Full Paper

Fabrication of PEOT/PBT nanofibers by atmospheric pressure plasma jet treatment of electrospinning solutions for tissue engineering

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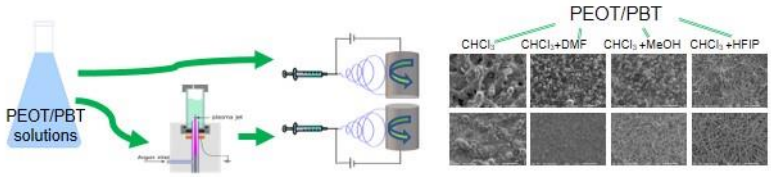
Abstract

This study focuses on the enhanced electrospinning of 300-Polyethylene oxide-polyethylene oxide terephthalate/polybutylene terephthalate. An atmospheric pressure plasma jet for liquid treatment is applied to a solution with 9(w/v)% PEOT/PBT dissolved in either $CHCl_3$, $CHCl_3$+DMF, $CHCl_3$+MeOH or $CHCl_3$+HFIP. For all conditions, the plasma-treated samples present better-quality fibers: less or no-beads and uniform fiber diameter distribution. Except for $CHCl_3$+DMF, no significant changes to the material bulk are detected, as shown with SEC. XPS spectra performed on nanofibers record an increase in C-C bonds for the $CHCl_3$+DMF combination upon plasma modification, while a shift and slight increase in oxygen-containing bonds is found for the $CHCl_3$+HFIP and $CHCl_3$+MeOH mixtures. MTT assay shows no-

cytotoxic effects for $CHCl_3$+DMF, while a better cellular adhesion is found on nanofibers from $CHCl_3$+MeOH and $CHCl_3$+HFIP. Among the examined additives, MeOH is preferable as it produces beadless electrospun nanofibers with an average diameter of 290±100 nm without causing significant changes to the final nanofiber surface properties.



1. Introduction

300-Polyethylene oxide-polyethylene oxide terephthalate/polybutylene terephthalate (300PEO PEOT/PBT or just PEOT/PBT) (Polyactive™) is a material showing high potential for tissue engineering applications, as its degradation rate can be tuned depending on its application and it is characterized by a higher wettability compared to other biodegradable thermoplastics such as poly-L-lactic acid (PLLA) and poly-ε-caprolactone (PCL) [1-3]. It has been extensively studied and has proven to be biocompatible both in vitro and in vivo [4,5]. Currently, the material has reached the clinical trials to be used as a bone filler and dermal substitute [6,7]. More recently, it has been considered as a suitable material for support structure manufacturing in tissue engineering. Here, it showed to be a suitable material for 3D additive manufacturing of interporous scaffolds and is currently under consideration for tissue engineered clinical treatments of bone and cartilage defects [8-10].

Besides 3D additive manufacturing, its potential to be spun into nanofiber mats via electrospinning for soft tissue engineering applications has also been investigated by several groups [11,12]. Whereas the 3D additive manufacturing process was relatively straightforward, the electrospinnability of PEOT/PBT is challenging due to the limited solubility [13-15]. In general, a polymer solution suitable for electrospinning should meet strict requirements in terms of conductivity, surface tension and rheology [16-18]. The most commonly used methodology to

improve the spinnability of a polymer solution, is enhancing its conductivity, either via the addition of salts or by increasing the polarity of the solvent, i.e. using solvent mixtures [19-21]. To improve the fiber morphology of PEOT/PBT nanofibers, it has been opted in the past to use the latter by adding hexafluoroisopropanol (HFIP) to chloroform ($CHCl_3$), which is an excellent solvent for PEOT/PBT [13-15]. HFIP is a polar solvent with strong hydrogen bonding properties, which can increase the conductivity of the PEOT/PBT solutions in chloroform, thereby resulting into enhanced PEOT/PBT fiber morphology. Unfortunately, HFIP is also harmful to handle, expensive and preferably not used for biomedical applications as unwanted trace amounts could strongly influence the fiber mat biocompatibility[22]. Without the addition of HFIP to chloroform, independent of the used electrospinning parameters, it was not possible so far to obtain continuous bead-free nanofibers. Driven by the need to find more effective, eco-friendly and biomedically benign strategies to improve the electrospinning process, several research groups have been investigating the potential of using atmospheric pressure plasma to improve polymer solution properties prior to electrospinning [23-25]. The exposure of solids to non-thermal plasma (NTP) is well-described and has found a widespread use in many disciplines [26-30]. However, the use of NTP to alter the properties of polymeric solutions (for electrospinning) is a field of research that has barely been touched upon. So far, to the best of the author's knowledge, only 5 research papers have been published that deal with this subject[23-25,31,32]. Shi et al. were the first to expose an aqueous solution of polyethylene oxide to an NTP and found remarkable improvements in fiber morphology [24]. Colombo et al. followed a similar strategy, using an atmospheric pressure plasma jet to treat polylactic acid dissolved in dichloromethane and observed 100% bead-free fibers [23]. In our research group, an extensive study on the effects of NTP exposure on a solution of PCL or PLLA in chloroform ($CHCl_3$) and N,N-dimethylformamide (DMF) was recently performed [25,32]. It was found that the plasma was responsible for improving the conductivity of the polymer solution as well as

changing the viscosity and pH via the generation of new chemical species without damaging the macromolecular structure of the polymer [31].

Considering the positive results obtained in the above-mentioned innovative papers, this work intend was to examine the potential of pre-electrospinning plasma treatment of PEOT/PBT solutions for the fabrication of continuous bead-free PEOT/PBT nanofibers. For this purpose, a fixed concentration of 9 w/v% PEOT/PBT was dissolved in 4 different solvents: pure $CHCl_3$, $CHCl_3$+ DMF, $CHCl_3$ +MeOH and $CHCl_3$ + HFIP. The solution containing only chloroform as solvent was selected to investigate whether plasma modification is able to alter the polymer solution parameters to such an extent that beadless PEOT/PBT nanofibers could obtained without the need of extra additives. Successively, a mixture of chloroform and DMF (10 v/v%) were also explored as DMF has a high dielectric constant and aprotic properties, which could positively affect PEOT/PBT electrospinnability. In addition, the mixture $CHCl_3$+DMF has already been extensively studied in our previous works focusing on PCL and PLA with successful electrospinning results [25,32]. Thirdly, a mixture of chloroform and MeOH (10 v/v%) was examined as methanol is also characterized by a high dielectric constant and presents a protic behavior and may thus positively affect PEOT/PBT electrospinnability. Finally, the widely used mixture of chloroform and HFIP was also explored to compare the PEOT/PBT nanofibers already obtained in literature with the electrospun nanofibers obtained after the plasma treatments performed in this work. In literature, the PEOT/PBT solutions containing HFIP typically possess polymer concentrations over 20% resulting into continuous beadless nanofibers with an average diameter of 2 μm [11]. The aim of the performed plasma treatments is to work with lower PEOT/PBT concentrations, which could in turn result into reduced fiber diameters, mimicking more closely the natural extracellular matrix. All polymer solutions under study were subjected to plasma modification with selected time intervals. The plasma-modified solutions were characterized in terms of viscosity, conductivity and possible changes in

polymer molecular weight. All pristine and plasma-modified solutions were electrospun to study the effects of the plasma exposure on nanofiber morphology, chemical composition and wettability. Finally, the electrospun structures obtained from pristine and plasma-modified PEOT/PBT solutions were subjected to in vitro cell studies to examine their cytotoxicity.

2. Experimental Methods

2.1.Materials

300PEO PEOT/PBT 55/45 (Polyactive™) block co-polymer pellets with a PEOT/PBT ratio of 55/45 in weight percentage were purchased from PolyVation B.V. (Groningen, The Netherlands) and used without further purification. In what follows, the used polymer will be consequently abbreviated as PEOT/PBT.

$CHCl_3$, MeOH, HFIP, DMF, dimethyl sulfoxide (DMSO) and ethanol with a purity >99% were purchased from Sigma-Aldrich (Belgium) and used as such. Argon (Ar) gas (Alphagaz 1) was ordered from Air Liquide (Belgium).

The culture medium used for cell culturing is Dulbecco's modified Eagle's glutamax medium (DMEM) (Gibco Invitrogen, Belgium) enriched with 15% foetal calf serum (Gibco Invitrogen, Belgium), 2 mM L-glutamine (Sigma-Aldrich, Belgium), 10 U/mL penicillin, 10 mg/mL streptomycin and 100 mM sodium-pyruvate (all from Gibco Invitrogen). For the MTT assay, the yellow tetrazolium dye 3-(4, 5-dimethyldiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Merck Promega, Belgium) was used. For the live/dead staining, calcein-acetylmethoxyester (AnaSpec, Belgium) and propidium iodide (Sigma-Aldrich, Belgium) were used as supplied. For the SEM imaging, glutaraldehyde (Sigma-Adrich, Belgium) and hexamethyldisilazane (Acros-organics, Germany) were used.

2.2.Preparation of PEOT/PBT polymer solutions

As previously mentioned, PEOT/PBT solutions were prepared by dissolving polymer pellets in pure $CHCl_3$ and in three different solvents mixtures: 1) $CHCl_3$+DMF, 2) $CHCl_3$+MeOH and 3) $CHCl_3$ +HFIP at a ratio of 9:1 v/v. For pure chloroform, PEOT/PBT polymer was initially dissolved in pure $CHCl_3$, by mechanically stirring the solution for 2 h at room temperature to obtain a solution with a polymer concentration of 9 w/v%. For the solvent mixtures, 10 v/v% of secondary solvent was added to pure chloroform after which PEOT/PBT polymer was completely dissolved in such an amount that polymer solutions with a final concentration of 9 w/v% were obtained.

