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Effects of pre- and post-electrospinning plasma treatments on electrospun PCL nanofibers to improve cell interactions

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Abstract. In this study, liquid plasma treatment was used to improve the morphology of Poly-ϵ-CaproLactone (PCL) NanoFibers (NFs), followed by performing a Dielectric Barrier Discharge (DBD) plasma surface modification to enhance the hydrophilicity of electrospun mats generated from plasma-modified PCL solutions. Cell interaction studies performed after 1 day and 7 days clearly revealed the highly increased cellular interactions on the double plasma-treated nanofibers compared to the pristine ones due to the combination of (1) a better NF morphology and (2) an increased surface hydrophilicity.

1. Introduction

The application of electrospun nanofibers (NFs) in tissue engineering is increasing owing to their morphological similarity to the ExtraCellular Matrix (ECM) as most of the ECM proteins have a fibrous structure with diameters in the nanometer or sub-micrometer range [1]. Nevertheless, a great challenge in electrospinning is the preparation of a suitable polymer solution because the system requires a balance of forces controlled by rheological behavior, conductivity and surface tension. In many cases, researchers have used mixed solvents or even additives to make solutions compatible to the electrospinning process [2]. However, within this work, a non-thermal Atmospheric Pressure Plasma Jet (APPJ) directly generated in a polymer solution will be explored trying to improve the electrospinnability and final NF quality. The applied APPJ set-up allows a close, intense contact between the plasma and the polymer solution which can lead to a very efficient effect on the quality of the resultant fibers [3 - 5]. As PCL is a good polymeric candidate for tissue engineering applications due to its numerous advantages such as good mechanical properties (highly elastic), chemical stability, excellent biocompatibility, research will be focused on PCL in this study. Nonetheless, due to its highly hydrophobic structure, PCL shows very poor cellular interactions. To cope with this problem, a Dielectric Barrier Discharge (DBD) plasma surface treatment will also be performed on selected nanofibrous samples after

the electrospinning process [6, 7]. For this purpose, PCL-NFs will be exposed to an argon plasma in an effort to improve their hydrophilicity as this is known to positively affect cellular interactions [8]. Plasma surface modification is a very attractive technique for this purpose as it does not significantly change the scaffold morphology nor its mechanical properties [9, 10]. In this study, the PCL solution viscosity and conductivity will be monitored before and after APPJ treatment, while Scanning Electron microscopy (SEM) will be used to evaluate the morphology of the resultant NFs. Furthermore, PCL-NFs will also be seeded with HFFs (Human foreskin fibroblasts) to perform cell adhesion tests after 1 and 7 days. For this purpose, 4 different samples will be examined: (1) untreated scaffolds, (2) scaffolds plasma-treated before the electrospinning process, (3) scaffolds plasma-treated after the electrospinning process and (4) scaffolds plasma-treated before and after the electrospinning step. Cell interactions on the different samples will be assessed by fluorescence microscopy and SEM.

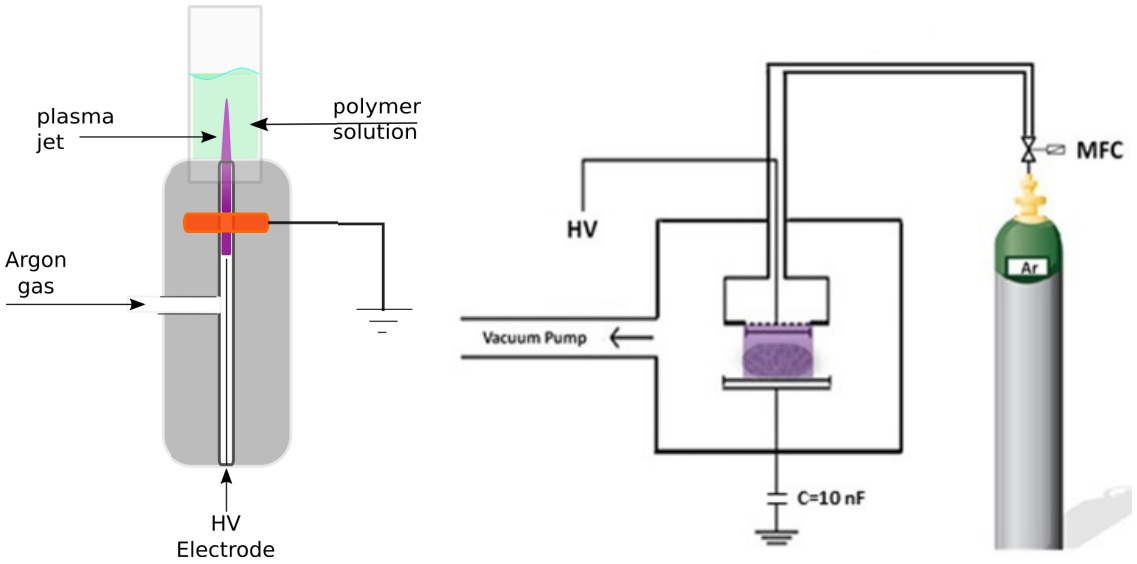

Figure 1. A schematic of the APPJ (left) to treat the PCL solution and the DBD (right) to improve the hydrophilicity of the PCL nanofibers.

