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POLYSTYRENE SURFACE MODIFICATION FOR SERUM-FREE CELL CULTURE USING AN ATMOSPHERIC PRESSURE DIELECTRIC BARRIER DISCHARGE

E.C. STANCU[1,2*], A. QUADE[1], K.-D. WELTMANN[1]

[1]Leibniz Institute for Plasma Science and Technology (INP Greifswald),
Felix-Hausdorff-Str. 2, 17489 Greifswald, Germany
[*]E-mail: *claudia.stancu@inp-greifswald.de*
[2]National Institute for Laser, Plasma and Radiation Physics (INFLPR),
Atomistilor Str. 409, 77125 Magurele-Bucharest, Romania

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Abstract. Surface treatments that are required to achieve polystyrene surface suitable for cell culture applications were performed using an atmospheric pressure dielectric barrier discharge generated in helium. Short time plasma treatments affected the polystyrene surface hydrophobicity and surface free energy due to insertion of oxygen-containing polar groups such as -OH, -COOH as proofed by X-ray photoelectron spectroscopy. These changes in surface properties were studied in relation to cellular behavior of human keratinocytes (HaCaT) *in vitro* and in serum-free medium conditions. Plasma treated polystyrene surfaces promoted cell growth in culture, after several hours.

Key words: biomaterials, atmospheric pressure discharge, dielectric barrier discharge, helium, polystyrene, keratinocytes, cell culture, Alamar blue.

1. INTRODUCTION

Dielectric barrier discharges (DBDs) are used widely for surface treatment of materials applied in fields ranging from biomaterials to automobile components [1, 2]. DBDs are plasma generated in configurations of one or more insulating layers with a discharge gap in the current path between the metal electrodes [3]. For industrial applications, such discharges have attracted interest due to the high flexibility with respect to their geometry and electrode configuration, size requirements ranging from a few millimeters to meters, working gas and type of materials to be treated, low cost and simple operation without vacuum equipment [4]. In particular, helium DBDs [5] are typically used for plasma ignition producing in most cases a uniform filament-free glow discharge with low degradation effect, high properties of crosslinking and functionalization onto the surface [6, 7]. Various papers exist in literature regarding helium DBD processes for surface treatment of polymers including polypropylene [8, 9], high density polyethylene [10], polyethylene terephthalate [11], polymethyl-methacrylate [12], etc. As with other polymers, polystyrene surface can be prepared by appropriate plasma treatment [13]. Polystyrene is one of the most extensively used

polymer material in cell culture applications [14]. Most of the medical vessels are made by polystyrene due to its excellent optical clarity, non-toxicity and durability. The freshly molded polystyrene, as pressed by manufacturer, have its limitations, especially for growing cells in serum-free medium and for maintaining differentiated cell functions in primary cultures and cell lines [15].

In this paper, polystyrene surfaces were prepared by atmospheric pressure dielectric barrier discharge generated in helium to support cell growth in a serum-free environment. In serum containing medium, the cellular attachment to surface is modulated by the interactions between cell surface receptors (*i.e.*, integrins) or cell membrane glycoproteins to serum protein [16, 17]. The surface investigations were performed by complementary analytical techniques like contact angle goniometry (CA) and X-ray photoelectron spectroscopy (XPS). Cell culture experiments were carried out *in vitro* using a human keratinocyte cell line (HaCaT). It was found that plasma treatment increased cell metabolic activity and promoted cell growth. This effect is attributed to the presence of oxygen-containing functional groups on the treated polystyrene surfaces.

2. EXPERIMENTAL

2.1. PROCEDURE FOR PLASMA TREATMENT

The atmospheric pressure DBD set-up is shown in Fig. 1. It is a conventional planar DBD with the upper electrode made of two parallel metallic stripes covered by a glass plate of 1.2 mm thickness that serves as dielectric barrier.

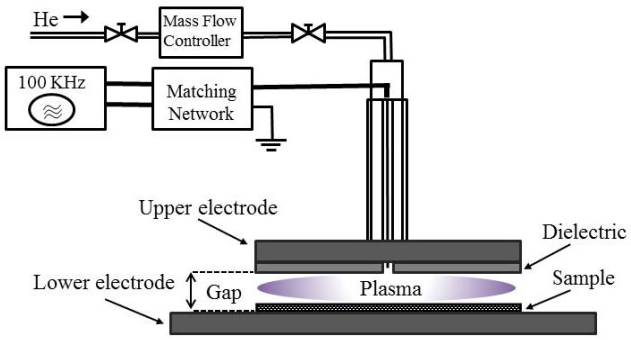

Fig. 1 – Schematic view of the DBD configuration.

