

Research Article

Cell Adhesion on Polycaprolactone Modified by Plasma Treatment

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We have investigated the influence of various plasma treatments of electrospun polycaprolactone (PCL) scaffolds on the adhesion and proliferation of human umbilical endothelial cells (HUVEC). The PCL scaffolds were treated in plasmas created in O₂, NH₃ or SO₂ gas at identical conditions. Surface functionalization of plasma-treated samples was determined using X-ray photoelectron spectroscopy. Cell adhesion and morphology were investigated by scanning electron microscopy and the influence of plasma treatment on cell adhesion and viability was evaluated with cell viability assay (MTT assay). The results showed the highest metabolic activity of HUVEC on PCL samples treated with O₂ and NH₃ plasma. Accordingly, the cells reflected the best adhesion and morphology on O₂ and NH₃ plasma-treated PCL samples already at 3 h. Moreover, treatment with O₂ and NH₃ plasma even stimulated endothelial cell proliferation on PCL surfaces by 60% as measured at 24 h, showing significant improvement in endothelialization of this material. Contrarily, SO₂ plasma appeared to be less promising in comparison with O₂ and NH₃ plasma; however, it was still better than without any plasma treatment. Thus, our results importantly contribute to the biocompatibility improvement of the PCL polymer, commonly used for scaffolds in tissue engineering.

1. Introduction

Polymeric materials are nowadays frequently used in various medical applications including artificial implants, tissue engineering scaffolds, wound dressings, and drug delivery systems [1–4]. Many of such applications usually require modification of surface properties of the polymer to improve its biocompatibility, cell adhesion and proliferation, and attachment of bioactive functional groups [5–8]. Various methods like plasma treatment, grafting of polymer brushes, or applying different coatings can be used for polymer surface modification.

For the past few decades, nanofibers have gained much importance due to the potential applications in broad areas of technological applications. Many of the potential uses of nanofibrous membranes are related to high porosity, large surface area, and small pore distribution. There are many methods for the fabrication of nanofibrous membranes; however, the most successful method is the electrospinning. Electrospinning can produce continuous nanofibers from submicron diameter scale down to nanometer diameter scale through an electrically charged jet of polymer solution [9, 10].

Electrospun three-dimensional polymeric scaffolds are becoming important in tissue engineering applications,

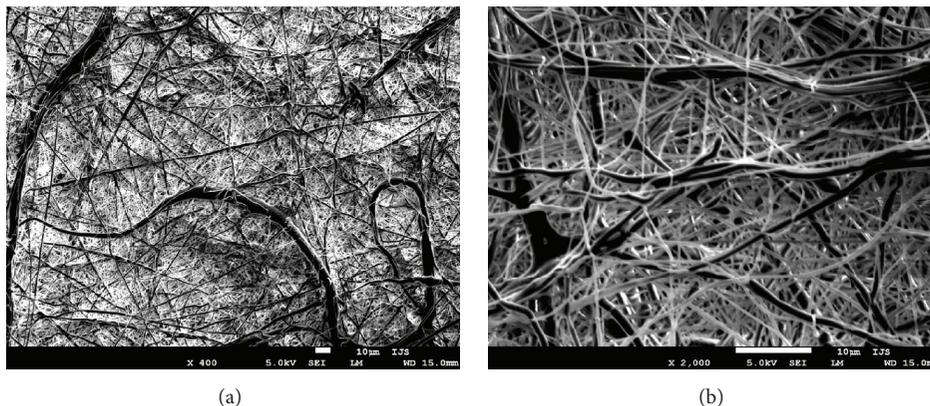


FIGURE 1: SEM image of electrospun PCL membranes at two different magnifications: (a) 400x magnification and (b) 2,000x magnification.

especially in bone regeneration [11, 12] and skin reconstruction [13, 14]. When cells attach to the scaffold, the scaffold has to offer optimal support and conditions for cell growth that leads to the formation of a new tissue. Tissue engineering scaffolds are often made of polymers like polycaprolactone (PCL) or polylactic acid (PLA) [15, 16]. Such polymeric scaffolds are biocompatible and biodegradable, which means that it is not necessary to remove them from the human body after implantation, because *in vivo* they slowly degrade to nontoxic products [17]. However, such scaffolds require surface modification to improve cell adhesion and proliferation [18]. Plasma treatment is one of the most useful techniques, because it allows for incorporation of different functional groups on the surface of the treated polymer. By proper choice of gas it is possible to manipulate these functional groups and change their nature, this way altering their surface wettability and surface energy, which both have a drastic impact on protein and cell adhesion [19–21].