2.3.Atmospheric pressure plasma jet (APPJ) treatment of PEOT/PBT polymer solutions

The plasma source used in this work to treat PEOT/PBT polymer solutions is an atmospheric pressure plasma jet (APPJ) specifically designed for liquid treatment and is schematically represented in Figure 1. The set-up was already described in detail in previous work [25]. In short, the plasma is generated inside a thin quartz capillary fed by an argon flow. A tungsten needle is placed within the capillary and acts as high-voltage electrode, while a ring-shaped copper grounded electrode is placed around the quartz capillary at 4.5 cm from the tip of

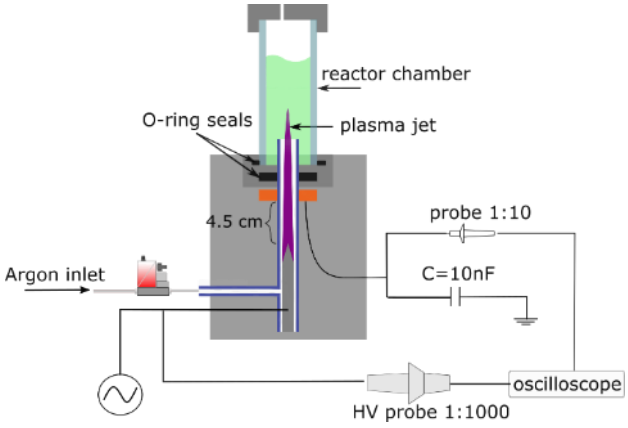


Figure 1. Schematic representation of the APPJ set-up used for PEOT/PBT solution treatment.

tungsten needle. A constant argon flow at a rate of 800 standard cubic centimetre per minute (sccm) is sent through the capillary after which the discharge is ignited by applying an AC high voltage (fixed frequency of 50 kHz) to the high-voltage electrode with a peak-to-peak value of

7.6 kV. A small reactor chamber, which can contain the polymer solution, is obtained on top of the capillary exit by fixing a quartz tube with an inside and outside diameter of 13 mm and 20 mm respectively to a stainless-steel flange possessing a small opening where the APPJ quartz capillary can be inserted, as shown in Figure 1. The distance between the top of the grounded electrode and the bottom of the stainless-steel flange is set to 0.5 cm to ensure electrical isolation. For all experiments, a fixed liquid sample volume of 10 mL is inserted in the reactor chamber using a glass syringe. Afterwards, the top of the reactor chamber is covered with another stainless-steel flange containing a small round opening with a diameter of 2 mm acting as gas outlet, thereby limiting solvent evaporation during plasma treatment. Plasma treatments in this work are performed with plasma exposure times varying between 3 and 7 minutes. To electrically characterize the plasma, the voltage applied to the needle electrode is measured using a high voltage probe (Tektronix P6015A) while the charge on the electrodes is obtained by measuring the voltage over a capacitor of 10 nF placed in series with the ground electrode. By visualizing the obtained voltage-versus-charge plot using a PC oscilloscope (Picoscope 3204A), a Lissajous figure can be constructed [33]. From this figure, the electrical energy consumed per voltage cycle E_{el} can be estimated since this value is equal to the area enclosed by the Lissajous figure. The electrical power P_{el} can be obtained by multiplying the electrical energy with the frequency of the feeding voltage, which is 50 kHz, and is found to be 4.8 W in this work [33].

2.4.Electrospinning of PEOT/PBT polymer solutions

In this study, a bottom-up electrospinning process was performed using a customized Nanospinner 24 electrospinning machine (Inovenso, Turkey), schematically represented in Figure 2. In a first step, the PEOT/PBT polymer solution under study was loaded into a 5-mL standard syringe connected to a blunt-ended copper needle, after which the syringe was placed into a syringe pump (NE-300 Just Infusion™ syringe pump). This syringe pump controls the flow rate of the polymer solution through a polyethylene tube (inner diameter: 2 mm) ending in an aluminium pipe containing a single brass nozzle with an inner diameter of 0.8 mm. During

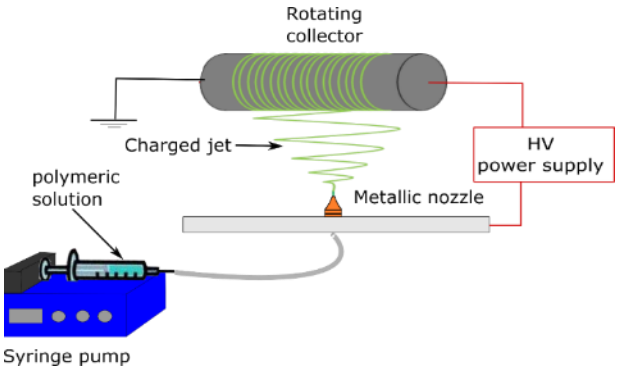


Figure 2. Electrospinning set-up.

the electrospinning process, the flow rate of the polymer solution was maintained at 0.1 mL/min. The metallic nozzle was placed vertically below a rotating stainless-steel collector (100 rpm) at a distance of 20 cm. During electrospinning, a DC high voltage of 30 kV was supplied to the nozzle, while the rotating cylinder was grounded. PEOT/PBT nanofibers were subsequently collected on an aluminium sheet placed on top of the collecting cylinder. Control samples were electrospun without any plasma modification, whereas the other samples were exposed to plasma for a varying amount of time prior to electrospinning, which was started immediately after plasma exposure.

2.5.PEOT/PBT solution characterization before and after plasma modification

The electrical conductivity and viscosity of a polymer solution are key features influencing the electrospinning process and the resultant nanofibers morphology [34]. Therefore, in a first step, the PEOT/PBT solutions were physically characterized before and after plasma modification.

The solution conductivity was determined using a Five Easy Mettler Toledo conductivity meter equipped with an InLab720 conductivity probe operating in a range of 0.1-500 µS/cm. The solution viscosity was measured with a Brookfield DV2T EXTRA viscometer.

To examine whether the plasma modification step is not degrading the PEOT/PBT polymer chains, size-exclusion chromatography (SEC) experiments were also performed in this work as it is an excellent chromatographic technique to reveal information on the molecular weight (MW) distribution of a polymer [35]. In this work, SEC measurements were conducted on a Waters instrument fitted with a Styragel HR column (IR+UV detection) using chloroform as eluent with a flow rate fixed at 1 mL min^{-1} (with polystyrene standards).

2.6.Characterization of electrospun PEOT/PBT nanofibers

The surface morphology of the electrospun PEOT/PBT nanofibers was imaged using a JSM-6010 PLUS/LV scanning electron microscope (SEM) (JEOL, Tokyo, Japan). Prior to the analysis, the samples were coated with a thin layer of gold to enhance their conductivity using a sputter coater (JFC-1300 Auto Fine Coater, JEOL, Tokyo, Japan). SEM images were acquired with an accelerating voltage of 7 kV and a spot size of 30 nm, while magnifications were varied between 500X and 5000X. The average diameter of the nanofibers was determined through the measurement of 40 different fibers, making use of ImageJ analysis software (v1.51j8, National Institute of Health, USA). Fiber diameters were however not determined when a lot of beads are present in the electrospun mats.

Furthermore, the surface chemical composition of the generated nanofibers was also examined. For this purpose, X-ray photoelectron spectroscopy (XPS) spectra were recorded with a PHI 5000 VersaProbe II spectrometer employing a monochromatic Al K_α X-ray source ($h\nu$ = 1486.6 eV) operating at a power of 25 W (beam size of 50 µm). Firstly, survey scans were recorded on 2 independent samples with 5 analysis points per sample using a pass energy of 187.85 eV (eV

step = 0.8 eV) at a take-off angle of 45° relative to the sample surface to identify the elements present on the PEOT/PBT nanofibers. The element quantification, based on these survey scans, was subsequently performed using MultiPak software (V 9.6) with a Shirley background by applying the relative sensitivity factors supplied by the manufacturer of the instrument. In the next step, high-resolution C1s and O1s peaks were recorded with a pass energy of 23.5 eV (eV step = 0.1 eV) to gain knowledge on the different chemical groups present on the surface of the nanofibers. MultiPak software was used to curve fit these peaks after the energy scale was calibrated with respect to the hydrocarbon component of the C1s spectrum (285.0 eV). Afterwards, Gaussian−Lorentzian peak shapes were utilized for the deconvolution of the peaks, and the full-width at half maximum of each line shape was restricted to less than 1.5 eV while an iterated Shirley background was employed.

To evaluate the hydrophilicity/hydrophobicity of the electrospun PEOT/PBT nanofibers obtained from different conditions, water contact angle (WCA) goniometry was also performed at room temperature. In this case, electrospun PEOT/PBT nanofibers were not collected on aluminium foil but on round glass cover slips (12 mm diameter). When using aluminium foil as a substrate, the nanofibrous mesh easily bended, making it very difficult to correctly determine the water contact angle values. 2 µL drops of distilled water were used for WCA analysis and 2 drops were placed on 6 independent samples (12 droplets per condition in total). For each drop, the WCA value was calculated based on Laplace-Young curve fitting making use of an EasyDrop (KRÜSS GmbH, Germany) device.

2.7.Cytotoxicity tests on electrospun PEOT/PBT nanofibers

2.7.1. Cell seeding

To examine the effects of the performed plasma treatments and the various solvent mixtures on the toxicity of the electrospun PEOT/PBT nanofibers, cell adhesion studies were also performed in this work. For cell seeding, the electrospun nanofibers were deposited onto coverslip glasses

(Ø: 12mm) which were stick on the mandrill making use of double sided tape. The human foreskin fibroblast (HFF) cells were supplied from ATCC (Belgium) and were taken from passage number 7. Prior to cell seeding, the electrospun samples were sterilized using UV light (Sylvania; 254 nm wavelength) for 30 min. UV sterilization was selected as it was previously observed that this sterilization method does not alter the physico-chemical properties of polymers [36]. After sterilization, the samples were placed in a 24-well plate and HFF cells were seeded onto the samples at a density of 60 000 cells/mL of culture medium, using a total of 1 mL of medium per sample. The seeded samples were subsequently incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 24 hours. Cells cultured on tissue culture polystyrene (TCP) plates were used as a positive control.

2.7.2. MTT assay

A colorimetric assay, using the yellow tetrazolium dye MTT was also performed in this work in triplicate to quantify cell viability by colorimetrically measuring the amount of metabolically active HFFs. In viable cells, the tetrazolium component was reduced by mitochondrial dehydrogenase enzymes into blue-purple water-insoluble formazan, which could be solubilized by addition of a mixture of DMSO/ethanol (1:1) and measured by a spectrophotometer. Cell viability was quantified in this study 1 day after cell seeding by replacing the culture medium by 0.5 mL (0.5 mg/mL) MTT reagent. Subsequently, the samples were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 4 h, after which the samples were removed from the MTT reagent and placed in a lysis buffer mixture of DMSO/ethanol (1:1) at 37°C for 30 minutes to solubilize the water-insoluble formazan. Afterwards, 200 µl of the formazan solution was transferred to a 96-well plate and the absorbance of the coloured solution at 580 nm was measured using a spectrophotometer (Universal microplate reader EL 800, BioTek Instruments). The background absorbance at 750 nm was subtracted from the measured absorbance and the obtained optical density of the

coloured solution is reported as a percentage compared to the normalized TCPs positive control.

