



Article

Improved Surface Properties and Enhanced Cell Adhesion on Poly- ϵ -Caprolactone for Heart Valve Tissue Engineering Applications via H₂-N₂ Plasma Treatment

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Abstract

A tissue-engineered heart valve is a fully functional tissue facilitated through the cultivation of autologous cells on appropriate scaffolds. Scaffold's surface charge and wettability are the main factors that significantly affect cell adhesion, which is known to be favourable on hydrophilic surfaces. Moreover, biocompatible scaffolds that induce minimal immunogenic response are also essential for successful tissue engineering (TE). However, commonly used biocompatible polymers with preferable bulk properties lack desirable surface properties. For example, poly- ϵ -caprolactone (PCL), which is widely used as a scaffold in TE, is known for its satisfying structural and mechanical properties, but due to its surface characteristics, cell attachment and, consequently, cell growth on this polymer are limited. In this study, we investigated the possible effect of H₂-N₂ plasma treatment on the surface wettability of electrospun PCL nanofibres to see the feasibility of improvement in cell adhesion and proliferation. Our results showed an increase in the hydrophilicity of the 650 nm PCL specimens after plasma treatment, which was followed by a significant enhancement in cell attachment without altering PCL mechanical properties. Plasma surface modification is a promising approach that can be used to improve hiMSCs growth without altering the desired bulk properties and fibre morphology of 650 nm PCL specimens.



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Keywords: heart valve tissue engineering; biocompatible polymer; plasma treatment; wettability; cell attachment

1. Introduction

Along with the growing and aging population, health care encountered a higher demand for organ transplantation or implants. While it is an essential medical intervention for patients with heart valvular disease, heart valve replacement encounters significant challenges, such as lifelong anticoagulant treatment of mechanical heart valve prosthesis with high thromboembolism risk [1,2] or necessary replacement of the bioprosthetic valve every 10–15 years [3]. In contrast to mechanical or bioprosthetic heart valves, tissue-engineered heart valves are similar to native valves and have integration and growth capacity. The latter makes them a promising replacement, particularly for pediatric patients [3–5]. Tissue engineering, a multidisciplinary alternative solution, provides a sustainable and reliable substitution of damaged or injured organs/tissues by combining engineering with biomedicine [6]. This substitute, for example, a tissue-engineered heart valve, is a fully functional tissue facilitated through cultivation of autologous cells on appropriate scaffolds [7]. Scaffolds, as the main component, provide a platform for cell attachment [8]. Therefore, in the absence of native extracellular matrix (ECM), an artificial 3D porous scaffold for cell attachment and proliferation is indispensable [6,9]. Cell growth, migration, and differentiation can be started only after successful cell adhesion, which consequently will result in a natural ECM biosynthesis [10–12].

Cells' interactions with the scaffold depend on the scaffold's surface energy and charge, its morphology, topography, hydrophilicity, and chemical composition [10–13]. Surface charge and wettability are known factors to significantly affect cell adhesion [12,14], which is known to be favourable on hydrophilic surfaces in comparison to hydrophobic surfaces [12,15]. Moreover, biocompatible scaffolds that induce minimal immunogenic response are also essential for successful tissue engineering [4,5]. Hence, biocompatible scaffold selection with optimal surface bulk properties plays an essential role [6,12]. However, finding a material that meets all crucial needs is unfeasible. Commonly used biocompatible polymers in industry with preferable bulk properties lack desirable surface properties [6]. For example, biocompatible biomedical polymers, such as poly- ϵ -caprolactone (PCL), poly (lactic-co-glycolic acid) (PLGA), and polylactic acid (PLA), which are widely used as scaffolds in tissue engineering, are known for their satisfying structural and mechanical properties, but due to their surface characteristics, cell attachment and, consequently, cell growth on these polymers are limited [6,16,17].

