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Biocompatibility of oxygen-plasma treated polystyrene substrates

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Running head: Biocompatibility of O_2-plasma treated PS

Abstract

The biocompatibility of polystyrene (PS) samples has been improved by treatment with weakly ionized highly non-equilibrium oxygen plasma. Samples were exposed to plasma for 30 s so they have received a dose of ions of 4.5×10^{17} m^{-2} and a neutral oxygen atom dose of 3×10^{23} m^{-2}. Both untreated and plasma treated samples were tested for biocompatibility according to the same procedure. Proliferation of human mammary epithelial cell (HMEC) cells on samples revealed a dramatically improved biocompatibility of polystyrene treated by oxygen plasma. The HMEC cells were deposited on all samples and incubated for 1, 2 and 6 days. MTT test revealed about 2 – times higher activity of cell enzymes after 48 hour incubation. The activity for plasma treated samples remained much higher than for untreated samples even after 6 days of incubation when the samples were already covered with a dense film of MHEC cells.

Keywords: plasma modification, oxygen plasma, polymer, polystyrene, cell proliferation, HMEC cells

1. Introduction

Polymer materials are nowadays widely used in medicine both in vitro and in vivo [1-3]. A material that is frequently used is polystyrene (PS). Examples of in vitro applications include containers for a variety of liquids, cells and bacteria while an example of in vivo application are microspheres used as carriers of drugs and magnetic particles. Although polystyrene is known for its moderate biocompatibility, the properties are not always optimal so in some cases they should be improved.

A popular method for improvement of polymer biocompatibility is treatment by non-equilibrium gaseous plasma [4-8]. Depending on particular requirements, a variety of different plasmas can be applied for surface functionalization. The functional groups that could be formed on the surface of the polystyrene include oxygen rich groups such as C-O, C=O, O=C-O, nitrogen rich groups such as C-N, C=N, C≡N, sulphur, chlorine and fluorine groups [9-13]. The type and quantity of surface functional groups influence the surface free energy of a material. Oxygen containing functional groups are very polar, so the surface free energy of a material functionalized by such groups is much higher than the surface energy of untreated materials. A high surface energy often results in a good adhesion of different materials onto the substrate. Although, many works have been published on surface functionalization of polymer materials, much less work has been devoted to proliferation of human cells on modified polymers [14-21]. The scope of the present paper touches the proliferation of HMEC cells on plasma modified polystyrene.

2. Experimental

Samples of polystyrene PS (from Goodfellow) in the form of thin disks with the thickness of 0.25 mm and diameter of 10 mm were exposed to oxygen plasma. We used a plasma reactor that has been described to details in our previous paper [22]. Due to the completeness let us briefly summarize particularities of the reactor. Plasma is created in a glass discharge tube with diameter of 4 cm and length of about 60 cm. A rather uniform glow discharge is created within a RF coil which is 15 cm long. The coil is connected to RF generator operating at frequency 27.12 MHz and the nominal power of about 200 W. The impedance of the generator was optimized for such a configuration using a vacuum capacitor in parallel with the RF coil. Commercially available oxygen is leaked into the

glass tube on one side, while on the other one it is continuously pumped with a two-stage rotary pump. Continuous pumping allows for rapid removal of any reaction products that might otherwise accumulate in the plasma reactor and distort the original gas composition. Plasma parameters are estimated with a double electrical probe and a nickel catalytic probe. At the pressure of 75 Pa where the dissociation fraction of oxygen molecules is the highest, the density of oxygen ions is about 3×10^{15} m^{-3} while the density of neutral oxygen atoms is 2×10^{21} m^{-3}. Samples of PS were kept in plasma at floating potential for 30 s. The resultant doses of positively charged ions and neutral oxygen atoms the samples received during plasma treatment were about 1×10^{19} and 9.5×10^{24} m^{-2}, respectively. These doses were calculated from the following equation: $D = j \cdot t = ¼ \cdot n \cdot v \cdot t$, where j is the flux of oxygen atoms or ions to the surface, n is their density, v is their average velocity and t is the exposure time.

The surface functionalization of the polystyrene was studied by high resolution X-ray photoelectron spectroscopy. We used XPS instrument TFA XPS Physical Electronics. The base pressure in the XPS analysis chamber was about 6×10^{-8} Pa. The samples were excited with X-rays over a 400-µm spot area with a monochromatic Al $K_{\alpha 1,2}$ radiation at 1486.6 eV. The photoelectrons were detected with a hemispherical analyzer positioned at an angle of 45° with respect to the normal to the sample surface. The energy resolution was about 0.5 eV. Survey-scan spectra were made at a pass energy of 187.85 eV, while for C1s individual high-resolution spectra were taken at a pass energy of 23.5 eV and a 0.1 eV energy step. Since the samples were insulators, we used an additional electron gun to allow for surface neutralization during the measurements. All spectra were referenced to the main C 1s peak of the carbon atoms which was assigned a value of 284.6 eV. The XPS spectra were measured at an untreated sample and sample treated by oxygen plasma for 30 s. The concentration of different chemical states of carbon in the C 1s peak was determined by fitting the curves with symmetrical Gauss-Lorentz functions. The spectra were fitted using MultiPak v8.1c software from Physical Electronics, which was supplied with the spectrometer.