2.7.3. Live/dead staining and fluorescence microscopy

A live/dead cell staining was also used to evaluate cell viability 1 day after cell seeding by fluorescence microscopy. Prior to the staining step, the supernatant was removed and the samples were rinsed twice with phosphate buffered saline (PBS). In a next step, staining was performed by placing the samples in a mixture of 2 μL (1 mg/mL) calcein –acetoxymethyl ester, 2 μL (1 mg/mL) propidium iodide and 1 mL PBS for 10 min at room temperature in the dark. Afterwards, the samples were removed from the solution, rinsed twice with PBS and were then visualized with a fluorescence microscope (Olympus; IX 81) using appropriate filters. To visualize the morphology of the cells adhering on the fabricated nanofibrous substrates, SEM images were also acquired 1 day after cell seeding after performing a cell dehydration and fixation step. In a first step, the cell-loaded nanofibrous structures were gently removed from the culture medium and rinsed 2 times with PBS to remove non-adhered cells. Subsequently, the cells were fixed by soaking the samples in a fixative solution of 2.5% glutaraldehyde in cacodylate buffer for 1 h at room temperature. Afterwards, the samples were washed in cacodylate buffer after which the cells seeded on the nanofibers are dehydrated by immersing them in increasing concentrations of ethanol (50%, 75%, 85%, 95% and 100%; 10 minutes immersion in each solution). As a subsequent step, the samples were immersed for complete dehydration in a 100% hexamethyldisilazane (HMDS) solution for 10 minutes, removed from the solution and subsequently again immersed in HMDS for 10 minutes. After that, the samples were left to dry in open air and viewed with SEM according to the procedure previously described.

3. Results and discussion

3.1. Plasma treatment of PEOT/PBT solutions in pure chloroform: effect on nanofiber morphology

In a first effort to generate bead-free, continuous PEOT/PBT nanofibers, electrospinning was performed starting from pristine and plasma-modified 9 w/v% PEOT/PBT solutions in pure chloroform. In this case, the morphology of the resultant nanofibers was examined making use of SEM and the results are shown in Figure 3.

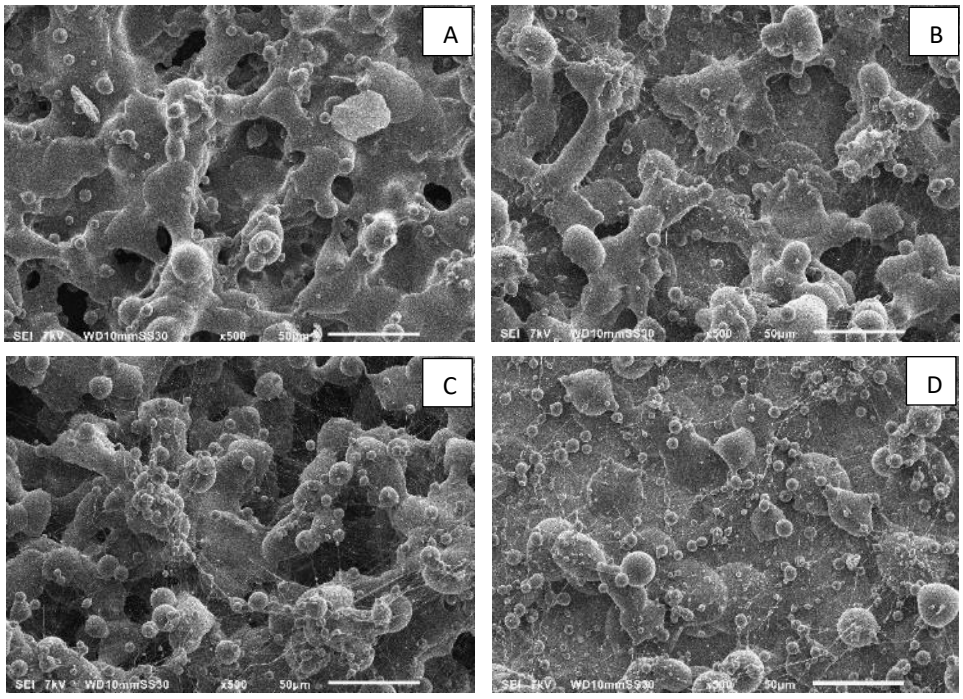


Figure 3. SEM images of PEOT/PBT nanofibers obtained from 9% w/v PEOT/PBT solutions in $CHCl_3$: A) untreated solution, B) 3 min plasma-treated solution, C) 5 min plasma-treated solution and D) 7 min plasma-treated solution

Figure 3 clearly reveals that spinning of the untreated PEOT/PBT solution results in a polymeric film consisting of small droplets (fig 3.A), while treating the polymer solution with plasma, with exposure times varying between 3 and 7 minutes (fig 3.B-D), results in the formation of some fibers. However, their number was considered too low to categorize these samples as nanofiber meshes. The SEM images thus clearly show that the solvent $CHCl_3$ as such did not

result in a proper spinning process, which could be attributed to the very low conductivity of the solution (which was zero for all conditions) and the low solution viscosity, even after the performed plasma modification steps. In literature, it is stated that solution conductivity is one of the key parameters in electrospinning since the applied electrical field between the tip and the collector acts on ions present in the polymer solution to start the electrospinning jet. As such, charge carriers will have to be added to chloroform to enhance the PEOT/PBT solution electrospinnability as they enhance solution conductivity. As previously mentioned, in this study, DMF, MeOH and HFIP were added to chloroform and the results are described in the following paragraphs.

3.2.Plasma treatment of PEOT/PBT solutions in $CHCl_3$+DMF: effect on solution physical parameters and nanofiber morphology

To enhance the electrospinnability of the PEOT/PBT solution, 10% DMF was first added, resulting in a final polymer solution of 9 w/v% of PEOT/PBT in $CHCl_3$ + DMF (9:1). DMF was selected because of its high boiling point (156°C), which could limit the fast evaporation of $CHCl_3$ and because of its strong polarity, thereby increasing the solution conductivity [19,37]. The obtained polymer solutions were subsequently electrospun as such or were subjected to a plasma modification step prior to electrospinning. It is however important to mention that unfortunately, during the plasma modification process, the solvents were partly evaporated due to the set-up configuration, which requires a fast argon flow through the liquid, as explained in more detail in previous work [25]. As the solvent progressively evaporates, the initial polymer concentration within the solution as well as the initial solvent ratio was changed. Therefore, to objectively compare the plasma-treated solution to an untreated one and to distinguish the effects solely caused by the plasma treatment, an additional set of control samples was required. These control samples were obtained by sending the same argon flow as used during the plasma

modification step through the polymer solution without igniting the plasma for a certain amount of time until similar solution volumes as after the conducted plasma modifications were reached.

In what follows, each plasma-treated solution is thus compared with a control solution streamed with argon, thereby guaranteeing the same final solution volume and polymer concentration. In table 1, the applied plasma exposure times can be found in combination with the resultant solution volumes after plasma exposure and the according final polymer concentrations. The time of argon streaming through the solution to reach the same final polymer concentrations can also be found in this table as well as the recorded viscosity and conductivity values for the solutions under study.

Table 1. PEOT/PBT in $CHCl_3$+DMF: Physical parameters for argon-streamed control solutions and plasma-modified solutions as well as plasma exposure (Tplasma) and argon streaming times (Targon).

Argon-streamed solutions						
V (mL)	**C_{polym} (w/v %)**	**T_{argon} (min)**	**Conductivity ($\mu S \cdot cm^{-1}$)**	**Viscosity (cP)**	**Fiber diameter (µm)**	**Corresponding SEM image**
10	9.0	0	0.75 ± 0.10	31 ± 5	/	Figure. 5 A
8	11.0	6	1.14 ± 0.15	120 ± 5	/	Figure 5 B
6	14.4	13	1.28 ± 0.15	140 ± 5	/	Figure 5 D
5	17.0	16	1.05 ± 0.10	560 ± 5	/	Figure 5 F
Plasma-modified solutions						

V (mL)	C_{polym} (w/v %)	T_{plasma} (min)	Conductivity ($\mu S \cdot cm^{-1}$)	Viscosity (cP)	Fiber diameter (µm)	Corresponding SEM image
10	9.0	0	0.75 ± 0.10	31 ± 5	/	Figure 5 A
8	11.0	3	4.02 ± 0.10	75 ± 5	/	Figure 5 C
6	14.4	5	4.70 ± 0.15	133 ± 5	0.28 ± 0.08	Figure 5 E
5	17.0	7	7.40 ± 0.15	>2000	0.25 ± 0.06	Figure 5 G

Based on the results reported in table 1, a progressive increase in solution conductivity as a function of plasma treatment time could distinguished. This was in sharp contrast with the argon-streamed samples where only a minor increase in conductivity could observed. Analysis of the viscosity showed a small difference in viscosity between the 3min plasma-modified sample and the corresponding argon-streamed sample, which could have been induced by small differences in solution temperatures. No distinctive differences in viscosity between the control and 5 min plasma-modified sample were observed. For the longest plasma treatment time (17 w/v % PEOT/PBT), a phase change however occurred during the plasma modification step, resulting in gelation of the liquid and thus a steep increase in viscosity.

The gelation phase change was recorded for all samples containing DMF. The untreated solution, when stored in a dark bottle, required on average 12 hours to gelate. The argon-streamed control samples, depending on the chosen argon streaming time, were found to gelate between 30 minutes and 1 hour. This decrease in gelation time could be attributed to the loss of solvents due to evaporation, resulting in higher concentrations of PEOT/PBT. For the plasma-exposed samples, it was observed that the speed of gelation was proportional to the plasma exposure time. Polymer solutions treated for 3 or 5 minutes were found to be still liquid at the

end of the experiment. This allowed transferring them from the reactor chamber and made it possible to characterize these solutions in terms of viscosity. However, within 5 and 10 minutes post-treatment for the 3 min and 5 min plasma-treated sample respectively, spontaneous gelation occurred, at a rate 3-6 times faster compared to the Ar-streamed controls. To demonstrate the difference in gelation rate, images were made of the control Ar-streamed solution (Figure 4, left) and the 5 min plasma-treated solution (Figure 4, right) 5 minutes post-treatment. While the Ar-streamed sample was still in its liquid form, the corresponding plasma-treated sample had already gelated. For plasma treatments lasting longer than 5 min, the solution turned into a gel almost instantaneously, as also evidenced by the very large viscosity value shown in Table 1. Since the gelation occurred for all samples with DMF as additive, we hypothesized that this phase transition was caused by a physically induced cross-linking reaction between the polymer and DMF, which was accelerated when the polymer concentration increased and the solvent ratio was altered. The plasma treatments seemed to accelerate the process even more, suggesting that the generated plasma species activated the DMF, thereby making it more reactive. For this reason, during the first few minutes after plasma exposure (approximately 20-30 min) the solution is electrospinnable, however after that time, the viscosity of solution increased and thus clogging of the electrospinning needle occurred more often. Consequently, in this case, it was very difficult to have a continuous jet and a stable electrospinning process.