A promising strategy is to select a biomaterial with ideal bulk properties and optimize the surface characteristics (such as hydrophilicity, charge, and energy). Plasma surface modification or so-called 'plasma treatment' is a reassuring approach that has been widely used in the last two decades to improve cell adhesion and, consequently, cell growth on a wide range of biomaterials without altering their bulk properties [6,12,16–21]. In plasma treatment, the polymer surface is bombarded with reactive species, leading to radical formation. Subsequently, different functional groups, depending on the plasma gas, will be added to the polymer surface by the reaction of these radicals with other species in the plasma. The incorporated groups ultimately modify surface properties such as energy and wettability [6,12,21–23]. For example, low-pressure microwave plasmas may introduce high densities of primary amino groups onto polymer surfaces for biomaterial applications. Already more than 20 years ago, Schröder et al. [24] used a downstream microwave plasma source using ammonia (NH₃) for high-performance amino functional-

ization [24]. By adding hydrogen to the ammonia discharge, the selectivity and density of the amino groups could be controlled to a certain extent. A common treatment sequence of the polymer involved a first step of functionalization (e.g., NH_3 plasma to add amino groups) followed by a second passivation step (e.g., hydrogen plasma) to create chemical micropatterns. The process could achieve an amino group surface coverage where up to 50% of the total nitrogen functionalities were primary amino groups. With the methods developed by Schröder et al. [24], a wide range of polymers, including polystyrene (PS), Poly(ether ether ketone) (PEEK), Poly(ethylene terephthalate) (PET), Polycarbonate (PC), Polyethylene (PE), and Poly(methyl methacrylate) (PMMA), could be successfully modified. These functionalized surfaces provided a stable basis for the covalent immobilization of biomolecules, which improved cell adhesion and spreading for osteoblasts [24].

PCL is known as one of the favoured biocompatible and biodegradable polymers with desirable mechanical properties used in tissue engineering [17,21], specifically in heart valve tissue engineering (HVTE) (e.g., [25–29]). However, due to the hydrophobic surface properties of PCL, cell attachment and proliferation are limited and require improvement [17,19,21,30]. Recently, a study by Matschegewski et al. [31] using ammonia plasma showed over 60% enhanced wettability and improved cell colonization on PCL used for general cardiovascular implant applications, including biomimetic cardiac implants. To our knowledge, plasma treatment has not yet been investigated on PCL-based biomaterials specifically in the context of HVTE scaffolds. Additionally, whereas most experimental studies related to PCL functionalization by plasma methods use the “top-down” approach (e.g., dissociation of ammonia (NH_3) to generate the amino groups), we prefer the “bottom-up” method (e.g., generation of amino groups by the “simple” gases nitrogen and hydrogen). Hence, in this present study, we investigate for the first time the effect of $\text{H}_2\text{-N}_2$ plasma treatment of electrospun PCL nanofibres on cell adhesion and proliferation for its application in HVTE scaffolds. In our experiments, 650 nm PCL (with desired mechanical properties close to those of native valves but with high hydrophobicity) and 1680 nm PCL (with low hydrophilicity but acceptable mechanical properties) were used. Our results showed an increase in the hydrophilicity of the 650 nm PCL specimens after plasma treatment, leading to a better adhesion of the cells without altering their mechanical properties.

2. Results

2.1. Morphology of PCL Fibres

Scanning electron microscopy (SEM) images (Figure 1 shows representative images of untreated PCL) were analyzed to investigate the possible effect of plasma treatment on fibre size and morphology. Our results showed that plasma treatment did not affect fibre diameter and fibre morphology (e.g., warping and shrinking) in both 650 and 1680 nm PCL specimens (Table 1 summarizes fibre diameters after different plasma treatment times).

Table 1. Fibre diameter [μm] of untreated and H_2 - N_2 plasma-treated PCL. For each setting, 9 images were analyzed by measuring 50 fibres per image manually. The differences between the mean values of untreated and treated PCL are shown, along with the lower limits of their corresponding one-sided 95% confidence intervals (LL CI 95%) to show non-inferiority. The values of LL CI 95% show the worst-case scenario. From the authors' perspective (see section for statistical analysis), plasma treatment did not affect fibre diameter.

