance [4]. The interaction of living cells with materials is strongly dependent on physical and chemical properties of material surface. The main properties decisive for the colonization of a material with cells are its polarity, wettability, electrical charge, roughness and topography [5–10]. Important may also be the presence of various chemical functional groups and biomolecules on the material surface [5, 6].

There are various ways of modifying the surfaces of the polymers to make them more suitable for cell cultivation. A modified polymer surface exhibits increased chemical reactivity, due to presence of free radicals and unsaturated bonds, and it can easily be grafted with suitable agents. For this purpose, the surfaces have been exposed to ultraviolet (UV) irradiation [7, 11], to beams of various ions (e.g., oxygen, nitrogen, noble gases, or halogens) for biological applications [12, 13] or to a plasma discharge [14–18]. For more pronounced changes in the physico-chemical properties of the modified surface, some of these processes are realised in a gas atmosphere, e.g., in acetylene or ammonia [7, 11]. These modifications change the stability, roughness, morphology, mechanical properties and chemical composition of the polymer surface due to creation of functional chemical groups containing oxygen or nitrogen, like carbonyl, carboxyl or amine groups, on the surface of the material [19–23]. These groups increase the surface wettability, support the adsorption of cell adhesion-mediating extracellular matrix proteins in an appropriate geometrical conformation and stimulate cell adhesion and growth [7, 12].

In this study high density polyethylene (PE) was modified by Ar plasma and subsequently grafted with poly(ethylene glycol) (PEG). The morphological, structural and compositional changes were characterized by different techniques: AFM (surface morphology, roughness), RBS (oxygen depth profile), goniometry (contact angle). The cell adhesion and proliferation on the modified polymers was studied by cultivation of rat vascular smooth muscle cells (VSMCs).

2. Experimental

2.1. Materials and Modification

Oriented high density polyethylene (PE, density 0.951 g/cm³) in the form 40 µm thick foils (supplied by Granitol Ltd., Czech Republic) was used. The samples were modified in Ar plasma discharge in a Balzers SCD 050 device. Exposure time was 50, 150 and 400 s, discharge power was 2 W and gas purity was 99.997%. Immediately after plasma treatment, the plasma activated PE was grafted by immersion to an aqueous solution (24 h, room temperature, 2 wt%) of poly(ethylene glycol) (PEG, molecular weight (M) 300 and 20 000 g/mol). Grafting is a chemical bonding of new structures on modified polymer surface [24]. The non-bound PEG was removed by immersion of the samples into distilled water for 24 h.
2.2. Characterization of Modified PE

Surface wettability was measured by goniometry, i.e., the static (sessile) water drop contact angle method. It was shown earlier that the contact angle of PE (exposed to plasma discharge) depends on the time elapsed from the moment of exposure [19]. In this experiment, the contact angle on the plasma-modified and grafted samples was measured over 54 days. Advancing water contact angles (error ± 5%) were measured at 10 different positions at room temperature using the Surface Energy Evaluation System (Advex Instruments, Czech Republic) [19, 27].

The concentration depth profile of oxygen in the modified PE surface layer (after plasma treatment and PEG grafting) was determined by Rutherford Backscattering Spectroscopy (RBS). The RBS analysis was performed in a vacuum chamber with 2.72 MeV He\(^+\) ions. Elemental depth profiles in the inspected samples were determined with the typical depth resolution less than 10 nm and an accessible depth of a few µm. The RBS spectra were evaluated by the GISA3.99 code [18]. The typical RBS detection limit is 0.1 at% for oxygen with an experimental error ±7%.

Surface morphology and roughness of the pristine and modified samples were determined using a VEECO CP II AFM working in the tapping mode. A Si probe, RTESPA-CP, with a spring constant 20-80 N/m was used. By repeated measurements in the same region (1 µm × 1 µm) it was shown that the surface morphology did not change after three consecutive scans. The roughness value \(R_a\) represents the arithmetic average of the deviations from the centre plane of the sample.

2.3. Cell Culture, Adhesion and Proliferation

The adhesion and proliferation of vascular smooth muscle cells (VSMCs) on pristine and modified PE substrates were investigated in vitro. Polymer samples were sterilized for 1 h in ethanol (70%) before cell cultivation. The samples were inserted into 24-well polystyrene multidishes (TPP, Switzerland; diameter 15 mm). Each sample was treated with 1.5 ml of Dulbecco’s modification of Eagle’s Minimum Essential Medium (DMEM, Sigma) containing 10% of fetal bovine serum (FBS, Sebak GmbH, Aidenbach, Germany) and 17 000 VSMCs per cm\(^{-2}\). Cells were cultivated at 37\(^\circ\)C in a humidified air atmosphere containing 5% of CO\(_2\). The adhered cells were counted 24 hours after seeding, and cell proliferation was determined on 2nd and 7th day after seeding. Cells were rinsed in phosphate-buffered saline (PBS), fixed in 70% ethanol and colored with Texas Red C\(_2\) maleimide, which conjugates with the cell membrane and cytoplasmatic proteins. Cell nuclei were visualized by Hoechst #33342 dye. The number and morphology of cells were then evaluated from pictures taken with an Olympus IX 51 microscope using an Olympus DP 70 digital camera.