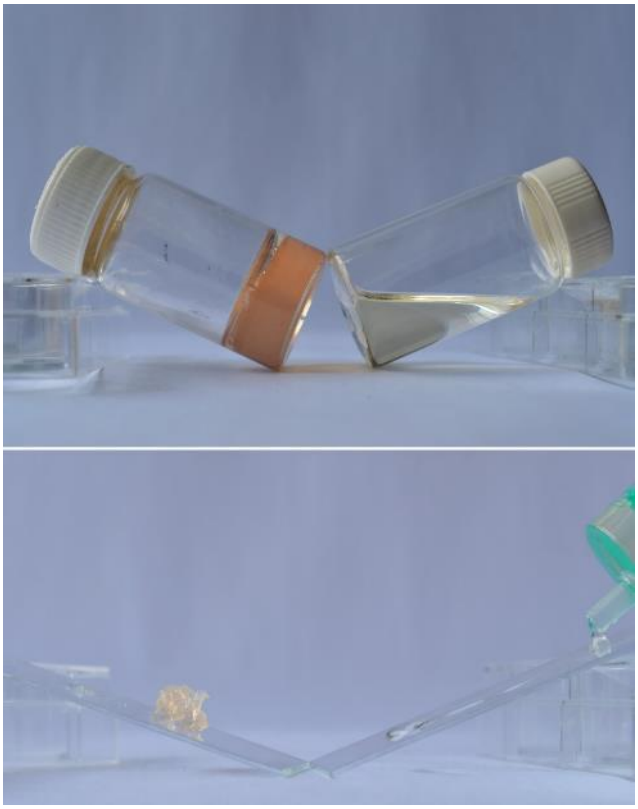


Figure 4. Picture of a PEOT/PBT polymer solution plasma-treated for 5 minutes (left) and the corresponding argon-streamed solution (right) in the top figure; a piece of gel and a drop of argon-streamed PEOT/PBT solution placed on tilted glass slips to highlight the differences in viscosity in the bottom figure. Due to the plasma treatment, the solution turns into a brown gel after 5 min in open air.

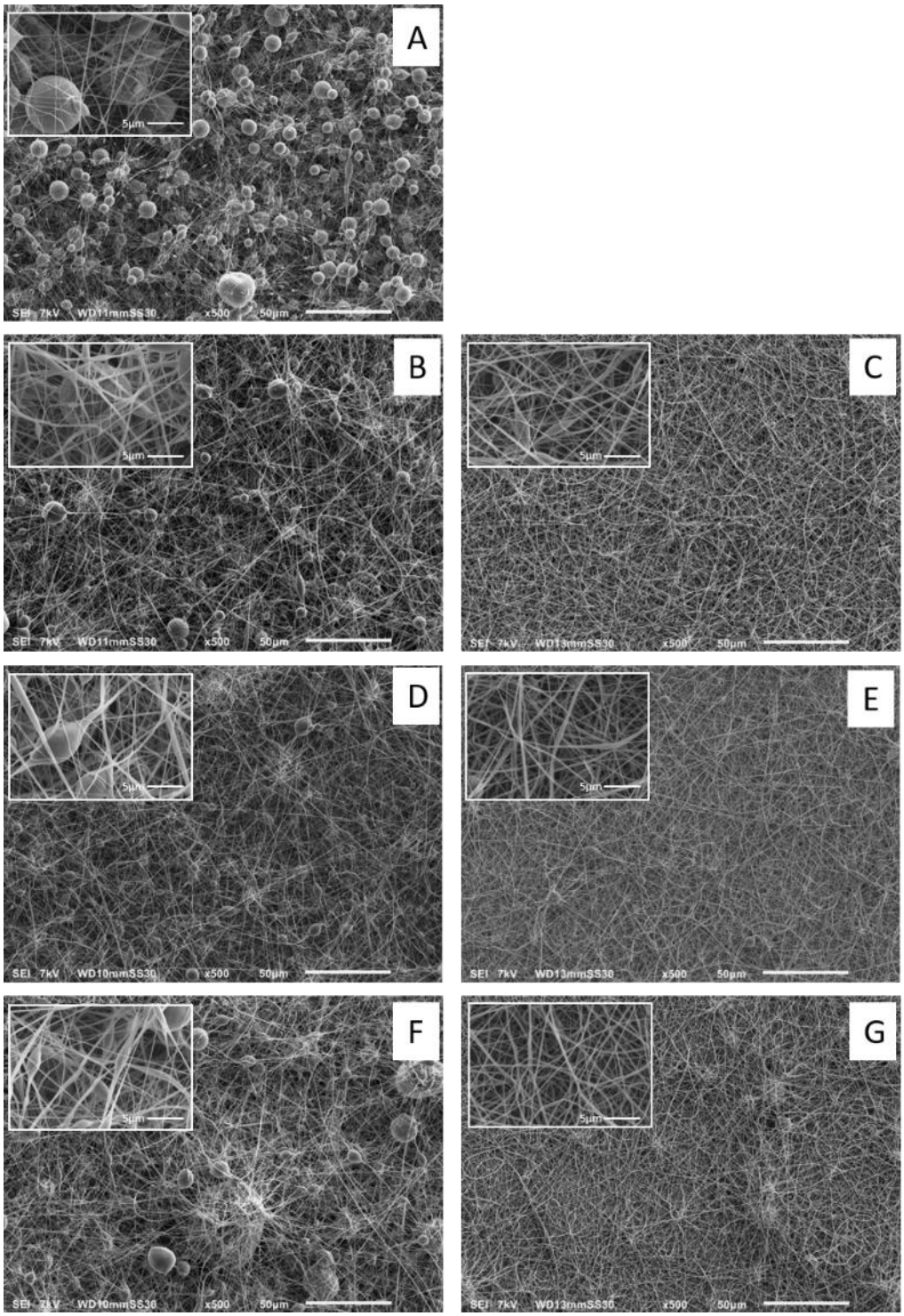


Figure 5. SEM images of electrospun PEOT/PBT nanofibers obtained from an untreated 9.0 w/v% solution (A), argon-streamed control solutions (left column) and plasma-treated solutions (right column) at different final PEOT/PBT polymer concentrations: 11.0 w/v% (B,

C), 14.4 w/v% (D, E) and 17.0 w/v% (F, G). PEOT/PBT is dissolved in a mixture of $CHCl_3$ and DMF.

Considering the observed large changes in solution conductivity because of the plasma modification, it was anticipated that the morphology of the electrospun PEOT/PBT nanofibers would also be strongly affected by the performed plasma treatments. In figure 5, these changes in electrospun nanofiber morphology are clearly depicted. When comparing the fiber morphology between the control samples in the left column and the plasma-treated samples in the right column, it was clear that the plasma treatments strongly improved the overall mesh quality. After 3 minutes (Figure 5.C) of plasma treatment, a significant improvement in fiber morphology was observed compared to the argon-streamed sample (Figure 5.B), but a significant number of beads were still present as could be observed in the inset of Figure 5.C. Figure 5.E represents the nanofiber sheet electrospun from the 5 min plasma-treated solution. In this case, the mesh was completely bead-free, which was in sharp contrast to the argon-streamed sample (Figure 5.D) where numerous µm-sized beads could be distinguished. Among all conditions depicted in Figure 5, the 5 min plasma-treated condition gave the highest quality nanofiber sheet. After 7 minutes of plasma treatment the nanofiber quality seemed to decrease with the formation of beads and unwanted fiber fusing. This altered behaviour could be assigned to the gelation occurring almost instantly after the plasma modification process as mentioned earlier. Due to the change in physical state, the polymer solution gave issues during the electrospinning step where the polymer jet was alternated by spraying, resulting in a heterogeneous mesh, which was observed for both the argon-flushed (Figure 5.F) and the plasma-treated solution (Figure 5.G). The fiber diameters for the plasma-treated solutions after 5 and 7 minutes were also calculated and were found to be equal to 0.28 and 0.25 µm, respectively (Table 1). The diameter dispersion could also be considered quite narrow since the standard deviation over 40 measured fibers was less or equal to 0.08 µm. These results could

be considered an improvement compared to literature, where PEOT/PBT nanofibers electrospun from the solvent mixture $CHCl_3$ + HFIP typically presented diameters in the range of micrometres [11,38]. Therefore, this work already presents a remarkable improvement in PEOT/PBT fibrous mats for tissue engineering applications. The diameters obtained in this study were considered to be more favourable for cell adhesion as these nanofibrous sheets closely mimicked the physical properties of the natural extracellular matrix [39]. Based on the results presented in this section it could thus be concluded that the plasma treatment of the polymer solutions had a significant effect on the solution conductivity, which lead to bead-free and uniform PEOT/PBT nanofibers possessing small fiber diameters. Unfortunately, in open air, a gelation process of the polymer solutions occurred for all samples where DMF was added with a considerable shorter time to reach gelation for the plasma-treated solutions. To overcome this limitation, methanol and HFIP have also been tested as alternative additives in the next paragraphs.

3.3.Plasma treatment of PEOT/PBT solutions in $CHCl_3$+MeOH: effect on solution physical parameters and nanofiber morphology

Methanol is an alcohol with a high relative dielectric constant ($\varepsilon_r(\omega)$= 33), is completely soluble in $CHCl_3$ and is a protic solvent, while DMF is aprotic, which efficiently interacts with the oxygen backbone of the PEO segment of the copolymer through hydrogen-bonding [40]. These characteristics made methanol an excellent choice as an alternative additive. Moreover, no gelation behaviour was registered during all conducted experiments, which was an advantage compared to the solvent mixture $CHCl_3$+DMF. Similar as for the latter solvent mixture, the solution conductivity and viscosity values were again obtained for control argon-streamed samples as well as for plasma-modified solutions and the results are shown in Table 2.

Table 2. PEOT/PBT in $CHCl_3$+MeOH: Physical parameters for argon-streamed control solutions and plasma-modified solutions as well as plasma exposure (T_{plasma}) and argon streaming times (T_{argon}).