Fibre Diameter [μm]		Untreated	1 min	3 min	5 min
650 nm	Mean $\pm$ SD	0.649 $\pm$ 0.08	0.712 $\pm$ 0.06	0.652 $\pm$ 0.11	0.624 $\pm$ 0.06
	Difference		0.063	0.003	−0.025
	LL CI 95%		(−0.018)	(−0.097)	(−0.113)
1680 nm	Mean $\pm$ SD	1.675 $\pm$ 0.19	1.652 $\pm$ 0.19	1.621 $\pm$ 0.13	1.605 $\pm$ 0.15
	Difference		−0.023	−0.054	−0.07
	LL CI 95%		(−0.233)	(−0.217)	(−0.246)

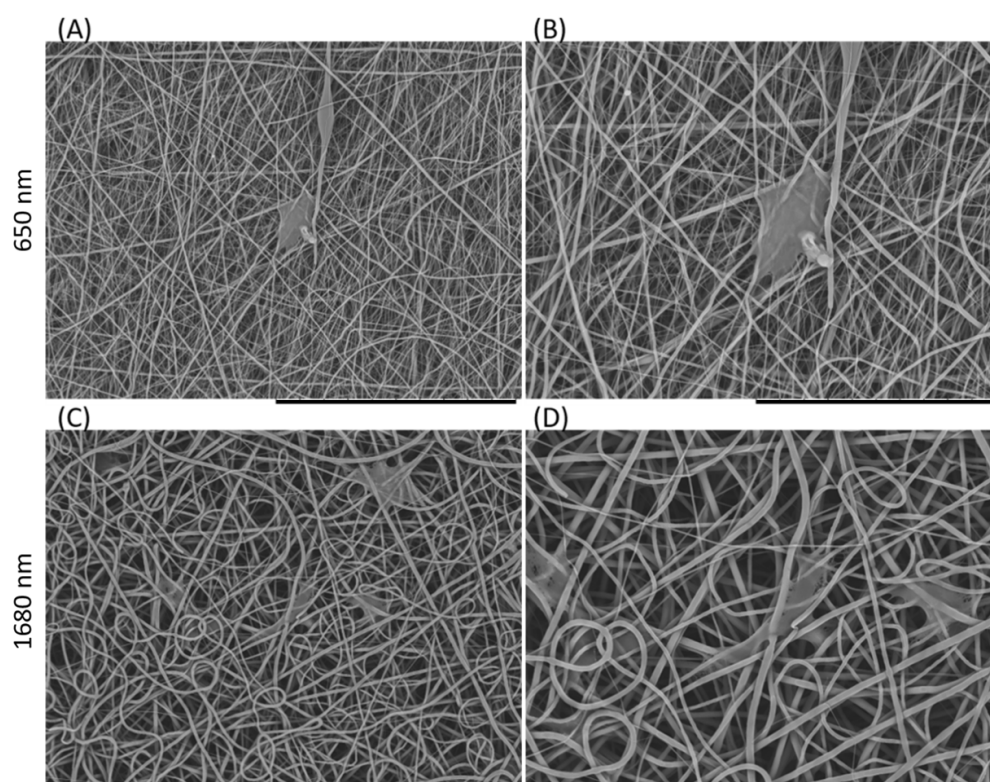


Figure 1. Representative scanning electron microscope images of PCL specimens with 650 nm (A,B) and 1680 nm (C,D) fibre diameter. The scale bar indicates 200 μm (A,C) and 100 μm (B,D).

2.2. Influence of Plasma Treatment on Hydrophilicity

Contact angles were used as an index to quantify the sample's hydrophilicity. The contact angles of different-treated PCL samples were measured using cell growth medium (DMEM/FBS/Pen/Str) as drops and were compared to untreated PCL samples. The untreated 650 nm PCL had a large contact angle (128.37 degrees, Figure 2A,C). Our results showed that an increase in plasma treatment duration led to a decrease in contact angle (Figure 2A, C). After 5 min of plasma treatment, the contact angle reached 0 degrees. In contrast to untreated 650 nm PCL, untreated 1680 nm PCL had a contact angle of 0 degrees, which was not affected by plasma treatment (Figure 2A,C) or could not be resolved with these measurements. We believe that the thicker fibres cause the PCL structure to form in such a way that the liquid penetrates very quickly into the interior of the tissue, making it impossible to measure the contact angle.

For comparison purposes and to rule out any influence of the test fluid on the contact angle, different polymers (Polypropylene (PP), Poly(vinyl chloride) (PVC), and Poly(tetrafluoroethylene) (PTFE)) were also examined with distilled water. The samples were treated with plasma for different lengths of time. No significant differences in the contact angles between measurements with distilled water and the cell medium (DMEM) were found (Table S1).