The numbers of viable and dead cells was evaluated by automated counting in a ViCell XR analyzer (Beckman Coulter, USA) after they had been detached by a trypsin–EDTA solution (Sigma, USA, Cat. No T4174) in PBS for 10 min at 37\(^\circ\)C. This automated analysis involved evaluation of cell viability by staining with trypan blue, which penetrated through the membrane of damaged and nonviable cells. As
revealed by trypan blue exclusion test performed automatically during cell counting in the cell viability analyser (ViCell, Beckman), the percentage of viable cells among cells detached from both PEG surfaces was relatively high, ranging from about 76 to 85%. It can be supposed that this percentage in the native culture before the cell detachment was even higher, and the cells were additionally damaged by trypsinization, resuspension in buffer and other procedures associated with the measurement in the ViCell Analyser.

3. Results and Discussion

3.1. Surface Properties of Modified PE

Treatment in the plasma discharge leads to cleavage of macromolecular chains, creation of free radicals and double bonds. Reaction with oxygen from ambient atmosphere in the reaction chamber or after exposing the samples to the atmosphere results in generation of various oxygen-containing structures which, in turn, increase surface wettability [19, 20]. The enhanced wettability is known to facilitate cell adhesion [7, 11, 18]. Contact angle as a function of time elapsed from the plasma treatment (aging) is shown in Fig. 1 for the samples exposed to the plasma discharge for 50, 150 and 400 s. Contact angle decreases dramatically after the plasma treatment and the decrease is inversely proportional to the exposure time. After about 5 days of aging the contact angle starts to increase and after some time (about 20 days) it reaches a saturated value [19]. Restoration of the contact angle is obviously connected with a rearrangement of degraded macromolecules on the plasma-treated PE surface in air [26–28]. On the contrary, contact angle measurements in water immersed samples lead to the conclusion that the surface polarity of the polymer increases continuously with time of water contact. This is attributed to the appearance at the surface of polar groups, initially ‘buried’ in the bulk of the polymer [29]. Reconstruction of the polymer surface may be ascribed to two stages: water adsorption, accompanied by diffusion into the bulk phase, and reorientation of the surface polar groups [30]. All biological experiments were done after sample aging time of 20 days or longer.

Dependence of the contact angle on the aging time for plasma-modified PE and plasma-modified PE and subsequently grafted with PEG (molecular weights 300 and 20 000) is shown in Fig. 2. Pronounced decrease of the contact angle after PEG grafting seen for both molecular weights indicates binding of oxygen-rich compounds onto the activated PE surface [7]. Saturated value of restored contact angle was achieved after about 25 days of aging when the reorientation of the oxygen-containing structures was completed [27, 28]. More pronounced decrease of the contact angle is observed for higher molecular weight PEG ($M = 20 000$) due to higher oxygen content in this PEG species.

Concentration depth profiles of oxygen from plasma-treated PE, plasma-treated and immediately immersed into distilled water (water etched PE) and plasma-treated and subsequently PEG-grafted PE, obtained from RBS measurements, are
Figure 1. Dependence of the contact angle of the plasma-modified PE on the aging time. Plasma exposure times were 50, 150 and 400 s.

shown in Fig. 3. Some of the samples were etched in water since the subsequent biological experiments are carried out in a physiological solution, so that the interaction of the plasma-modified PE with water is of crucial importance. The maximum oxygen concentration is observed on the plasma-treated PE. Water etching results in a decrease of the oxygen concentration in the surface layer. This decrease indicates dissolution of a part of the oxidized structures or low molecular weight fragments [19, 26]. An increase of the oxygen concentration after the PEG grafting shows chemical binding of a part of PEG molecules on the surface of the plasma-modified PE. This increase of oxygen concentration does not explicitly prove PEG grafting on plasma-modified PE. In our previous work aminoacids were grafted onto PE degraded surface. In case of such reaction the decrease of radicals and double bonds and increase of oxygen concentration in surface layer occurred [6]. Therefore, based on our already published results we assumed that grafting occurred also in case of PEG.

Surface morphology is well known to affect the interaction of cells with polymers [11, 18, 27]. AFM images of pristine, plasma-treated, plasma-treated and water-etched, and plasma-treated and subsequently grafted with PEG with differ-
Figure 2. Dependence on the aging time of the contact angle of PE plasma modified for 400 s (0) and PE plasma modified and subsequently grafted with PEG. The molecular weights of PEG were $M = 300$ and 20 000.

Different molecular weights PE samples are shown in Fig. 4. Plasma treatment results in increased surface roughness and leads to exposure of PE lamellar structure. This phenomenon is a typical demonstration of plasma stimulated ablation of PE [9]. After water etching (Fig. 4(c)) the morphology changes due to dissolution of low molecular weight degradation products [19]. Subsequent PEG grafting leads to a change in surface roughness and, regardless of PEG molecular weight, to a decrease in surface roughness (Fig. 4(d, e)). While the grafting with PEG of molecular weight $M = 300$ preserves PE lamellar structure, the grafting with PEG of molecular weight $M = 20 000$ results in significant change of the morphology and in creation of branched star-like formations (Fig. 4(e)). This might be a clear 2D PEG crystallization process [31].