Argon-streamed solutions						
V (mL)	**C_{polym} (w/v %)**	**T_{argon} (min)**	**Conductivity (µS·cm^{-1})**	**Viscosity (cP)**	**Fiber diameter (µm)**	**Corresponding SEM image**
10	9.0	0	1.13 ± 0.10	33 ± 5	/	Figure 6 A
8	11.2	5	1.16 ± 0.10	65 ± 5	/	Figure 6 B
6.5	13.5	10	1.05 ± 0.15	72 ± 5	/	Figure 6 D
6	14.6	12	1.10 ± 0.10	98 ± 5	0.50 ± 0.20	Figure 6 F
Plasma-modified solutions						
V (mL)	**C_{polym} (w/v %)**	**T_{plasma} (min)**	**Conductivity (µS·cm^{-1})**	**Viscosity (cP)**	**Fiber diameter (µm)**	**Corresponding SEM image**
10	9.0	0	1.13 ± 0.10	33 ± 5	/	Figure 6 A
8	11.2	3	1.60 ± 0.10	68 ± 5	/	Figure 6 C
6.5	13.5	5	1.81 ± 0.15	77 ± 5	0.29 ± 0.10	Figure 6 E
6	14.6	7	1.92 ± 0.15	100 ± 5	0.45 ± 0.17	Figure 6 G

Comparable to the solvent mixture $CHCl_3$+DMF, the electrical conductivity for the argon-streamed solutions remained constant around 1.10 ± 0.10 µS/cm, while an increasing trend could be observed for the plasma-modified samples, where the conductivity nearly doubled after 7 minutes of plasma treatment to 1.92 ± 0.15 µS/cm. The viscosity of the untreated solution was found to be around 33 cP and increased up to 100 cP after 7 min of plasma treatment, with

the same trends noted for both the plasma-treated and argon-streamed samples. The morphology of the PEOT/PBT nanofibers obtained after electrospinning of the control samples as well as the plasma-modified samples was also examined making use of SEM and the obtained images are depicted in Figure 6. As shown in this figure, all control samples (left column) were characterized by a high density of beads. Only the highest concentration sample corresponding to C_{polym}= 14.6 w/v% showed less beads with respect to the other control samples. For this case, the average fiber diameter was also determined and was found to be equal to 0.50 ± 0.20 µm (see Table 2). A strong improvement in fiber morphology quality could however be observed for the plasma-treated electrospun fibers (Figure 6, right column). After 3 minutes of plasma treatment (Figure 6.C), some beads could still be distinguished in the mesh, but their number was severely reduced compared to the corresponding argon-streamed control sample (Figure 6.B). After 5 minutes of plasma treatment, the electrospun sample still contained a few beads, but at the same time also nicely elongated PEOT/PBT nanofibers with an average fiber diameter of 0.29 ± 0.10 µm could be observed (see Table 2). At the maximum plasma exposure time (7 minutes, Figure 6.G), the mesh also presented a few beads with an estimated bead density of 200 beads/mm², which was however still a 3-fold reduction compared to the control sample (Figure 6.F). In this case, the average fiber diameter was found to be slightly larger compared to the 5 min plasma-treated sample: 0.45 ± 0.17 µm. Overall, when comparing the meshes electrospun from plasma-treated solutions to the control samples, a clear improvement in nanofiber quality was induced by the plasma modification step. This observation could again be assigned to the plasma-induced increase in solution conductivity, leading to a more stretched electrospinning jet, resulting in uniform and almost bead-free nanofibers [41].

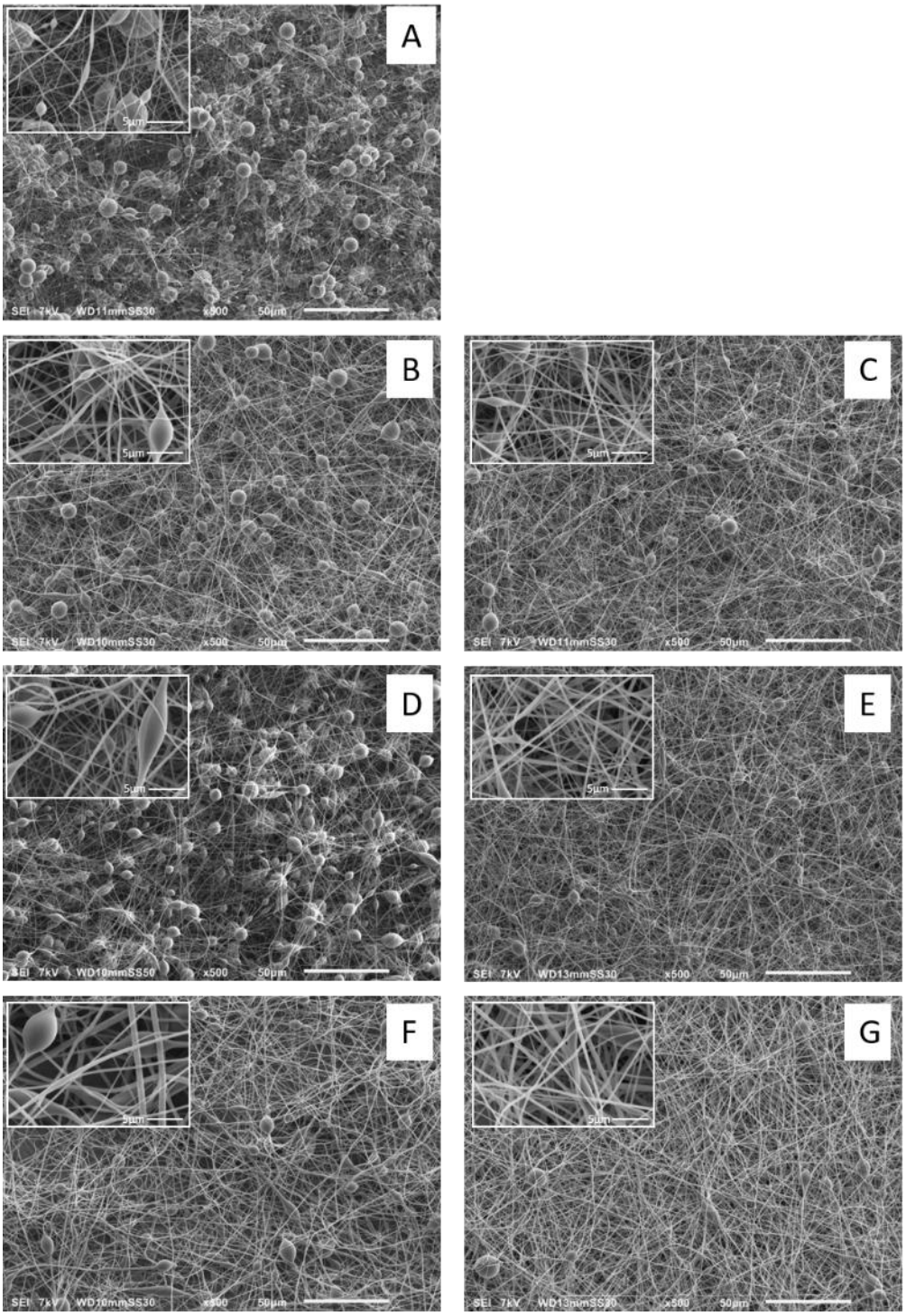


Figure 6. SEM images of electrospun PEOT/PBT nanofibers obtained from an untreated 9.0 w/v% solution (A), argon-streamed control solutions (left column) and plasma-treated solutions (right column) at different final PEOT/PBT polymer concentrations: 11.2 w/v% (B,

C), 13.5 w/v% (D, E) and 14.6 w/v% (F, G). PEOT/PBT is dissolved in a mixture of $CHCl_3$ and MeOH.

3.4.Plasma treatment of PEOT/PBT solutions in $CHCl_3$+HFIP: effect on solution physical parameters and nanofiber morphology

As mentioned in the introduction, HFIP is widely used in combination with $CHCl_3$ as electrospinning additive for PEOT/PBT due to its high polarity in combination with its excellent hydrogen bonding properties, enabling it to efficiently react with any hydrogen bond acceptor [11]. As such, HFIP was also used as an additive in this work and the obtained changes in solution conductivity and viscosity for argon-streamed control samples and plasma-treated samples can be found in Table 3. Comparing the viscosity and conductivity results of the plasma-exposed and argon-streamed samples to the other polymer solution mixtures under study in this work, the same trends could again be observed: an increased final polymer concentration through argon streaming did not increase the electrical conductivity. In contrast, for the plasma-treated solutions, the conductivity again increased progressively from 0.25 up to 1.80 µS/cm as a function of plasma exposure time. The viscosity results shown in Table 3 also confirmed the earlier observed trend of solvent evaporation leading to an increased polymer concentration, thus increasing the solution viscosity. No differences in solution viscosity could again be observed between the control and plasma-modified samples indicating that the plasma treatment was only causing a viscosity increase as a result of solvent evaporation.

The morphology of the PEOT/PBT nanofibers obtained after electrospinning of untreated, control samples and plasma-modified solutions was again analysed by SEM and the results are shown in Figure 7. The fibers electrospun from the untreated (C_{polym}= 9 w/v%) and the argon-streamed (C_{polym}=11.1 w/v%) solution are again characterized by a considerable number of large beads (Figure 7.A; B). When the polymer concentration was however further increased

through longer argon-streaming times (Figure 7.D; F), the electrospun fibers became completely bead-free, which is in close correlation with current literature [11,12,42]. From the plasma-treated solutions, bead-free fibers were already obtained after 3 minutes of plasma exposure and the meshes continued to be bead-free for the longer treatment times (Figure 7.C; E; G). As reported in Table 3, the average fiber diameter after argon streaming and plasma treatment was unchanged when the polymer concentration was equal to 11.1 w/v% (0.65 µm) and slightly different for samples with C_{polym}= 11.8 w/v% (1.27 µm for the argon-streamed and 1.14 µm for the plasma-treated solution) and 15 w/v% (1.70 µm for the argon-streamed and 1.40 µm for the plasma-treated solution). The best condition for this set of solvents was when the plasma treatment time equalled 3 minutes. In this case, the obtained nanofibers were bead-free with a diameter lower than 1 µm, while the corresponding argon-streamed nanofibers still presented quite some beads.

Table 3. PEOT/PBT in $CHCl_3$+HFIP: Physical parameters for argon-streamed control solutions and plasma-modified solutions as well as plasma exposure (T_{plasma}) and argon streaming times (T_{argon}).

Argon-streamed solutions						
V (mL)	**C_{polym} (w/v %)**	**T_{argon} (min)**	**Conductivity (µS·cm^{-1})**	**Viscosity (cP)**	**Fiber diameter (µm)**	**Corresponding SEM image**
10	9.0	0	0.25 ± 0.10	63 ± 5	/	Figure 6 A
7.5	11.1	8	0.27 ± 0.10	140 ± 5	0.65 ± 0.30	Figure 6 B
7	11.8	9	0.30 ± 0.15	164 ± 5	1.27 ± 0.37	Figure 6 D
5.5	15.0	12	0.28 ± 0.10	715 ± 5	1.70 ± 0.40	Figure 6 F
Plasma-modified solutions						

V (mL)	C_{polym} (w/v %)	T_{plasma} (min)	Conductivity ($\mu S \cdot cm^{-1}$)	Viscosity (cP)	Fiber diameter (µm)	Corresponding SEM image
10	9.0	0	0.25 ± 0.10	63 ± 5	/	Figure 6 A
7.5	11.1	3	1.60 ± 0.10	183 ± 5	0.63 ± 0.20	Figure 6 C
7	11.8	5	1.50 ± 0.15	203 ± 5	1.14 ± 0.41	Figure 6 E
5.5	15.0	7	1.80 ± 0.15	720 ± 5	1.40 ± 0.30	Figure 6 G

In the past, other research groups had also achieved the fabrication of bead-free electrospun meshes in the solvent mixture $CHCl_3$ + HFIP, but only when they worked with higher PEOT/PBT concentrations. As a result, these researchers typically only succeeded in the generation of nicely elongated thick PEOT/PBT nanofibers with average fiber diameters close to 2 µm [11,15]. After performing the morphological investigation of PEOT/PBT nanofibers electrospun from four different solvents (untreated and plasma-treated), substantial positive effects of the plasma exposure on PEOT/PBT nanofibrous mesh quality could be observed. The plasma jet treatment of the polymer solutions allowed us to strongly improve the nanofibers morphology, largely reducing or even eliminating the beads presence on the nanofibrous mats. In addition, smaller PEOT/PBT fiber diameters could also be obtained.