Some authors have used oxygen or air plasma for surface modification of PCL and found increased surface hydrophilicity, surface energy, surface roughness, and total amount of oxygen functional groups on PCL surface [22, 23]. They have also observed better attachment and proliferation of osteoblast cells [18, 24, 25]. Wulf et al. have used ammonia plasma and found that modified surface did not affect the mouse fibroblast cell viability [26]. Helium and argon plasma were used as well. Both plasmas led to incorporation of oxygen functional groups to PCL. In case of helium, the nitrogen functional groups were found on the surface of treated PCL as well. Unfortunately, the biological cell response to modified PCL was not tested [22].

Because different plasmas can influence cell adhesion differently and, furthermore, different cells can behave differently on the same surface [27], more systematic research of modified polymer surfaces is needed. In the present investigation we investigated the surface modifications of PCL polymer induced by oxygen (O_2), ammonia (NH_3), or sulphur dioxide (SO_2) plasma treatment. NH_3 plasma was chosen to introduce amino groups which are important in many biological processes. For SO_2 plasma there is a lack of scientific literature, although it is known that sulphate

groups can play an important role in antithrombogenicity of the surface [28, 29], whereas O_2 plasma was used as a control, because it is usually very efficient for achieving good endothelialization [27]. Therefore, we have tested the effect of these modifications on cell adhesion and proliferation of human umbilical vein endothelial cells (HUVEC).

2. Material and Methods

2.1. Fabrication of Electrospun PCL Scaffolds. Electrospinning was carried out using PCL solutions with a polymer concentration of 15 wt.% which were prepared in acetone. The clear solution without any turbidity was taken in a 15 mL plastic syringe with 20-gauge blunt tip needle and electrospun at a high direct current (DC) voltage of 18 kV, a flow rate of 1 mL/h, and a tip to collector distance of 15 cm. A thin aluminum sheet with an approximate dimension of 10 cm \times 7 cm was used as the collector. Upon applying a high DC voltage using a high-voltage power supply, a fluid jet was ejected from the tip of the needle. As the jet accelerated toward the target, the solvent evaporated and polymer nanofibers get deposited on the aluminum sheet as a fibrous membrane. All the electrospinning process was carried out at a temperature of 28°C and a relative humidity of 60%.

Scanning electron microscopic (SEM) images of the fabricated electrospun PCL membranes are shown in Figure 1.

2.2. Plasma Treatment. PCL samples were treated in a discharge tube made from Pyrex glass, 80 cm long and 4 cm in diameter. The tube was pumped with a rotary pump operating at a nominal pumping speed of 80 m³ h⁻¹. A coil of 6 turns was mounted in the centre of the tube. Plasma was created by an RF generator coupled to the coil via a matching network. The generator operated at the standard frequency of 13.56 MHz and its nominal power was set to 150 W. The plasma was ignited in the capacitive mode (E-mode). Samples were treated in plasmas created in different gases: oxygen (O_2), ammonia (NH_3), or sulphur dioxide (SO_2). The pressure was set to 50 Pa. Samples were treated in the afterglow 20 cm away from the coil. Treatment time was 10 s.

TABLE I: Surface composition of plasma-treated samples.