To show the temporal progression, the contact angles of 3 min plasma-treated PCL were measured directly after plasma treatment, after one hour, and after one day. Our results showed that the contact angle of the 650 nm PCL increased to 18.55 and to 30.91 degrees after 1 and 24 h, respectively (Figure 2B). This increase can be explained by the time development of the functional groups, which increases surface energy and adhesion [32,33]. However, these functional groups are not temporally stable, undergoing hydrophobic recovery (aging) over days or weeks as polar groups reorient into the bulk or react with surface contaminants [34,35]. Contact angles in 1680 nm PCL samples did not change from zero degrees (Figure 2B).

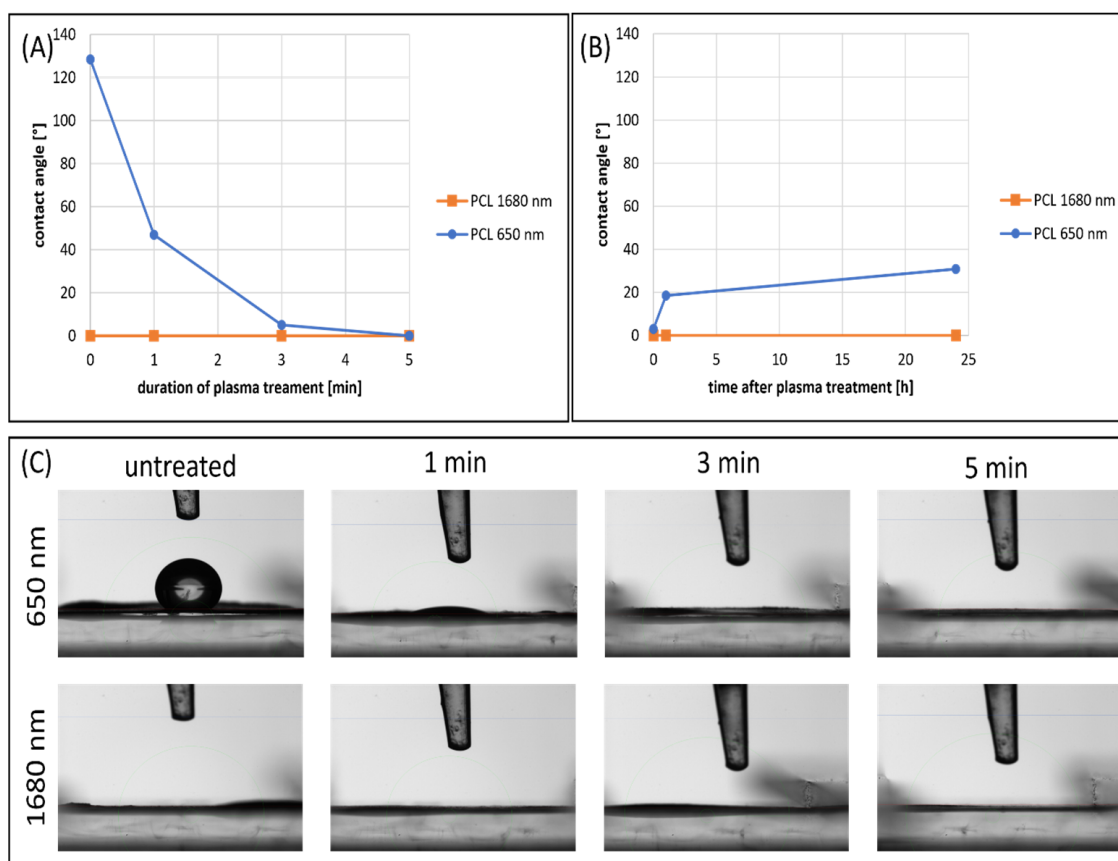


Figure 2. Contact angle of 650 nm and 1680 nm PCL specimens after different plasma treatment durations: 0 min (untreated), 1 min, 3 min, and 5 min (A). Progress of contact angle of 3 min plasma-treated PCL samples over time: immediately after plasma treatment, 1 h $\pm$ 0.1 later, and 24 h $\pm$ 0.1 later (B). Images of contact angle measurement after a few seconds using DMEM as medium (C). While an increase in plasma treatment duration led to a drastic decrease in the contact angle of 650 nm PCL specimens (or increased wettability), untreated 1680 nm PCL had a contact angle of 0 degrees and was not affected by plasma treatment.