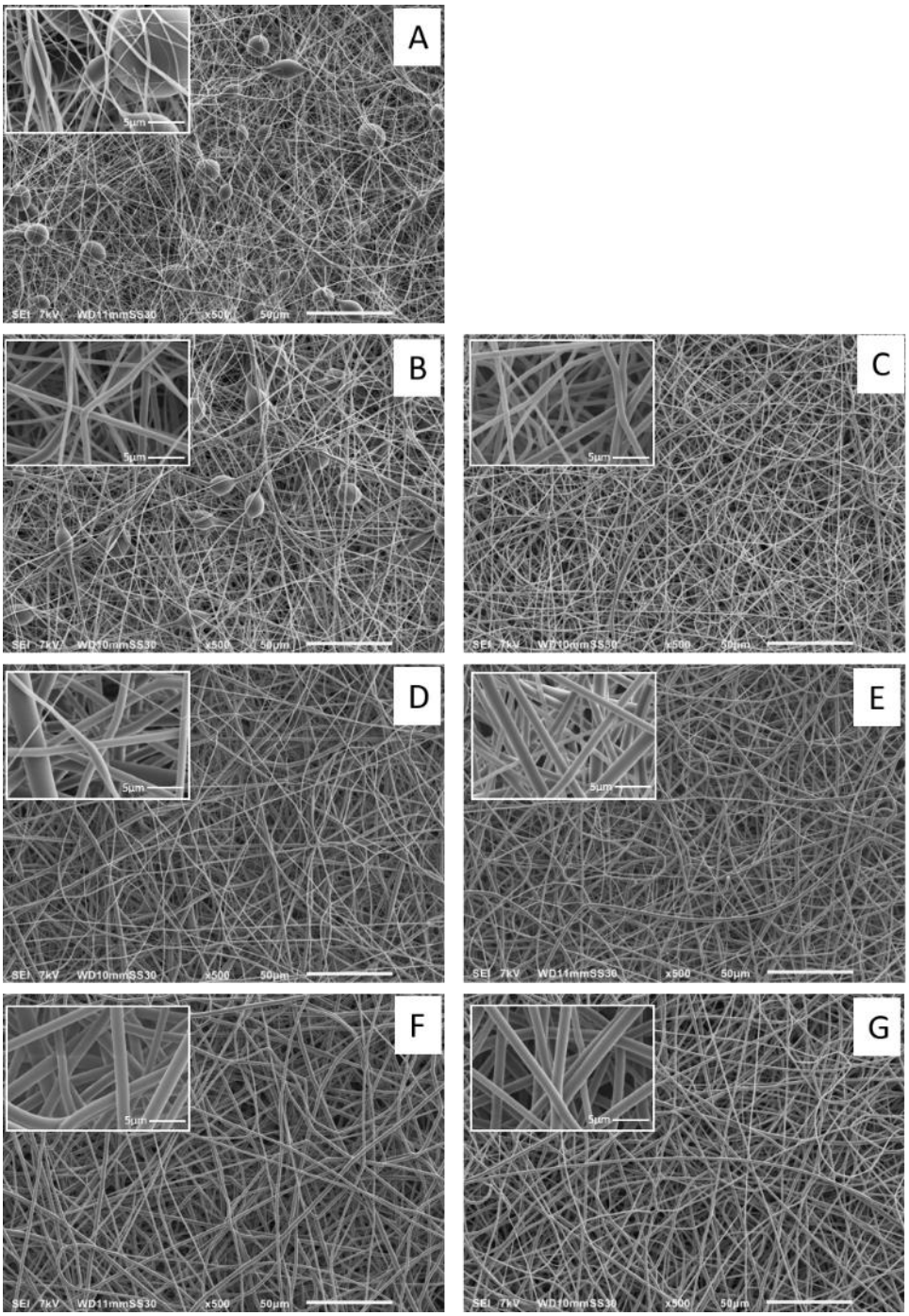


Figure 7. SEM images of electrospun PEOT/PBT nanofibers obtained from an untreated 9.0 w/v% solution (A), argon-streamed control solutions (left column) and plasma-treated solutions (right column) at different final PEOT/PBT polymer concentrations: 11.1 w/v% (B, C), 11.8 w/v% (D, E) and 15 w/v% (F, G). PEOT/PBT is dissolved in a mixture of $CHCl_3$ and HFIP.

3.5.Plasma treatment of PEOT/PBT solutions: effect on PEOT/PBT molecular weight

To investigate whether the plasma treatment had an (unwanted) influence on the polymer molecular weight (MW), SEC was performed on the PEOT/PBT solutions before and after plasma treatment. Any change in MW could have lead to an alteration of the polymeric nanofibers characteristics such as their mechanical properties and surface wettability, thus altering possible cell-surface interactions. As PEOT/PBT consists of 3 components, PEO, PEOT and PBT, it is their ratio that controls the surface wettability. Therefore, if one of them

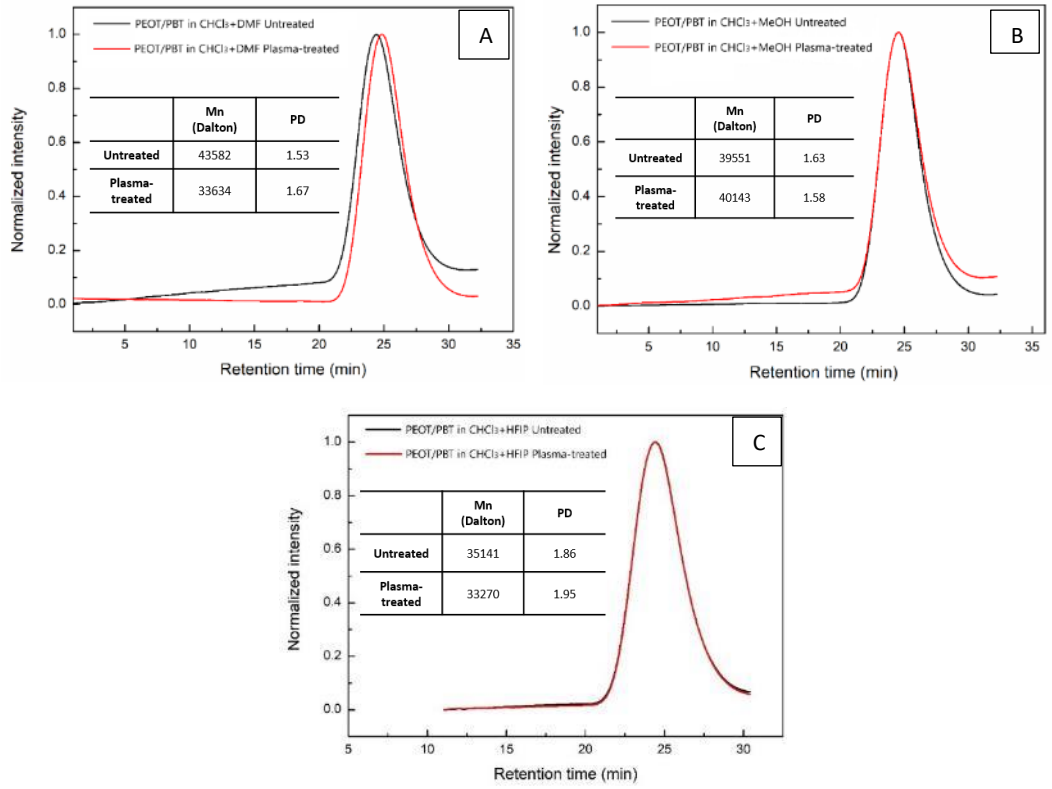


Figure 8. Size exclusion chromatographs of an untreated and plasma-modified (plasma exposure time: 5 min) PEOT/PBT solution in: A) $CHCl_3$+DMF, B) $CHCl_3$+MeOH and C) $CHCl_3$+HFIP.

would have been broken down by the plasma, the wettability may have considerably changed.

Figure 8 shows the obtained SEC chromatograms for the untreated sample and different plasma-treated PEOT/PBT solutions with a fixed plasma treatment time of 5 minutes. The argon-streamed samples are not shown in this figure as they perfectly overlapped with the untreated

sample. The chromatographs depicted in Figure 8 clearly showed that for the $CHCl_3$ + DMF solvent mixture the plasma modification induced a slight degradation of the polymer structure, as could be observed from the increased retention time. From the obtained curves, it was found that the MW decreased from 43.6 kDa to 33.6 kDa while the polydispersity (PD) value increased from 1.53 up to 1.67. These variations indicated that the PEOT/PBT polymer chains were modified during the conducted plasma treatment when using $CHCl_3$ and DMF as solvent mixture. Further studies will need to be conducted to explore in detail the occurring degradation process. In addition, it may be possible that this degradation behaviour was also causing gelation of the polymer solution upon plasma treatment as previously observed. When DMF is replaced by methanol or HFIP in the solvent mixture, the two SEC curves perfectly overlapped as shown in Figure 8, indicating that the plasma treatments in these solvent mixtures did not induce any modification of the MW of PEOT/PBT. As a result, these additives were preferably used instead of DMF.

3.6.Plasma treatment of PEOT/PBT solutions: effect on the surface chemical composition of PEOT/PBT nanofibers

To investigate whether the plasma modification of the polymer solutions was affecting the chemical surface composition of the nanofibers, XPS analysis has been performed on PEOT/PBT nanofibers generated from untreated, argon-streamed and plasma-modified solutions (plasma treatment time: 5 min). The XPS results of the nanofibers electrospun from the argon-streamed solutions were identical to the XPS results of the meshes electrospun from the untreated PEOT/PBT solution and are therefore not reported in this work. Based on the obtained XPS survey spectra, the surface elemental composition of the differently prepared nanofibers was determined and the obtained results are reported in Table 4. This table reveals that small differences in elemental composition could be observed on nanofibers electrospun from untreated and plasma-treated solutions containing DMF and MeOH (variations of

approximately 1.5 to 2 %). In contrast, no significant changes in elemental composition were observed for the PEOT/PBT nanofibers electrospun from untreated and plasma-modified solutions containing HFIP.