The gas gap can be varied between 1 and 5 mm, and during the experiments it was of 2 mm. The lower electrode is made of plain Al and used as substrate holder. The plasma is generated with a power supply using high AC peak-peak voltages *via* a matching network at a fixed frequency of 100 kHz. Typical electrical parameters according to oscilloscopic measurements involved sustaining voltages

of 3–5 kV (peak) corresponding to the same discharge power dissipated on the substrates (P = 65 W). Helium gas was fed into discharge by a slit of 2 mm between the metallic stripes.

Helium DBD is able to produce a non-equilibrium plasma with a gas temperature of 30°C being suitable for treatment of temperature-sensitive materials like polymers. Previously, this DBD was used to deposit thin silicon organic protective films on Al sheet metal using helium and small admixtures of hexamethyldisiloxane (HMDSO) [18].

2.2. SURFACE ANALYSIS

Static contact angle (CA) measurements were performed under ambient air at room temperature by the sessile drop method using a Digidrop contact angle analyzer (GBX Instrumentation Scientifique, FR). Drops of selected liquids with known surface tension (distilled water, ethylene glycol and diiodomethane) and defined volume (0.5 μL) were deposited on polymer surface with microsyringes designated for each liquid. The contact angle was determined utilizing the SCA20 imaging software and the surface free energy was estimated using the Owens, Wendt and Rabel model.

X-ray photoelectron spectroscopy (XPS) measurements were performed using an Axis Ultra DLD electron spectrometer (Kratos Analytical, GB) equipped with a monochromatic Al K_α irradiation at 1,486.6 eV (150 W), implemented charge neutralization and pass energy of 80 eV for the estimation of the chemical elemental composition or pass energy of 10 eV for highly resolved C 1s spectra. A peak fitting procedure was carry out with CasaXPS software version 2.3.15 (Casa Software Ltd., UK) using the Gauss-Lorentzian distribution and a linear baseline. The full width at half maximum of the C 1s components was 1.2 eV for high energy resolution measurements. All values are given in atomic percentage and corresponding element ratio.

2.3. CELL CULTURE

Human keratinocyte cells (HaCaT) were cultured in plastic flasks containing RPMI 1640 w/phenol red, supplemented with 10% FBS plus 1% L-glutamine and 1% penicillin/streptomycin. Cells were subcultured using PBS/EDTA and maintained at 37°C, 95% in a humidified air with 5% CO_2 atmosphere. For assays were used 24-well ultra-low attachment plate and serum-free cell culture medium RPMI 1640 without phenol red supplemented with 1% L-glutamine and 1% penicillin/streptomycin.

HaCaT cells were seeded on corresponding surfaces at a concentration of 1.5×10^5 cells/ml, 1 ml per well and allowed to adhere. Cells were washed 3 times in HBSS, fixed in 4% PFA, stained with 0.05% trypan blue solution in PBS and examined with an Olympus CK40 microscope.

The percent confluency of the cells on the surfaces was measured by eye under an optical microscope. To account for the subjective nature of this approach, the error in estimating the percent confluency was set at ±5% for all surfaces.

Cell viability was investigated using the non-toxic Alamar blue assay. Alamar blue is a tetrazolium-based dye, incorporating resazurin and resorufin as oxidation–reduction indicators that produce colorimetric changes and a fluorescent signal in response to metabolic activity where the blue non-fluorescent oxidized form turns to pink and fluorescent upon reduction [19, 20]. After seeding, the cell culture medium was replaced by 1 ml of RPMI 1640 without phenol red supplemented with 1% L-glutamine and 1% penicillin/streptomycin and 2% Alamar blue. The plates were incubated for 4 h at 37°C. After incubation, 1250 µl solution of color product was transferred into 24-well plate to measure the fluorescence intensity using a multi-plate reader (Tecan M200, Switzerland) at 530 nm excitation and 590 nm emission wavelength. The amount of fluorescence was directly proportional to the metabolic activity of living cells.

3. RESULTS AND DISCUSSION

3.1. CONTACT ANGLE MEASUREMENTS

The comparison of the water contact angle measurements as function of treatment time for different positions of the localized plasma treatment is presented in Fig. 2. The surface hydrophilicity profile after plasma treatment was investigated by measuring water contact angles along a line crossing the polystyrene surface with a step size of 2.5 mm. Position "0" represents the center of DBD set-up, which measures 5 cm in length. The initial contact angle of 89° was reduced to a contact angle of 36° after 1 s plasma treatment. The maximum contact angle reduction was obtained after 120 s of treatment and shortly after the contact angle reached a constant value. By increasing the treatment time (> 30 s) a broadening of the total modified area was observed (Fig. 2).