	C	N	O	S	O/C	N/C	S/C
Untreated	77.8		22.2		0.29		
Treated in O ₂	68.0		32.0		0.47		
Treated in NH ₃	81.0	3.8	15.2		0.19	0.05	
Treated in SO ₂	65.9	1.6	28.5	4.0	0.43		0.06

2.3. Surface Characterization. Surface functionalization of plasma-treated samples was determined by X-ray photoelectron spectroscopy (XPS). XPS characterization was performed using an XPS (TFA XPS, Physical Electronics, Munich, Germany). The samples were excited with monochromatic Al K $\alpha_{1,2}$ radiation at 1486.6 eV over an area with a diameter of 400 μm . Photoelectrons were detected with a hemispherical analyser positioned at an angle of 45° with respect to the normal of the sample surface. XPS survey spectra were measured at a pass energy of 187 eV using an energy step of 0.4 eV, whereas high-resolution spectra were measured at a pass energy of 23.5 eV using an energy step of 0.1 eV. An additional electron gun was used for surface neutralization during XPS measurements. All spectra were referenced to the main C1s peak of the carbon atoms, which was assigned a value of 284.8 eV. The measured spectra were analyzed using MultiPak v8.1c software (Ulvac-Phi Inc., Kanagawa, Japan, 2006) from Physical Electronics, which was supplied with the spectrometer.

2.4. Cell Adhesion and Morphology Studies. Human umbilical endothelial cells (HUVEC, ATTC, Manassas, VA, USA) were cultured in MEM medium (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, USA), 100 U penicillin, 1000 U streptomycin, and 2 mM L-glutamine and plated at density of 3000 cells/cm². For the cell adhesion and morphology investigations, the cells were seeded at a density of 2×10^4 cells in 100 μL drop of medium on the upper side of the polymers (concentration: 2.55×10^4 cells/cm²) and left for 3 h and 24 h to attach at 37°C in a humidified atmosphere of 5% CO₂. Cells were seeded onto modified polymer in duplicate for each time and plasma treatment condition. Cells were seeded to the plasma-treated polymer samples within 30 minutes, after plasma treatment.

Cell adhesion and morphology were assessed only after 3 and 24 hours of incubation to see differences in adhesion of cells in the first few hours after incubation, because after 24 h cells normally already adapt to different surface conditions and differences are not that pronounced anymore.

Cell adhesion and morphology were investigated by scanning electron microscopy (SEM). Briefly, the polymer samples with the attached cells were fixed in 2% glutaraldehyde (Sigma-Aldrich, USA) in phosphate buffer solution for 5 minutes, followed by dehydration through an increasing gradient of ethanol and then vacuum-dried by the critical point method. Finally the samples were covered by a thin layer of gold and analyzed by SEM. For gold evaporation PECS instrument (Model 682) from Gatan GmbH (Munich, Germany) was used. SEM analyses were performed using a

JEOL JSM-840 Scanning Electron Microscope (JEOL, Tokyo, Japan).

2.4.1. MTT Assay. HUVEC were seeded and cultured in the same manner as for the cell adhesion and morphology investigation by SEM. The MTT-related colorimetric assay (EZ4U; Biomedica, Austria) was used to determine cell growth and viability, according to the manufacturer's instructions. 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 3 and 24 hours of HUVEC culture on the differently modified polymer surfaces the medium was removed and the polymer samples were rinsed with phosphate buffer saline to remove for all nonattached cells. Then 200 μL of fresh Hanks' Balanced Salt Solution (HBSS) mixed with the tetrazolium agent was added to each well with the polymer sample of the 24-well plate. After 3 and 24 h of incubation, supernatants were transferred into 96-well plates and the absorbance was measured at OD 570/690 nm with Synergy™ HT Microplate Reader (Bio-Tech Instruments, Inc., USA).

2.5. Statistical Analysis. All the above experiments were performed in duplicate and independently repeated at least three times, unless otherwise stated. The results obtained are shown as the mean \pm SE for duplicates of cultures. Student's *t*-test was used to test the effect different plasma modifications of PCL have on the adhesion and metabolic activity of HUVEC and a value of $p < 0.05$ was considered significant.