3.2. Cell Adhesion and Proliferation

Adhesion and proliferation of rat VSMCs was studied on pristine PE, PE modified by plasma discharge for 50, 150 and 400 s and on PE modified by plasma discharge and subsequently grafted with PEG ($M = 300$ and 20 000). The anti-adhesive action of PEG is due to its very high hydrophilicity and mobility of its
Figure 3. Oxygen concentration depth profiles of the plasma-treated PE (400 s, PE/plasma), PE plasma treated and immersed into distilled water (water etched) (PE/plasma/H$_2$O), PE plasma treated and subsequently grafted with PEG (molecular weight $M = 20000$, PE/plasma/PEG).

chains, which hamper stable adsorption of the proteins that mediate cell adhesion. However, at the same time, the anti-adhesive action of PEG is strongly dependent on its concentration on the polymer surface and on the length of its chain [32]. For comparison, the same procedures were applied to tissue culture polystyrene (TCPS). All experiments were performed in vitro. Total number of adhered (after 1 day cultivation) and proliferated (after 2 and 7 days of cultivation) cells is shown in Fig. 5. During the first 24 h the cells adhere very well on pristine PE and PE modified by plasma discharge. Lowest adhesion was observed on the PE grafted with PEG ($M = 300$). Higher adhesion was found on the PE grafted with PEG ($M = 20000$) and on the TCPS. The situation changes 2 days after seeding. While the cell proliferation on pristine and plasma-modified PE is minimal, much higher proliferation is observed on the PEG-grafted PE. Dramatic increase of proliferating cells is observed on grafted samples 7 days after seeding. Much more cells grow on the samples grafted with PEG of higher molecular weight.

In Fig. 6 are shown images of adhered and proliferated VSMCs observed after 7 days of cultivation. Cells on pristine PE and PE modified by plasma discharge
are homogeneously spread over the sample area. After 7 days, the cells are still well spread but their distribution is not homogeneous and cells aggregates appear. On the contrary, on the plasma-modified and PEG-grafted PE a dense and homogeneous cell coverage is observed after 7 days of cultivation, in spite of the fact then grafting did not affect initial cell adhesion.

Similarly, the PEG chains used in the present study were either short ($M = 300$) or long ($M = 20\,000$), and good adhesion and growth of cells on the such PEG-modified surfaces were observed. In addition, the PEG chains in the present study were probably attached to the plasma-modified PE through several sites on one
Figure 5. Dependence of the number of VSMCs on the cultivation time (1st, 2nd and 7th day) for pristine PE (PE), PE plasma modified for 50, 150 and 400 s (PE/plasma/50–400) and subsequently grafted with PEG (molecular weight $M = 300$, PE/plasma/50–400/300) and PEG (molecular weight $M = 20000$, PE/plasma/50–400/20 000) and tissue culture polystyrene (TCPS).

PEG chain, and not only through the chain end, which hampered mobility of the PEG chain but did not prevent the protein adsorption and cell colonization. What is more, the oxygen-containing groups present in the PEG molecules might enhance the colonization of the material by VSMCs as indicated by the highest final cell number on PEG-grafted PE on day 7 after seeding.

4. Conclusion

The properties of the plasma-modified and PEG-grafted PE were investigated and the bioactivity of modified PE was studied with VSMCs in in vitro experiments. Plasma treatment leads to a rapid decrease of contact angle and corresponding increase of the PE surface polarity and wettability. Sample aging results in surface relaxation and restoration of the contact angle which achieves a saturated value after about 20 days of aging when reorientation of polar structures in the modified surface layer is completed. With increasing time of exposure to the plasma discharge the wettability of the modified PE decreases. Grafting with PEG leads to additional dramatic reduction of the contact angle. With decreasing chain length of PEG the
Figure 6. Photographs of VSMCs adhered (1st day) and proliferated (7th day) on pristine PE (PE), PE plasma treated for 50 s (PE/plasma) and subsequently grafted with PEG (molecular weight $M = 300$, PE/plasma/PEG 300) and PEG (molecular weight $M = 20\,000$, PE/plasma/PEG 20\,000).
polarity of the grafted PE decreases. Presence of oxygen-containing groups in the surface layer of the plasma-modified PE and subsequently grafted with PEG was evidenced by the RBS technique. Plasma treatment increases surface roughness of PE and exposes its lamellar structure.

The surface roughness of the plasma-modified PE is reduced by the etching in water. Grafting with short PEG chains preserves the PE lamellar structure but grafting with longer PEG chains produces branched star-like formations on the PE surface. The biological in vitro experiments with VSMCs show that PEG grafting does not increase cell adhesion but it increases dramatically cell proliferation.

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