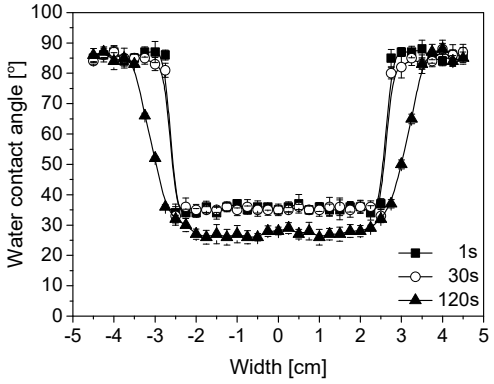

Fig. 2 – Surface profile of water contact angle of polystyrene as function of plasma treatment time.

Figure 3 shows the total surface free energy (γ) and its polar (γ^p) and dispersive (γ^d) components calculated from static contact angles with distilled water, ethylene glycol and diiodomethane as function of plasma treatment time. Untreated polystyrene has a low surface energy and a very low polarity. A drastic increase of surface energy from 29 mN/m to 55 mN/m occurred after the first second of polystyrene exposure to plasma. Further changes in the surface energy with time are slight. A maximum value (60 mN/m) of surface energy was obtained after 30 s of plasma treatment. Figure 3 also exhibits that the polar component for plasma treated polystyrene increased almost in the same way as the surface free energy changed while the dispersive component remained nearly unchanged. The reason of the observed behavior is due to the presence of oxygen-containing functional groups inserted by plasma into the polymer surface.

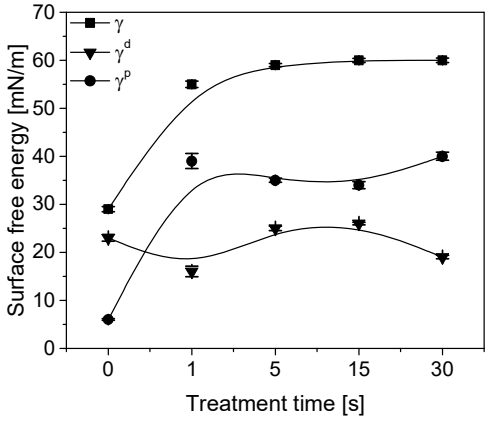

Fig. 3 – Variation of surface free energy and its components, polar and dispersive, for polystyrene as function of plasma treatment time.

3.2. SURFACE COMPOSITION ANALYSIS

An overview of elemental compositions and their quantifications based on high-resolution elemental spectra of untreated and plasma treated polystyrene is presented in Fig. 4. As expected, polystyrene contains mostly carbon (96.6 atomic %) and small amounts of oxygen (3.1 atomic %) and nitrogen (0.1 atomic %) associated with sterilization process and/or contaminations of the surrounding air. Primarily, oxygen (9.5 atomic %) and small amounts of nitrogen (1.5 atomic %) were incorporated into the polymer surface due to addition of oxygen and nitrogen from ambient air to helium discharge. In this work, the DBD uses only pure helium but the plasma chemistry is a mixture of helium-oxygen-nitrogen led by the presence of oxygen and nitrogen radicals from ambient air [21].

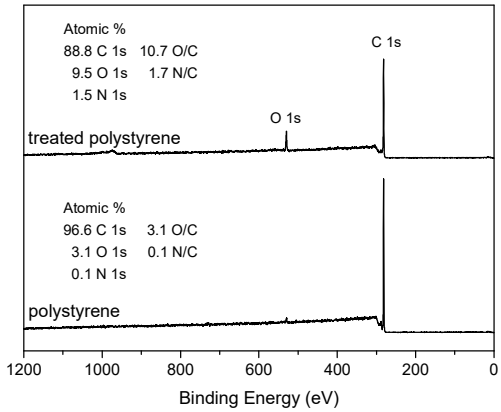

Fig. 4 – XPS survey for untreated and plasma treated polystyrene.

High-resolution XPS data in the C 1s region were acquired to identify the presence of carbon-based functional groups on the treated polystyrene. Figure 5a shows the highly resolved measured C 1s spectra of untreated polystyrene which is mainly composed of C-C_{arom} at 284.5 eV (carbon atoms in the phenyl ring), C-H/C-C_{aliph} at 285.0 eV (aliphatic carbon), and the characteristic $\pi \rightarrow \pi^*$ shake-up transition at 291.2 eV.