3. Results and Discussion

3.1. Surface Characterization of Plasma-Treated PCL. Surface composition of various plasma-treated samples as deduced from XPS survey spectra is shown in Table 1. Each gas used for plasma treatment of the polymer caused different modification of the surface. Oxygen plasma treatment caused oxidation of the PCL surface, where the concentration of oxygen increased from 22 atomic % to 32 atomic %. Contrarily, NH₃ plasma treatment of the PCL samples caused reduction of the amount of oxygen as oxygen concentration decreased to only 15 atomic %. This is due to dissociation of NH₃ in plasma to hydrogen atoms that react with oxygen from the polymer. NH₃ plasma treatment also caused incorporation of nearly 4 atomic % of nitrogen to the polymer surface.

Oxygen concentration on the surface of plasma-treated PCL sample also increased upon SO₂ plasma treatment.

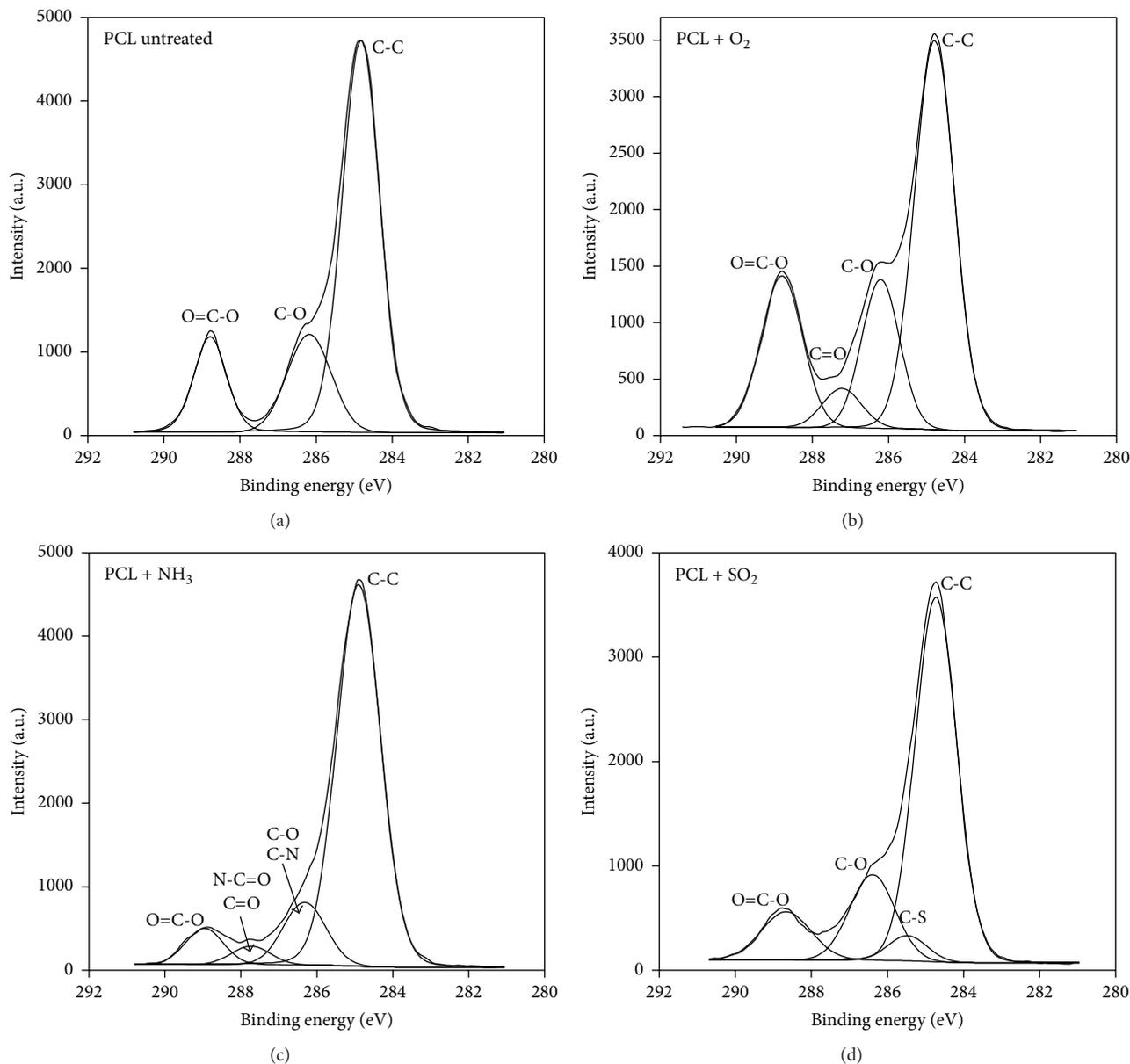


FIGURE 2: Carbon high-resolution peaks for various plasma-treated PCL samples.