Table 4. Elemental composition of PEOT/PBT nanofibers obtained from untreated and plasma-treated PEOT/PBT solutions in different solvent mixtures (plasma treatment time: 5 min).

	PEOT/PBT in $CHCl_3$+DMF		**PEOT/PBT in $CHCl_3$+MeOH**		**PEOT/PBT in $CHCl_3$+HFIP**	
	C (at %)	**O (at %)**	**C (at %)**	**O (at %)**	**C (at %)**	**O (at %)**
Untreated	72.5 ± 0.5	27.5 ± 0.5	77.0 ± 0.3	23.0 ± 0.3	78.8 ± 0.6	21.2 ± 0.6
Plasma-treated	74.7 ± 0.2	25.3 ± 0.2	75.6 ± 0.4	24.4 ± 0.4	78.2 ± 0.5	21.8 ± 0.5

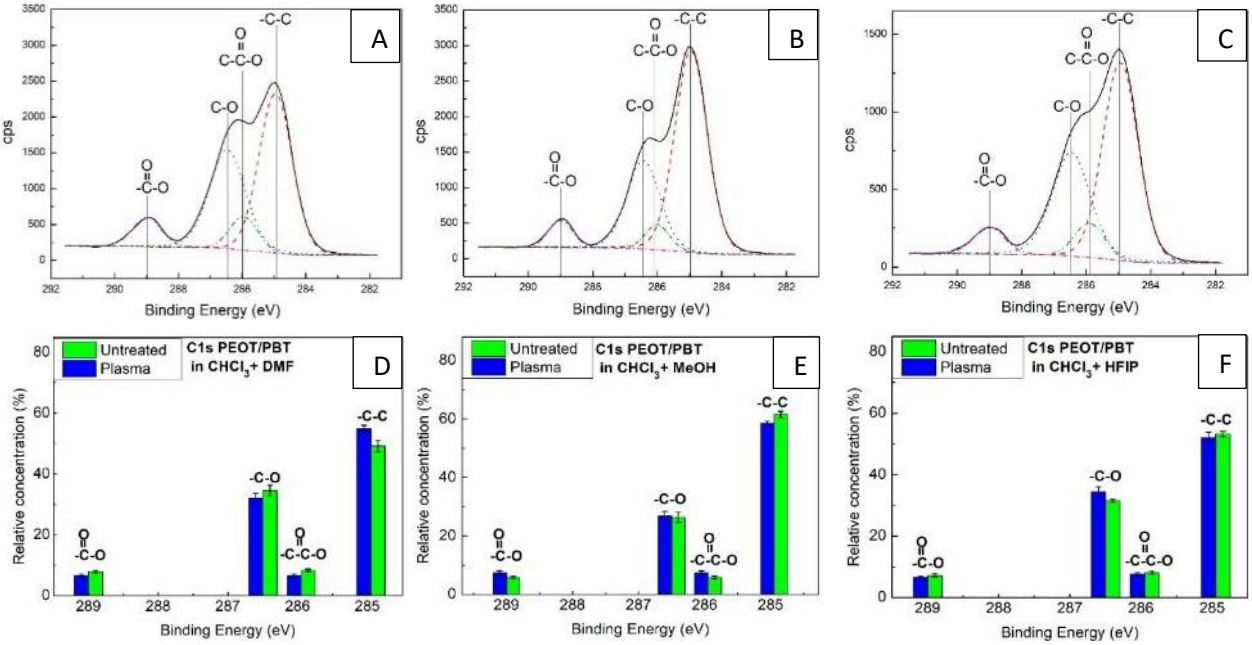


Figure 9. C1s deconvoluted peaks of nanofibers electrospun from untreated PEOT/PBT solutions using A) DMF, B) MeOH and C) HFIP as solution additives (top) and the relative concentration of the different carbon bonds for untreated and different plasma-treated solutions (bottom).

A high-resolution peak fitting of the obtained C1s peaks was also performed to check how the elemental composition variation affected the surface chemical groups because of the occurring plasma-liquid interactions. In Figure 9.A; B and C, the C1s peak deconvolutions are reported for the meshes electrospun from untreated solutions with the three different additives under study. The histograms (Figure 9.D; E and F) represent the relative amount of each chemical bond detected from the C1s spectra deconvolution. Four different carbon bonds were identified through the high-resolution fitting: 285.0 eV (C-C), 286.0 (C-COO), 286.6 eV (C-O), and 289.0 eV (O=C-O). When comparing the surface composition of the electrospun nanofibers before and after plasma treatment for each studied solution, some differences could be distinguished. When DMF was added to the solution, a significant difference before and after plasma treatment was recorded in the amount of C-C bonds, which changed from 50.2± 2.7% for the untreated solution up to 55.2 ± 1.2 % for the plasma-modified sample. At the same time, the relative concentration of most oxygen-containing bonds decreased because of the plasma treatment. The amount of C-COO in the pristine sample was 8.3 ± 0.2 % while it was reduced to 6.6 ± 0.5 %

after plasma modification. Following the same trend, the concentration of the bond O=C-O decreased from 7.8 ± 0.2 % to 6.6 ± 0.5 % after plasma treatment. In contrast, the last peak at 286.6 eV remained constant, within the error intervals, with a concentration of 34.6 ± 1.8 % for the nanofibers electrospun from the untreated solution and 31.4 ± 2.0 % for the nanofibers generated from the plasma-treated solution. The observed differences in surface chemical composition of the PEOT/PBT nanofibers were consistent with the observed decrease in total oxygen content (see Table 4) and could be explained by the polymer degradation as observed with SEC.

On the contrary, when MeOH or HFIP were used as solvent additives, only minor changes were observed in the relative concentration of the different carbon bonds on the PEOT/PBT nanofibers as a result of polymer solution plasma treatment. For the nanofibers electrospun from the polymer solution containing MeOH, the concentration of C-O bonds remained constant upon plasma treatment while the amount of C-COO and O=C-O bonds slightly increased, resulting in a slight reduction of the C-C concentration. These results were again in excellent agreement with the elemental composition results, where also a slight increase in oxygen content was found for the electrospun nanofibers generated from plasma-treated solutions. When HFIP was used as additive, a small increase in the amount of C-O bonds was observed, in combination with a slight decrease in the concentration of C-COO and O=C-O bonds. As a result, the total oxygen content at the surface can remained constant as observed in Table 4. These XPS results thus clearly showed that the plasma jet exposure caused some chemical changes to the surfaces of the electrospun PEOT/PBT nanofibers, however, these plasma-induced chemical changes were very minor and were thus considered to be not problematic.

3.7.Effect of solution plasma treatment on the surface wettability of PEOT/PBT nanofibers

The previously mentioned SEC and XPS results suggested that the performed plasma treatments could induce small changes in the MW and the surface chemical composition of the resultant PEOT/PBT nanofibers. In this section, the effect of the conducted plasma treatments on the wettability of the electrospun PEOT/PBT nanofibers was examined making use of WCA analysis. Table 5 shows the result of this analysis for PEOT/PBT nanofibers obtained from untreated and plasma-treated polymer solutions making use of different solvent mixtures.

Table 5. WCA values of electrospun nanofibers obtained from PEOT/PBT solutions in different solvent mixtures with and without pre-electrospinning plasma modification (plasma exposure time: 5 min).

	PEOT/PBT in $CHCl_3$+DMF	**PEOT/PBT in $CHCl_3$+MeOH**	**PEOT/PBT in $CHCl_3$+HFIP**
	WCA [°]	**WCA [°]**	**WCA [°]**
Untreated	137.8 ± 2.1	138.2 ± 0.9	147.8 ± 2.3
Plasma-treated	141.2 ± 2.3	129.5 ± 0.8	113.3 ± 0.8

Not only the surface chemistry could influence the contact angle, also the morphology of electrospun nanofibers strongly affected the hydrophobicity of the mesh. In fact, a change in surface wettability could be related to the contact area between the water droplet and the water surface, which also depended on surface roughness. On a rough surface, such as a nanofibrous mesh containing multiple beads, air was trapped in the apertures which could increase the

hydrophobicity as the water contact angle of air is considered to be around 180°. On the contrary, when the surface is bead-free, the WCA would decrease and would continue to decrease with increasing fiber diameters as higher diameters mean larger contact areas between the water droplets and the polymer surface, and hence lower WCA values [43]. The values reported in Table 5 were thus the result of two factors: surface chemistry and surface morphology (supporting information, Table S1, shows the results of WCA for spin-coated films for the same experimental conditions).

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The wettability of the nanofibers obtained from the solution with DMF, despite the improved morphology, decreased after plasma treatment. This could be explained with the increase of C-C bonds detected by XPS measurements, which confirmed that plasma induced some minor chemical changes to the fibers. On the contrary, when MeOH was used as additive, after 5 minutes of plasma treatment, more hydrophilic meshes could be electrospun, which could be explained by the slightly increased oxygen content on nanofibers electrospun from plasma-modified solutions as well as the presence of less beads. Finally, the last samples, involving HFIP, were characterized by a very low wettability for the untreated sample and a substantial increase in water affinity after 5 minutes of plasma modification. The XPS results suggested some small shifts in type of oxygen-containing functional groups before and after plasma treatment which could be responsible for this behaviour. In addition, also the morphological structure of the nanofibers could contribute to the enhanced hydrophilicity after plasma exposure. Indeed, the untreated sample presented quite a few beads which are known to increase the contact angle value, as mentioned before, while the plasma-treated mesh was completely bead-free therefore characterized by a significantly lower WCA value.

3.8.Effect of solution plasma treatment on the cytotoxicity of PEOT/PBT nanofibers

As PEOT/PBT is primarily used for biomedical applications, it was also important to investigate whether the plasma modification step affected the cell-surface interactions. Therefore, in vitro HFF cell adhesion tests were also conducted in this work.