Selected samples were prepared for biocompatibility testing. A drop of medium with FCS (fetal calf serum) containing human mammary epithelial cells (HMEC) with seeding density 20,000 cells/100 µl was applied to all samples. The cells were left to attach to the surface for 2 hours. After this short incubation 600 µl of medium with FCS was added to cover the polymer discs with the cells. The samples were then placed into an incubator to allow for proliferation of HMEC cells. Cell morphology was observed under light microscope (magnification 100x). Cell growth and viability was measured after 1, 2 and 6 days with the MTT-related colorimetric assay (EZ4U; Biomedica, Austria) according to the manufacturer's instructions and Jaganjac et al. [23]. The method is based on the fact that living cells are capable of reducing less colored tetrazolium salts into intensely colored formazan derivatives. This reduction process requires functional mitochondria, which are inactivated within a few minutes after cell death.

Briefly, after 1st, 2nd and 6th day of the cell culture on the polymer surfaces the medium was removed and 1 ml of fresh Hanks' Balanced Salt Solution (HBSS) and 100 µl of the tetrazolium agent were added to each culture. After 2 h incubation, supernatants were transferred into 96-well plates and measured in a microplate reader (Easy-Reader 400 FW, SLT Lab Instruments GmbH, Austria) at 450/620 nm.

3. Results

Numerous samples were prepared by plasma treatment. One sample was characterized by XPS. XPS spectra were obtained at several different spots on the sample. A typical survey spectrum is presented in Figure 1. There are two curves in Figure 1: the lower curve is for untreated polystyrene sample and the upper curve is for plasma treated sample. As expected, the plasma treated sample contains much oxygen while for untreated PS sample no oxygen was found on the surface (see insert in Figure 1). High resolution XPS C1s spectra are presented in Figure 2. Again, the lower curve is for untreated sample and the upper one for plasma treated sample.

Optical microscopy images were taken occasionally in order to monitor the proliferation of cells during the incubation. Figure 3 reveals an optical image of the untreated sample incubated for 6 days, while Figure 4 shows the image of an oxygen plasma treated sample incubated for the same time. The difference between two images is obvious: the

concentration of cells in Figure 4 is so large that they form a uniform and rather dense film. On the other hand, Figure 3 reveals small areas that are not covered by the cells. Furthermore, even in the areas covered by the cells their density is much smaller than in Figure 4.

The optical microscopy reveals important information on the shape and local density of the cells but does not tell much about viability of the cells. The viability is often determined by MTT assay that actually measures the activity of cell enzymes. The time consuming MTT test were performed for the cells incubated by 2 and 6 days. The results are summarized in Figure 5. The histograms show the enzyme activity for different samples normalized to untreated polystyrene samples. It is noticeable that the activity for plasma treated samples is much larger than those for untreated samples.

4. Discussion

Samples were treated by oxygen plasma with a low density of ions and a rather high density of neutral oxygen atoms. Plasma with such parameters was chosen because we did not want to modify bulk properties, but at the same time we wanted to saturate the surface of PS with surface functional groups. Namely, it is well known that ions cause radiation damage of materials, while neutral oxygen atoms react only chemically with a sample. The functionalization by oxygen is revealed in Figure 1. The original concentration of oxygen in polystyrene is negligible (lower curve in Figure 1). Exposure of a sample to oxygen plasma causes enrichment of surface with oxygen so the final concentration as revealed from the XPS survey spectrum (upper curve in Figure 1) is about 27.5 at.%. This value is averaged over the analyzing depth which is several nanometers. Since we used plasma with a very high density of atoms and a low density of ions we can assume that the concentration of oxygen on the surface is much larger than 27.5 at.% [24]. This effect has been elaborated in a separate paper recently [24]. The survey spectra presented in Figure 1 reveal the average composition in the surface film but do not tell much about the chemical structure. In order to find out the type of functional groups presented on the surface of polystyrene after plasma treatment we measured also high-resolution C1s spectra. The spectrum for plasma treated sample (upper curve in Figure 2) shows a variety of different functional groups including C-O,

C=O, O=C-O and CO_3^- groups as published recently for the same material [24]. Here we should also note that we may have formation of –C(O)OO (peracids and perester) groups as well which appear at a binding energy close to CO_3^- groups [25-27]. Furthermore, oxygen O1s peak can be deconvoluted into three peaks at binding energies of about 532.1 eV, 533.4 eV and 534.5 eV which can be attributed to C=O, C-O and C(O)OO- groups, respectively. Last but not least, the concentration of the shake-up peak in the C1s spectrum has decreased from 8.5 % to about 4% after the treatment indicating possible destruction of phenyl rings [24].