Furthermore, 4 atomic % of sulphur was found incorporated to the PCL surface. Oxygen found on the surface of SO_2 plasma-treated PCL sample was bound to sulphur groups rather than carbon atoms as explained later in the text. Additionally, small concentrations of nitrogen were found on the surface of the SO_2 plasma-treated PCL sample. This is not unusual, because many authors have found small amounts of nitrogen on SO_2 plasma-treated surfaces and its presence was explained by traces of nitrogen in the residual atmosphere [30, 31]. Collaud Coen et al. proposed stabilization of newly formed sulfur group via a hydrogen bond with amine group which can be formed in plasma [30], whereas Holländer and Kröpke proposed reaction (deactivation) of surface radicals with NO which is also formed in the plasma [31].

To get more details regarding surface functional groups induced by various plasma-treatment procedures, we performed deconvolution of high-resolution carbon peaks (Figure 2). Concentration of different chemical groups is shown in Table 2. For untreated PCL polymer (Figure 2(a)) we can find C-C (284.8 eV) as well as C-O (286.3 eV) and O=C-O groups (288.8 eV). Concentration of these oxygen-containing functional groups has increased after O_2 plasma treatment and a new peak at 287.3 eV due to C=O groups appeared as well, whereas the C-C peak decreased (Figure 2(b)).

For the case of NH_3 plasma treatment (Figure 2(c)) the intensity of the C-C peak increased, whereas the intensity of other peaks associated with oxygen decreased, which is in correlation with quite low oxygen concentration shown in

TABLE 2: Concentration of different functional groups for various plasma treated samples (* in the case of NH₃ treatment).

	C-C	C-S	C-O C-N*	C=O O=C-N*	O=C-O
Untreated	66.7		18.8		14.5
Treated in O ₂	54.3		19.2	5.3	21.2
Treated in NH ₃	78.4		12.1	3.3	6.2
Treated in SO ₂	67.8	4.3	17.1		10.8

Table 1. Furthermore, a new peak at 287.7 eV appeared, which is attributed to amide groups O=C-N; however C=O groups can be present as well. C-N groups appear close to C-O peak; therefore, the peak at approximately 286.3 eV was attributed to both C-O and C-N functional groups.

SO₂ plasma treatment (Figure 2(d)) gave rise to a new C-S peak at approximately 285.5 eV. Since concentration of carbon-oxygen functional groups did not change much or it even slightly decreased (Table 2), it is clear that increased oxygen concentration observed in Table 1 is due to the formation of oxygen rich sulphur groups such as C-SO₃ or C-SO₄. Namely, sulphur S2p peak appeared at approximately 168.6 eV, which is typical for highly oxidized sulphur groups [28].

Because plasma treatment and related surface modification can have significant influence on the surface wettability, we measured water contact angle on different plasma-treated PCL polymers. The contact angle of untreated sample was approximately 110°. The contact angle slightly increased in NH₃ plasma, whereas in oxygen and SO₂ plasma it decreased significantly and reached approximately 20°. This means that surface treated in oxygen and SO₂ plasma was very hydrophilic, while the one treated in NH₃ plasma was very hydrophobic.

3.2. Cell Adhesion Studies. Morphology and adhesion of HUVEC on different plasma-treated PCL polymers were investigated with SEM at two time intervals: after 3 and 24 hours (Figure 3). The SEM image after 3 hours of incubation was taken to capture the initial appearance of the cells getting adhered and adapted to the surface. Figure 3 shows that, already after 3 hours, the HUVEC were observed to best adhere to NH₃- and O₂-plasma-treated PCL samples, whereas there was nearly no difference between untreated and SO₂ plasma-treated PCL samples. Later, after 24 hours the cells already fully adhered to the surface and the substantial difference in the number of the adherent cells between the untreated sample and plasma-treated samples was observed. Again, the highest number of HUVEC was present on a PCL surface that has been treated with NH₃ and O₂ plasma, which both allowed for significant increase in cell adhesion and likely triggered different cellular signalling pathways, consequently resulting in improved metabolic activity and growth of HUVEC.