2. Experimental

PCL with a MW of 80000 g/mol, chloroform and N,N-Dimethylformamide were purchased from Sigma-Aldrich and used without further purification. A solution of 5% of PCL in $CHCl_3$+DMF (ratio of 9:1 (w/w)) was prepared at room temperature by stirring for 2 hours. Half of the obtained polymer solution was electrospun without any plasma pre-treatment step, while the other half of the polymer solutions was treated with the atmospheric pressure plasma jet (APPJ) schematically presented in Fig. 1 for 5 minutes making use of an argon flow rate of 0.9 slm (standard liters per minute) and an applied voltage of 7.5 kV. Before and after the APPJ modification step, the solution was characterized by measuring its conductivity and viscosity making use of a Mettler Toledo FiveEasy conductivity meter equipped with an InLab 720 probe and a Brookfield DV2T extra viscometer, respectively. After performing the APPJ modification step, untreated and plasma-modified PCL solutions were immediately electrospun making use

of a Nanospinner 24 electrospinning machine manufactured by Inovenso (Turkey). This machine consists of a copper tip which is fed with the PCL polymer solution at a rate of 3.5 ml/h. A high voltage of 25 kV is applied to this tip, while a grounded rotating drum (rotation speed: 100 rpm) placed at 20 cm from the tip acts as collector plate. Round cover glasses with a diameter of 12 mm were fixed to the rotating drum and used as collecting substrates.
In a next step, the obtained PCL nanofibers were exposed to a DBD plasma schematically presented in Fig. 1(right) to increase their hydrophilicity. The DBD treatments were performed in argon at a discharge power of 1.5 W at medium pressure (50 kPa). A treatment time of 30 seconds was selected since this treatment duration was found to result in the highest possible hydrophilicity combined with negligible changes in NF morphology. The morphology of the PCL-NFs before and after APPJ liquid treatment and DBD plasma surface treatment were characterized with a scanning electron microscope (SEM; JSM-6010 PLUS/LV; JEOL). SEM images are acquired with an accelerating voltage of 7 kV, after coating the samples with a thin layer of gold making use of a sputter coater (JFC-1300 autofine coater, JEOL). The average diameter of nanofibers is calculated over 20 fibers using ImageJ (National Institutes of Health, USA) analysis software.
In a final step, HFF cells were cultured on untreated, pre-electrospinning plasma treated, post-electrospinning plasma treated and pre- and post-electrospinning plasma treated NFs after exposure to UV light for 30 minutes as a sterilization step. For this purpose, a density of 40.000 cells/500 μl of culture medium per scaffold was used. The cell seeded scaffolds were incubated at 37°C under 5% CO2 atmosphere for 1 and 7 days and Tissue Culture PolyStyrene (TCPS) was used as positive control. Live/dead cell staining was applied to evaluate cell viability: the cell seeded scaffolds were incubated in 2 μl (1 mg/ml) of calcein-AM and 2 μl (1mg/ml) propidium iodide in 1 ml phosphate buffered saline (PBS), after which they were imaged with a fluorescence microscope (Olympus; IX 81) with appropriate filters [8]. The morphology of the HFF cells on the scaffolds after 1 and 7 days was also evaluated by SEM after performing a cell fixation and dehydration step. The preparation procedure included cell washing with PBS followed by fixation with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 1 hour. The cells were subsequently dehydrated by immersion through increasing ethanol series (50%, 70%, 85%, 95% and 100%) for 10 min. Afterwards, samples were immersed in hexamethyldisilazane (HDMS) for 10 min.

3. Results and discussion

3.1. APPJ treatment of the PCL polymer solution: solution properties and PCL-NF morphology

The conductivity and viscosity of the PCL polymer solution before and after APPJ treatment can be found in Table 1: from reported values it is evident that plasma exposure can strongly increase these parameters. SEM images of electrospun PCL-NFs generated from untreated and plasma treated PCL solutions are also displayed in Fig. 2. This figure clearly shows that the nanofibrous scaffold obtained from the untreated polymer solution presents a high density of beads (on average 2000 beads per mm^2) and an average fiber diameter of approximately 300 nm (high standard deviation due to the presence of beads). After 5 minutes of treatment with the plasma jet, the beads however completely disappear resulting in a network of elongated, uniform fibers with an average fiber diameter of approximately 500 nm.