Comparison of upper curves in both Figures 1 and 2 shows a virtual discrepancy. Namely, Figure 1 reveals only about 27.5 at.% of oxygen, while Figure 2 clearly shows existence of functional groups containing two or more oxygen atoms per carbon atom. Such discrepancy is often explained by the particularities of the XPS method as well as the fact that each oxygen atom can be bonded to two carbon atoms. In any case, the surface of plasma treated sample is densely covered with polar functional groups and likely to attract foreign coating materials.

A foreign material could be substances that biological cells use to adhere onto surfaces. It is well known that most biological cells are rather inactive if not firmly bond to a substrate. Most biological cells are capable of reproduction only if properly adhered to a scaffold. The adhesion is therefore of crucial importance in cell proliferation. Figures 3 and 4 reveal important differences in the proliferation between untreated and plasma treated polystyrene. In both cases, spherical cells are not adhered on the surface and are inactive. Cells with more complex shape are adhered well on the surface. Figure 3 represents an optical image for untreated sample. We can observe numerous spherical cells indicating that many cells are not well adhered onto the surface. Furthermore, the cells of more complex shape are not very dense and there are clearly distinguishable areas not covered by the cells. The largest area of this type is in the bottom left in Figure 3. As expected, untreated polystyrene has a rather moderate biocompatibility in terms of proliferation of HMEC cells. Figure 4, on the other hand, reveals dense cells that seem to overlap and are not spherical. Only few spherical cells are observed, which can represent the cells in the process of division. According to the upper discussion the plasma treated polystyrene represents a good environment for adhesion and proliferation of the cells.

From this point of view, the biocompatibility of plasma treated polystyrene is improved significantly.

Qualitative results presented in Figure 3 and 4 are confirmed by a quantitative technique – MTT assay. Figure 5 summarizes the results of the cell enzyme activity which, as mentioned above, is an indicator of the cell viability. The histograms presented in Figure 5 indicate that the proliferation of cells is increased by a factor of 2 after incubating for two days. The ratio is somehow lower after 6 days of incubation. This effect is probably due to the fact that the sample proliferation occurs already even on the untreated material.

5. Conclusions

Samples of polystyrene were modified by oxygen plasma treatment in order to improve their biocompatibility. XPS results showed formation of oxygen rich functional groups on the surface that allow for rather good adhesion of MHEC cells. Since they are incubated properly, the proliferation occurs as demonstrated by both optical microscopy and MTT assay. The results clearly demonstrate highly approved biocompatibility of the material treated by non-equilibrium oxygen plasma.

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FIGURE CAPTIONS

Figure 1: Survey XPS spectra of untreated sample (lower curve) and plasma treated sample (upper curve).

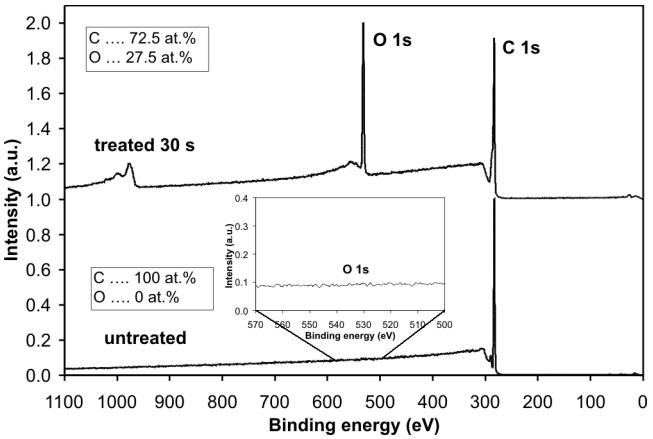
Figure 2: High-resolution XPS C1s spectra of untreated sample (lower curve) and plasma treated sample (upper curve) showing concentration of newly formed various oxygen functional groups.

Figure 3: Optical microscopy image of HMEC cells on PS polymer after 6 days of incubation for untreated sample.

Figure 4: Optical microscopy image of HMEC cells on PS polymer after 6 days of incubation for treated PS sample.

Figure 5: Results of MTT assay – comparison of untreated and plasma treated sample after 2 and 6 days of incubation.