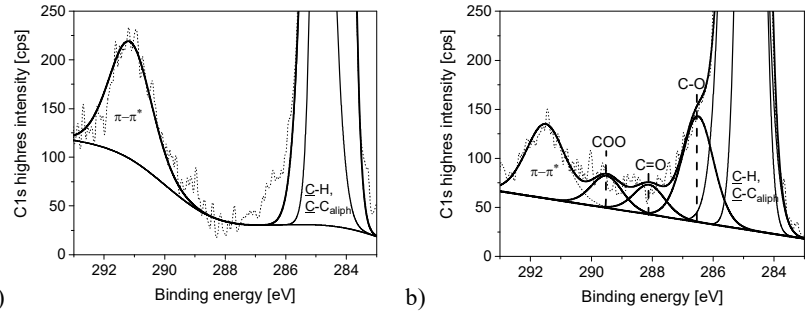

Fig. 5 – XPS C 1s high-resolution spectra of (a) untreated and (b) plasma treated polystyrene.

Curve fitting of the highly resolved C 1s peak of plasma treated polystyrene (Fig. 5b) exhibited three additional components positioned at BE of 286.5, 288.1 and 289.5 eV assigned to carbon-oxygen single bond (hydroxyl (C-OH)), carbonyl group C=O, and carboxyl group (COOH), respectively. Carboxyl group and hydroxyl group are polar groups and the greatest promoters to surface wettability [22].

3.3. CELL MORPHOLOGY AND VIABILITY

Cell experiments were performed *in vitro* and in serum-free medium conditions. Figure 6 shows the optical micrographs of HaCaT cells grown on untreated and

plasma treated polystyrene after 24 h of incubation. A distinct difference in cell morphology and spreading on corresponding samples is observed. HaCaT cells were fully spread out with good attachment, exhibiting 100% confluency on treated polystyrene (Fig. 6b). In contrast, cells on untreated polystyrene exhibited 40-50% confluency with cells rounded and lighter in appearance that indicate a poorly adherence (Fig. 6a). The observed increase in cell attachment may result from insertion of oxygen-containing functional groups on surface after plasma treatment. The preference exhibited by keratinocyte cells for treated polystyrene containing mixtures of -COOH and -OH groups confirms other findings with fibroblast [23], osteoblast [24] and endothelial [25] cells. Moreover, keratinocyte cells have shown a strong preference for surfaces with low amounts of carboxylic acid functional groups [26].

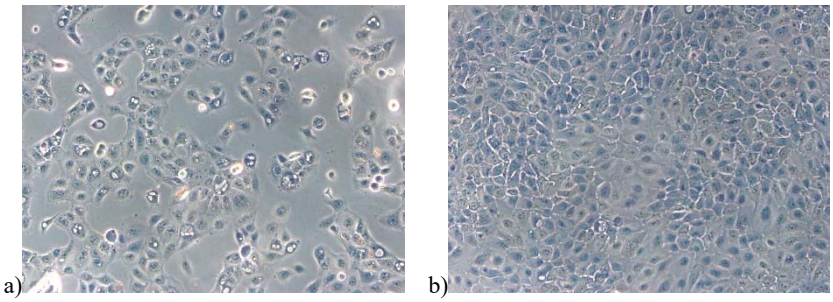

Fig. 6 – Optical micrographs of HaCaT cells after 24h of seeding in a serum-free environment on: a) untreated polystyrene and b) plasma treated polystyrene (magnification 200×, fixed and stained).

The metabolic activity of HaCaT cells on untreated and plasma treated polystyrene was measured by Alamar blue assay. The measured metabolic activity of cells at 5 min, 30 min, 3 h and 24 h after placement of cells in the wells is presented in Fig. 7. At these specific points, Alamar blue was added and fluorescence intensity was measured at 4 h after incubation.

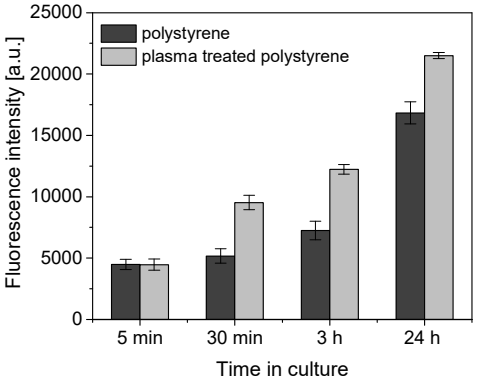

Fig. 7 – Fluorescence intensity of HaCaT cells cultured on untreated and plasma treated polystyrene.

An increase in metabolic activity occurred on both untreated and treated samples. Nevertheless, a difference in increased cell number with cell culture time on corresponding samples is observed. The metabolic activity of the HaCaT cells growing on the plasma treated polystyrene is significantly higher than on untreated sample.