Table 1. Polymer solution characteristics before and after APPJ treatment

	Untreated	**APPJ for 5 min**
Conductivity (μS)/cm	0.25 ± 0.05	5.1 ± 0.1
Viscosity (cP)	85 ± 5	320 ± 5
Fibers diameter (nm)	300 ± 150	500 ± 50

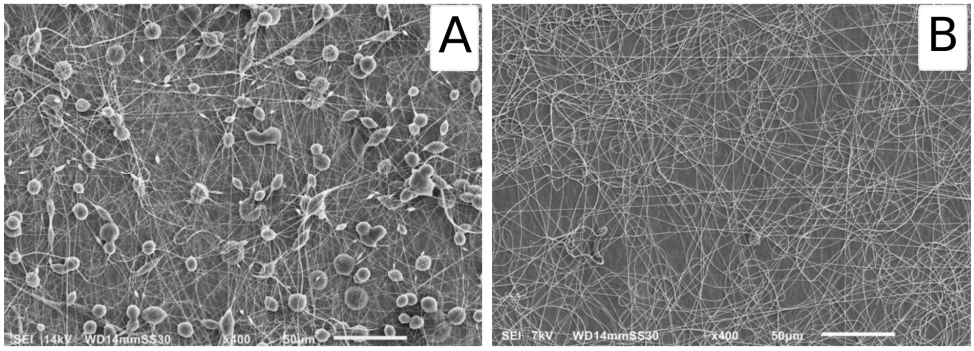

Figure 2. SEM images of nanofibers generated from A) an untreated PCL solution and B) a PCL solution treated with an APPJ for 5 minutes.

As mentioned before, plasma treatment is a very useful method to modify the chemical properties of a polymer surface. Plasma incorporated polar chemical groups are known to increase the surface energy of polymers and thus increase the surface wettability. The untreated nanofibers have a contact angle of approximately 127° which decrease to values of 55°, 64° and 95° for the nanofibers plasma treated with combined plasma treatments, DBD treated and APPJ treated respectively.

3.2. Live/dead immune microscopy and cell morphology using SEM

The cellular interactions on differently prepared PCL scaffolds have also been investigated 1 day and 7 days after cell seeding and the images obtained by SEM and fluorescent microscopy are presented in Figs. 3 and 4 respectively. These images clearly demonstrate the differences in cellular interactions between untreated, APPJ treated, DBD treated and combined plasma treatments on PCL-NFs. On untreated samples, after 1 day of cell seeding, mostly round and thus poorly attached cells can be observed. In addition, after 7 days of cell seeding, most of the present cells still show a round morphology while also quite a few cells appear to be dead (red color in Fig. 4). Cellular interactions on untreated samples are thus very poor. On samples which have been treated with the DBD, one can see that after 1 day and 7 days of cell seeding, more live cells are present on the samples and the cells adhere better to the sample surface, which can be evidenced from their spread morphology. These better cellular interactions compared to the untreated sample is most likely due to the enhanced hydrophilicity, which is known to positively affect cell-material interactions. When looking at the sample which has been treated with the APPJ alone, one can see that also in this case, cells nicely adhere to the sample as they present an elongated morphology, which is most likely due to the presence of uniform fibers instead of beads. However, the best cell-material interactions can be observed on the combined

APPJ and DBD plasma-treated samples: in this case, a high number of nicely elongated cells can be observed on the surface, even after only 1 day after cell seeding. After 7 days, a layer of cells is almost completely covering the nanofibers (as can be nicely observed in Fig. 3), which was not at all the case for the other investigated samples. These enhanced cellular interactions compared to the APPJ treated samples can again be related to the enhanced hydrophilicity upon DBD treatment. One can therefore conclude that a double plasma treatment is able to strongly enhance the cellular interactions on PCL nanofibers.