Moreover, similar morphology of the PCL samples treated with O₂ and NH₃ plasma, consisting of porous fibrous structure with very thin crisscrossed fibers, was observed by SEM. It is well known that polymer sample morphology plays

a crucial role in cell-surface adhesion process [32]. Another important effect on cell adhesion has a chemical composition of the surface and surface functional groups, created by plasma treatment [33]. Based on the SEM and XPS analyses, the conditions created by O₂ and NH₃ plasma on PCL surface represent optimal condition for cell adhesion and proliferation. Cells exhibit long protrusions, for spreading all over the fibrous surface to cover the maximum surface area for sufficient attachment, which would signal them then to proceed with their proliferation to form the tissue. Thus, great adhesion was achieved after O₂ and NH₃ plasma treatment. The integrity of endothelialization depends on cell-matrix adhesion and the transmembrane proteins called integrins play a crucial role in this process [34].

On the SO₂ plasma-treated PCL samples, the surface morphology changed significantly after plasma treatment. The fibers became denser and thicker, and the surface of the PCL samples appeared less porous. This could be the potential reason for the impaired anchoring of the integrins and subsequently reduced adhesion of cells to the SO₂ treated PCL surface.

With SO₂ plasma treatment sulphur functional groups were introduced to the surface. As reported in the literature by various authors [28, 29], sulphur functional groups display antithrombogenic effect by reducing adhesion and activation of platelets. This is extremely important for the production of the prosthetic implants such as synthetic vascular grafts, which are always in direct contact with the blood. However, adhesion of cells to polymer surfaces containing sulphur functional groups does not appear promising, because the SEM images showed poor and impaired adhesion of the cells, although it appears that once the cell is attached to the surface it grows normally. This is supported by the relatively high signal of MTT assay obtained for the SO₂ plasma-treated PCL samples, as explained later in the text.

On the untreated polymer most of the HUVEC appeared shrunken and rounded, with the morphology not typical for HUVEC. Furthermore, a very few attached cells observed on the untreated PCL surface indicate adhesion problems of this surface, which per se possibly represents unfavourable environment for cells. Cells on the untreated PCL surface were not firmly attached to the surface; their morphology changed significantly because of the PLC fibres, which were twisted or captured in between them.

To get additional data on the metabolic activity of the adhered cells and their proliferation on plasma-treated PCL samples, the MTT assay was performed (Figure 4). Three hours after HUVEC seeding no difference in their metabolic