XPS analysis already demonstrated that no traces of solvents remained on the surface of the nanofibrous mesh after the electrospinning step. To have this confirmed and to investigate whether some traces of solvents might still have been present in the bulk material, MTT assays were performed on all three polymer solutions under study before and after plasma modification. The cell viability analysis 1 day after cell seeding, presented in Figure 10 gave promising results. All examined conditions gave cause to a cell viability higher than 80 %,

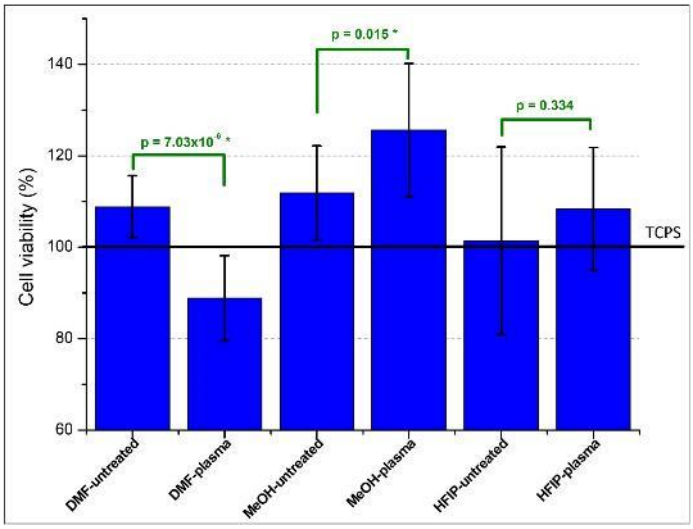

Figure 10. Cell viability 1 day after cell seeding on differently prepared PEOT/PBT nanofibers. Samples marked with * are significantly different with a 95% certainty.

thereby confirming the non-toxicity of the nanofibrous mats. For methanol the plasma treatment even increased the cell viability to 125% compared to the untreated samples (p-value=0.01). This might be explained by the combination of an improved morphology of the nanofibers, as it is known that cells tend to avoid beads, in combination with the slightly increased surface wettability recorded during WCA measurements [44]. For HFIP the average cell viabilities were 101 and 108% untreated and plasma treated samples, respectively, however this difference

could not be considered statistically significant since the p-value was bigger that 0.05 as reported in the in Figure 10. Moreover, the average fiber diameter in samples with MeOH as additive was significantly smaller than the one with HFIP, which could explain the higher cell viability values recorded for both untreated and plasma-treated substrates electrospun from $CHCl_3$ + MeOH mixtures. For instance, Christopherson *et al*.[45] showed that cells stretched in multidirectional direction followed the underlying smaller nanofibers (diameter of 283 nm), but when grown on larger fibers, expanded along a single fiber axis. Furthermore, for decreasing fiber diameters, a higher degree of proliferation and cell spreading and a lower degree of cell aggregation were also observed [45].

For the DMF-based meshes, the cell viability decreased from 108 % for the untreated mesh to 88 % for the mesh electrospun from the plasma-treated solution (p-value=$7.03*10^{-6}$). When this result was correlated with the obtained SEC and XPS results, this decrease in cell viability could be assigned to the slight degradation of the polymer backbone which resulted in a decrease of the oxygen-containing surface chemical groups. It is well known that the presence of polar functional groups increases the hydrophilicity of a surface, allowing cells to adhere more easily. Besides the loss of oxygen, also small residues originating from the polymer backbone might have been incorporated into the nanofibers which are also known to have a negative influence on cell viability [46]. However, even for these DMF conditions, cell viability still remained above the standard non-toxicity threshold of 80 % [47].

To have an accurate observation regarding the cell morphology and spreading, life/dead staining fluorescence images and SEM micrographs were recorded for the different solvent combinations (Figure 11). The fluorescence images showed that there were no significant differences between the untreated conditions for all solvents used for the electrospinning solutions. In addition, SEM images revealed that cells grown on untreated samples avoided the beaded nanofibers, preferring the bead-free areas. This finding was in agreement with the

results reported by other researchers, for instance Chen et al., who investigated how nanofibers morphology affect cell attachment [44]. Fluorescence and SEM imaging also revealed that cells

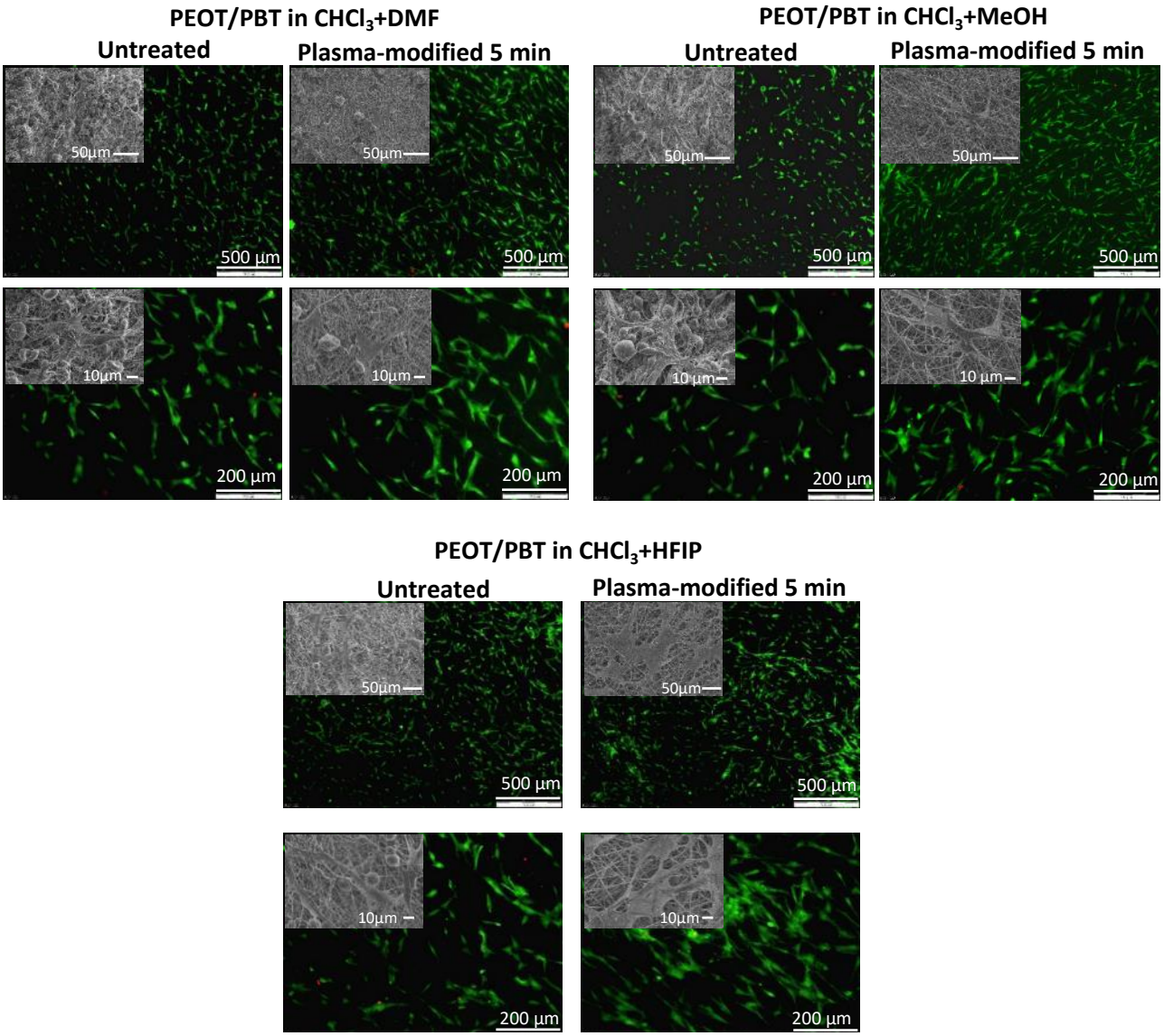

Figure 11. Life/dead fluorescent and SEM images of HFFs seeded on differently prepared PEOT/PBT nanofibers 1 day after cell seeding.

adhere homogeneously to the PEOT/PBT nanofibers electrospun from all plasma-treated solutions under study, most likely due to the fact that cells preferred to grow on bead-free areas. Overall, it could be concluded that all fabricated meshes, regardless of their solvent composition or pre-treatment, were not inducing cell toxicity 1 day after cell seeding.

4. Concusions

In this work, an APPJ has been successfully applied to improve the electrospinnability of PEOT/PBT polymer solutions in three different sets of solvent mixtures ($CHCl_3$+DMF, $CHCl_3$+MeOH and $CHCl_3$+HFIP). Plasma treatment exposure times were varied from 1 minute up to 7 minutes and after 5 minutes of treatment, the resultant electrospun nanofibers presented an almost beadless morphology with an average fiber diameter smaller than the equivalent control samples. However, for DMF and MeOH as additives, the fabrication process will still require a further optimization step to obtain completely bead-free meshes while not increasing polymer concentration (and thus fiber diameter). With HFIP, the best solvent additive, a plasma treatment time of 3 minutes was found to give the highest quality nanofibers. In this case, the obtained nanofibers are completely bead-free with an average fiber diameter of 0.65 µm. The corresponding argon-streamed nanofibers still presented quite some beads, showing that plasma treatment can further improve the current golden standard for PEOT/PBT electrospinning. The enhanced electrospinnability was found to be the result of an increased conductivity of the plasma-exposed solutions. However, the polymer solution containing DMF was found to gelate upon exposure in open air, which limits practical applications. The mechanism of gelation in the polymer solution is currently still under investigation.

SEC measurements revealed a slight degradation of the polymer backbone after plasma treatment of PEOT/PBT solutions in the solvent mixture $CHCl_3$+DMF. However, this degradation was not recorded when DMF was substituted with MeOH or HFIP. Contrasting results between the three additives under study were also found after WCA analysis: the plasma modification induced a higher hydrophilicity compared to the corresponding pristine samples when MeOH and HFIP are used as additives. The opposite trend was observed when DMF was used as additive: the untreated sample presented a higher hydrophilicity compared to the nanofiber meshes obtained from the plasma pre-treated solution. This result was also confirmed

by XPS high-resolution C1s curve fitting: the chemical bonds consisting of oxygen-rich groups diminished after plasma modification of the polymer solution containing DMF, compared to the nanofibers electrospun from the untreated solution. For the MeOH system, the carbon-carbon bonds slightly decreased, while a small increase in carbon-oxygen bonds was noted after plasma exposure. For the HFIP solvent mixture, some small shifts in the concentration of the different oxygen-rich groups could be observed, while the total amount of carbon-carbon bonds remained constant.

The cytotoxicity of the differently prepared nanofiber meshes was also tested by examining the behaviour of HFF cells on the meshes 1 day after cell seeding. MTT assays and live/dead fluorescence and SEM microscopy confirmed the non-toxicity of the electrospun nanofibers for all conditions (pristine and plasma-modified). In addition, the MTT assay revealed a better cell adhesion on plasma-modified samples obtained from the polymer solution containing MeOH or HFIP as additive. As a final conclusion, it can be stated that plasma modification of PEOT/PBT polymer solutions can strongly increase the quality of the final nanofibers, even at low polymer concentrations, leading to the formation of thin nanofibers more closely resembling the human ECM. Moreover, among the three examined solvent additives, MeOH is preferable as it has the ability to produce beadless electrospun PEOT/PBT nanofibers with an average diameter of 290 ± 100 nm after 5 minutes of plasma exposure without causing significant changes to the final nanofiber surface properties.

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