4. CONCLUSIONS

Polystyrene surfaces were prepared by helium plasma using a dielectric barrier discharge configuration to improve keratinocyte cell growth in a serum-free medium. Few seconds of plasma treatment produced significant changes in surface chemistry, noticeably lowering the contact angle and increasing surface free energy due to the formation of oxygen-containing polar groups, such as hydroxyl and carboxyl groups, on the active sites. Visual assessment of confluency and measured metabolic activity showed that plasma treated surfaces are preferable for cell growth compared to untreated ones.

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REFERENCES

1. H.-E. Wagner, R. Brandenburg, K.V. Kozlov, A. Sonnenfeld, P. Michela, J.F. Behnke, Vacuum **71**, 417–436 (2003).
2. N.N. Morgan, Int. J. Phys. Sci. **4**, 885–892 (2009).
3. R. Brandenburg, Plasma Sources Sci. Technol. **26**, 29 (2017).
4. U. Kogelschatz, Plasma Chem. Plasma Process. **23**, 46 (2003).
5. T. Yokoyama, M. Kogoma, T. Moriwaki, S. Okazaki, J. Phys. D. **23**, 1125–28 (1990).
6. F. Massines, A. Rabehi, P. Décomps, R. Ben Gadri, P. Séguer, C. Mayoux, J. Appl. Phys. **83**, 2950–7 (1998).
7. G. Placinta, F. Arefi-Khonsari, M. Gheorghiu, J. Amouroux, G. Popa, J. Appl. Polym. Sci. **66**, 1367–75 (1997).
8. J.H. Choi, E.S. Lee, H.K. Baik, S.-J. Lee, K.M. Song, Y.S. Lim, Plasma Sources Sci. Technol. **14**, 9 (2005).
9. F. Massines, R. Messaoudi, C. Mayoux, Plasmas Polym. **3**, 43–59 (1998).
10. G. Borcia, A. Chiper, I. Rusu, Plasma Sources Sci. Technol. **15**, 849–857 (2006).
11. A.V. Nastuta, G.B. Rusu, I. Topala, A.S. Chiper, G. Popa, J. Optoelectron. Adv. M. **10**, 2038–42 (2008).
12. A. Chiper, G. Borcia, M. Dobromir, G. Popa, J. Adv. Res. Phys. **2** (2012).
13. K.G. Doherty, J.-S. Oh, P. Unsworth, A. Bowfield, C.M. Sheridan, P. Weightman, J.W. Bradley, R.L. Williams, Plasma Process. Polym. **10**, 978–989 (2013).
14. H. Rubin, Public Health Rep. **81**, 843–844 (1966).
15. J.A. Ryan, BioFiles **3**, 8–11 (2008).
16. A.V. Janorkar, E.W. Fritz, K.J.L. Burg, A.T. Metters, D.E. Hirt, J. Biomed. Mater. Res., Part B: Appl. Biomater. **81**, 142–152 (2007).
17. H.J. Griesser, R.C. Chatelier, T.R. Gengenbach, G. Johnson, J.G. Steele, J. Biomater. Sci. Polym. **5**, 531–554 (1994).

18. R. Foest, F. Adler, F. Sigeneger, M. Schmidt, Surf. Coat. Technol. **163**–**164**, 323–330 (2003).
19. R.M. DeBaun, G. de Stevens, Arch. Biochem. Biophys. **31**, 300–308 (1951).
20. B. Page, M. Page, C. Noel, Int. J. Oncol. **30**, 473–476 (1993).
21. V. Milosavljevic, M. Donegan, P.J. Cullen, D.P. Dowling, J. Phys. Soc. Japan **83**, 014501 (2014).
22. G. Zhou, C. Xu, W. Cheng, Q. Zhang, W. Nie, J. Anal. Methods Chem. **2015**, 8 (2015).
23. E. Cooper, R. Wiggs, D.A. Hutt, L. Parker, G.J. Leggett, T.L. Parker, J. Mater. Chem. **7**, 435–441 (1997).
24. C.A. Scotchford, C.P. Gilmore, E. Cooper, G.J. Leggett, S. Downes, J. Biomed. Mater Res. **59**, 84–99 (2002).
25. C.D. Tidwell, S.I. Ertel, B.D. Ratner, B.J. Tarasevich, S. Atre, D.L. Allara, Langmuir **13**, 3404–13 (1997).
26. R.M. France, R.D. Short, R.A. Dawsonb, S. MacNeilb, J. Mater. Chem. **8**, 37–42 (1998).