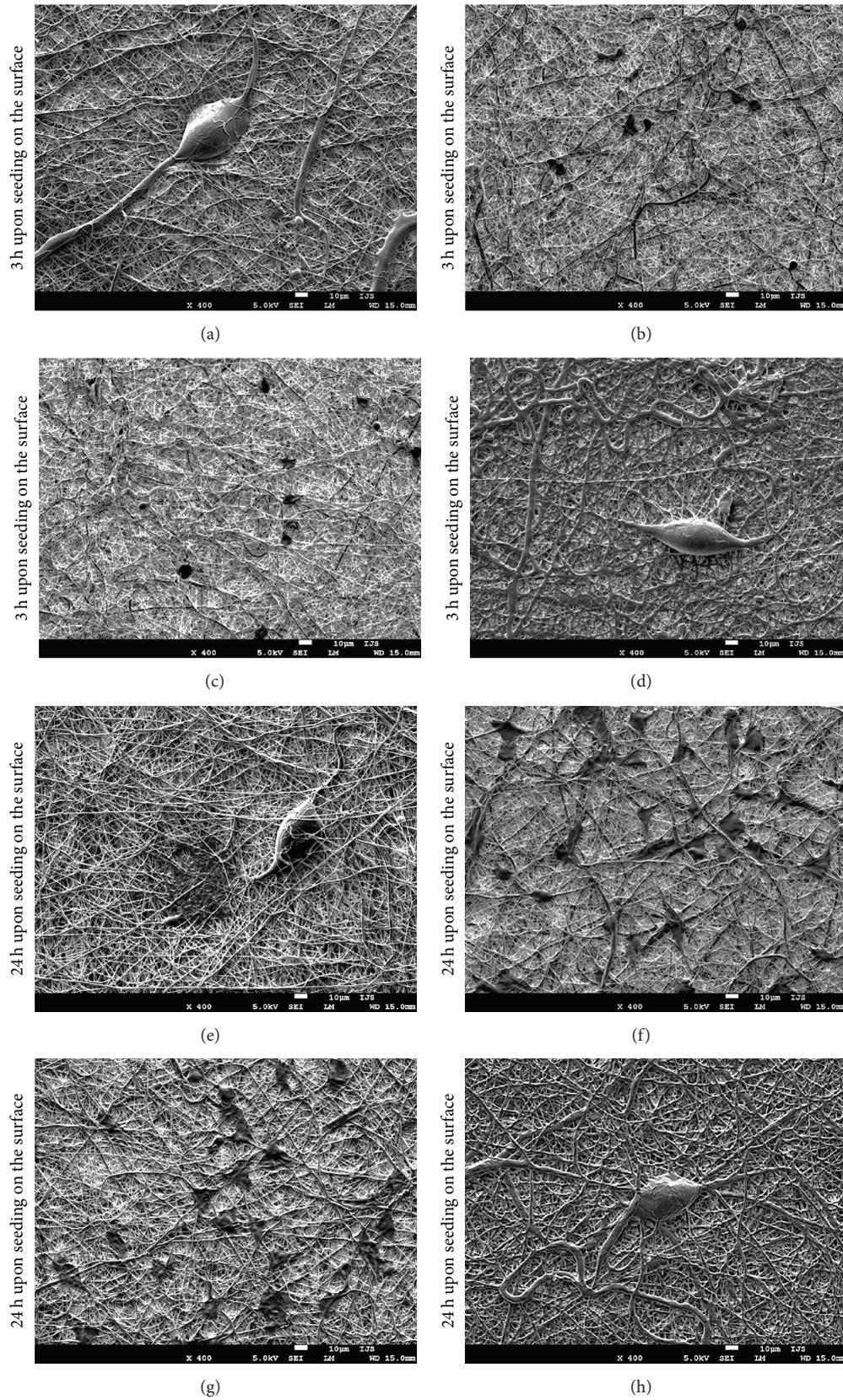


FIGURE 3: SEM images of plasma-treated PCL samples with adhered HUVEC 3 and 24 h upon seeding: (a, e) untreated, (b, f) treated in O_2 plasma, (c, g) treated in NH_3 plasma, and (f, h) treated in SO_2 plasma.

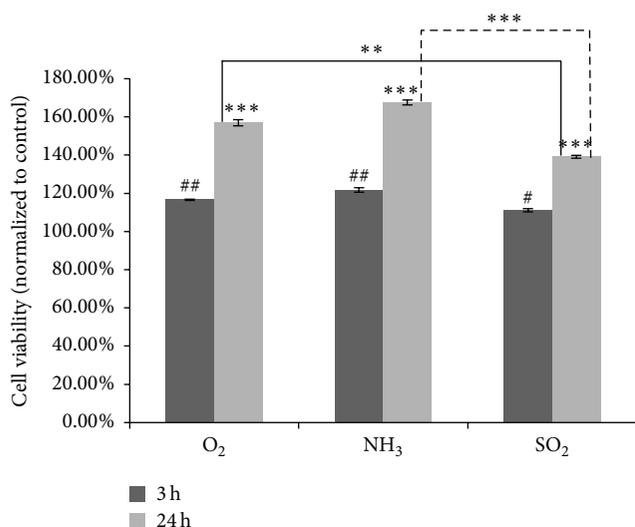


FIGURE 4: Effect of chemical surface composition on HUVEC proliferation. Proliferation of HUVEC on plasma untreated and treated PCL polymers determined by MTT assay. Student's *t*-test was used to check the statistical significance. *Statistically significant at $p < 0.05$ for 3 hours compared with control. **Statistically significant at $p < 0.01$ for 3 hours compared with control. ***Statistically significant at $p < 0.001$ for 24 hours compared with control and/or between NH₃ and SO₂ treated samples. Mean values (\pm SE) for the respective duplicates are given.