FIGURE 1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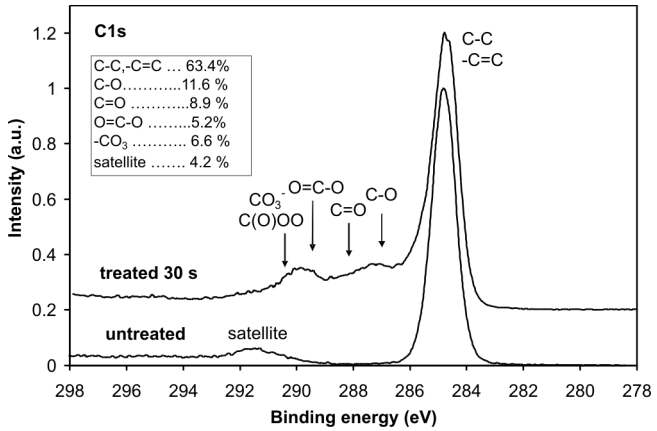
O … 27.5 at.%
O 1s
C 1s
treated 30 s
C …. 100 at.%
O …. 0 at.%
untreated
Intensity (a.u.)
Binding energy (eV)
O 1s
Intensity (a.u.)
Binding energy (eV)

FIGURE 2


C1s
C-C,-C=C … 63.4%
C-O……..11.6 %
C=O ……..8.9 %
O=C-O ……..5.2%
-CO3 …….. 6.6 %
satellite ……. 4.2 %
C-C
-C=C
CO3-
C(O)OO
O=C-O
C=O
C-O
treated 30 s
untreated
satellite
Intensity (a.u.)
Binding energy (eV)
1.2
1
0.8
0.6
0.4
0.2
0
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296
294
292
290
288
286
284
282
280
278

FIGURE 3

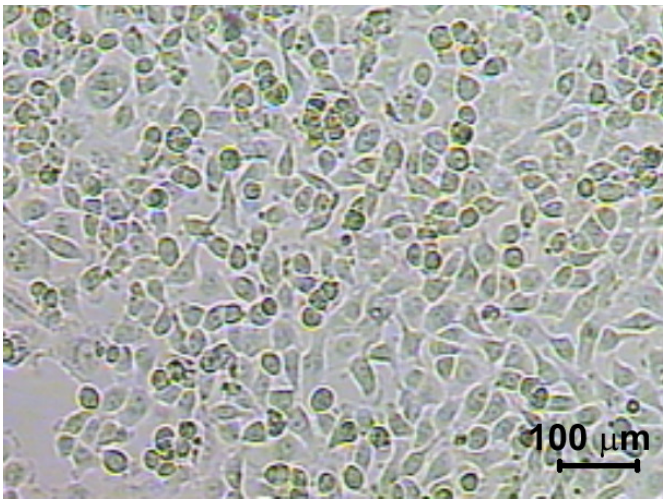


FIGURE 4

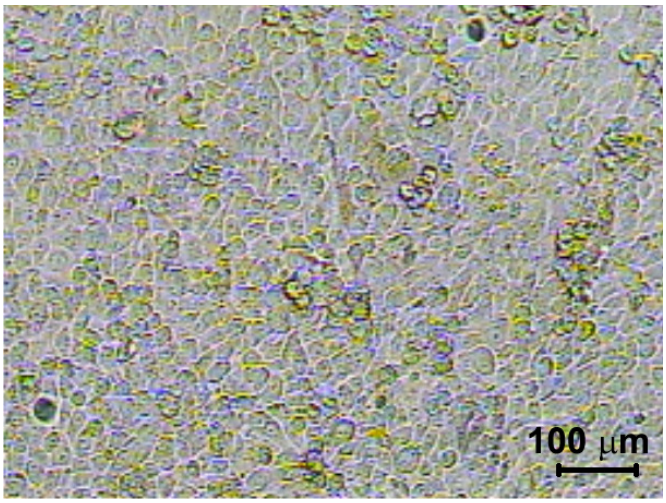
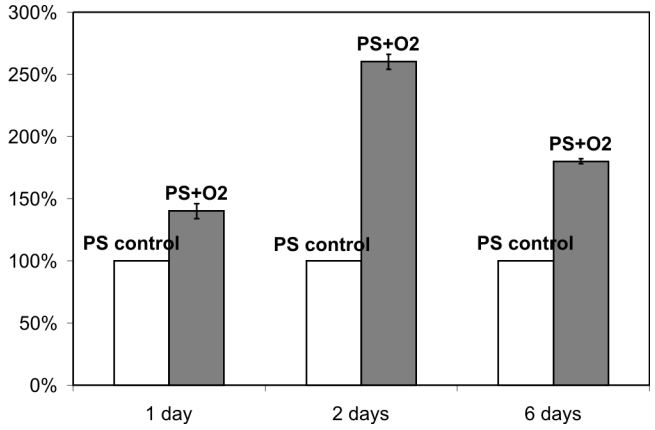


FIGURE 5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PS+O2
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1 day
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6 days