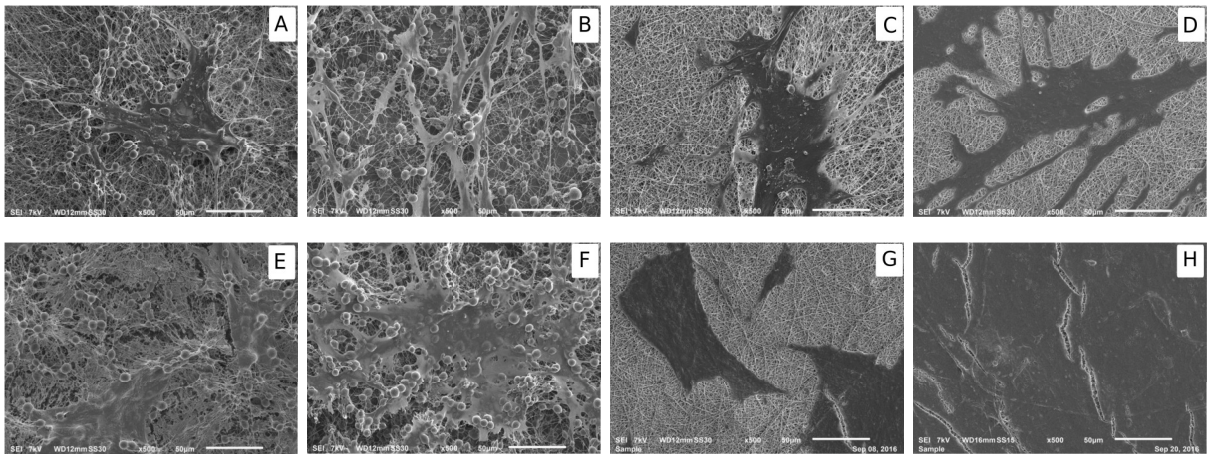

Figure 3. SEM images. First row: after 1 day, A) untreated samples, B) fibers with a DBD plasma treatment, C) APPJ plasma treatment, D) APPJ+DBD plasma treatment. Second row: after 7 days of seeding, E) untreated, F) DBD treatment, G) APPJ treatment, H) APPJ+DBD plasma treatment.

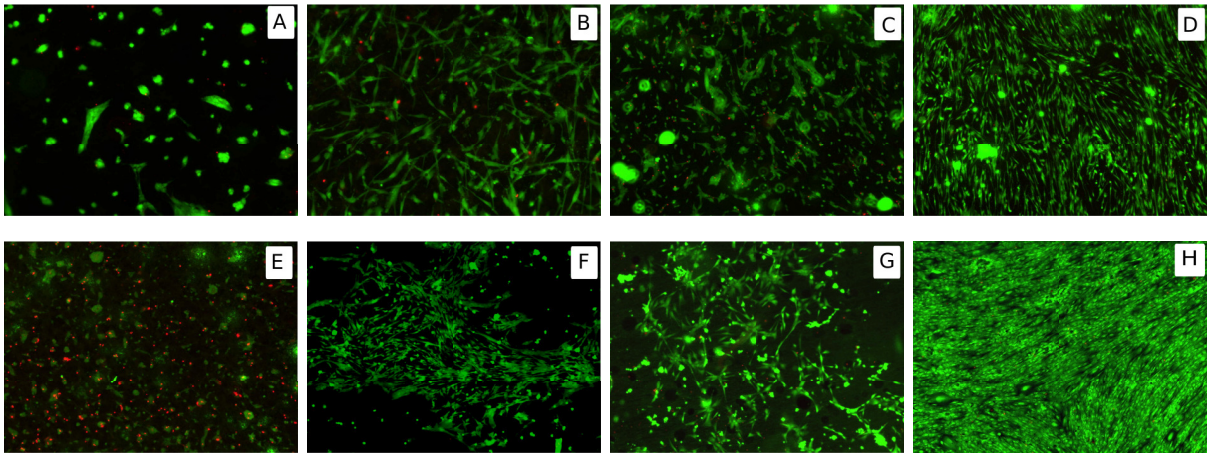

Figure 4. Fluorescence microscopy images. First row: after 1 day, A) untreated samples, B) fibers with a DBD plasma treatment, C) APPJ plasma treatment, D) APPJ+DBD plasma treatment. Second row: after 7 days of seeding, E) untreated, F) DBD treatment, G) APPJ treatment, H) APPJ+DBD plasma treatment.

4. Conclusion

After electrospinning an untreated PCL solution, the generated NFs presented a large amount of beads. It was however found that in the liquid plasma-treated fiber mesh, beads completely disappeared leading to more uniform fibers in diameter and in morphology. However, the resultant, elongated NFs still showed to have a strong hydrophobic behavior which is unwanted in the foreseen biomedical applications. DBD plasma treatment in argon of the NFs is therefore also performed to significantly improve their hydrophilicity. Cell studies revealed that HFFs cultured on PCL-NFs present a considerably higher cell adhesion and proliferation on combined APPJ and DBD plasma-treated NFs compared to pristine NFs. These results suggest that double plasma-modified NFs may have a large potential for the efficient replacement of damaged tissues in the future.

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