activity was observed for SO₂ plasma-treated PCL samples as compared to the untreated one. However, after 3 hours the significant increase of metabolic activity was already observed for O₂ and NH₃ plasma-treated PCL surfaces, compared to control ($p < 0.05$). After 24 hours, proliferation of HUVEC on these two surfaces increased by more than 60% in comparison to the untreated sample ($p < 0.05$). Interestingly, polymers treated with SO₂ plasma also showed 40% increase in cell viability as compared to the untreated sample, although SEM images showed poor adhesion of HUVEC to these surfaces.

Moreover, after 24 hours the significant differences in cell viability can also be observed in between different plasma-treated samples. One can observe decrease in cell viability on PCL surfaces treated with SO₂ plasma, when compared to NH₃ and O₂ plasma-treated surfaces ($p < 0.01$).

To summarize, our results clearly show that plasma treatment has an important effect on cell adhesion and viability, as well as cell morphology. NH₃ and O₂ treatment led to strong HUVEC adhesion and viability. HUVEC did also adhere to SO₂ plasma treated-surface; however, their adhesion was poorer and aggravated. Yet, once the cells managed to adhere to the surface they were capable of growing and proliferating further normally. Contrarily, untreated PCL sample represents the least suitable environment for HUVEC adhesion and growth. Enhanced cell proliferation in the case of NH₃ and O₂ plasma treatment was explained by the presence of functional groups that are beneficial for cell proliferation and by different surface morphology.

4. Conclusions

PLC polymer was treated with NH₃, O₂, and SO₂ plasma in order to change surface properties of the sample (i.e., morphology, chemistry, and roughness). We were seeking for the best modification of the PCL surface that would allow for the best cell adhesion and proliferation. Plasma treatment led to changes in surface chemical composition and morphology as well as related hydrophobicity of the polymer, reflecting different adhesion characteristics of cells to the polymer surface. According to literature, the best cell proliferation is normally observed at moderate hydrophobic polymers. Our results show that surface morphology and surface chemistry can be even more important than surface hydrophobicity, because none of our plasma-treated surfaces was moderately hydrophobic. Using various plasmas allowed for incorporation of different functionalities to the surface: various carbon-oxygen functional groups after O₂ plasma treatment, formation of nitrogen groups after NH₃ plasma treatment, and oxidized sulphur groups after SO₂ plasma treatment. MTT assay showed that modification of polymers with O₂ and NH₃ plasmas significantly increased cell adhesion and viability as compared to the untreated polymer, proving that the characteristics of NH₃ and O₂ plasma-treated surfaces are the most favourable for cell adhesion and proliferation. Interestingly, increased viability of HUVEC was also observed on SO₂ plasma-treated surfaces; however, SEM images of SO₂ plasma-treated surfaces showed unfavourable conditions for HUVEC adhesion. Lower cell adhesion can be attributed to changed fibrous morphology of the PCL samples, after treatment in SO₂ plasma. Nevertheless, once the cells managed to adhere to this surface, they were capable of growing and proliferating further normally that indicated the high signal of our MTT assay for SO₂ plasma-treated PCL surfaces.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Robin Augustine fabricated electrospun PCL scaffolds, Matic Resnik carried out plasma treatment, Nina Recek carried out XPS analysis, and Nina Recek, Helena Motaln, and Tamara Lah-Turnšek evaluated the cell viability and cell proliferation after plasma modification. Robin Augustine, Miran Mozetič, Nandakumar Kalarikkal, and Sabu Thomas were involved in the discussions to outline and implement the work. Nina Recek and Miran Mozetič wrote the paper. All authors have read and made necessary modifications before submission of the paper.

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