2.3. Plasma Treatment Effects on Cell Attachment and Morphology

Plasma-treated 650 nm PCL samples in comparison to untreated ones showed drastically more cells and consequently a larger area covered with cells (Figure 3). Generally, a distinct improvement in cell attachment was seen in all 650 nm PCL samples. However,

comparing different durations of plasma treatment, results showed that one- and three-minute plasma treatment were most effective in enhancing cell attachment in comparison to 5 min plasma treatment. In contrast to 650 nm samples, our results from plasma treatment of 1680 nm PCL specimens showed that cell attachment was significantly higher on untreated samples (Figure 3).

For a better comparison of the number of cells attached to each PCL specimen subjected to different durations of plasma treatment, in a separate experiment, only 125,000 cells were used to seed PCL specimens (Figure 4). Similarly, the results of this experiment also confirm the positive effect of plasma treatment on the number of cells attaching to 650 nm PCL. On the contrary, fewer cells were attached to plasma-treated 1680 nm PCL specimens compared with the untreated ones (Figure 4).

Apart from cell number, cell morphology was also considered as another important factor for cell attachment. After MSC's successful attachment to a surface, its spherical morphology changes to a range of potential morphologies, like polygonal or elongated spread morphology [36]. In a separate experiment, MSCs seeded on native heart valve exhibited an elongated morphology (Figure 5). This morphology was considered an indication of optimal cell attachment and used as a reference for comparing the morphology of MSCs on both treated and untreated 650 nm PCL specimens (Figure 5). Our results suggested that cells on the plasma-treated samples have more complete attachment to the fibres in comparison to the loose attachment of cells on untreated samples. Moreover, cells seeded on the 650 nm samples showed a closer morphology to that on the native valve with elongated spread morphology (Figure 5).

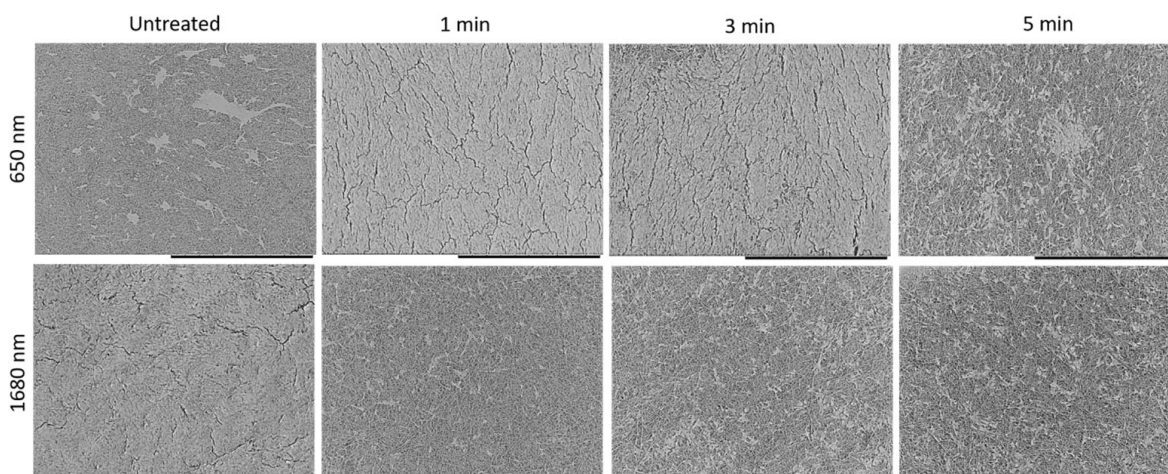


Figure 3. Representative SEM images of the PCL fibres with fibre diameters of 650 nm and 1680 nm that were seeded with ca. 250,000 cells after 2 days of incubation (untreated, 1 min, 3 min, and 5 min plasma treatment). The scale bar indicates 1 mm. Plasma treatment significantly enhanced cell adhesion in 650 nm PCL specimens (light grey areas), while the 1680 nm PCL specimens seem to have the best results in the untreated case.

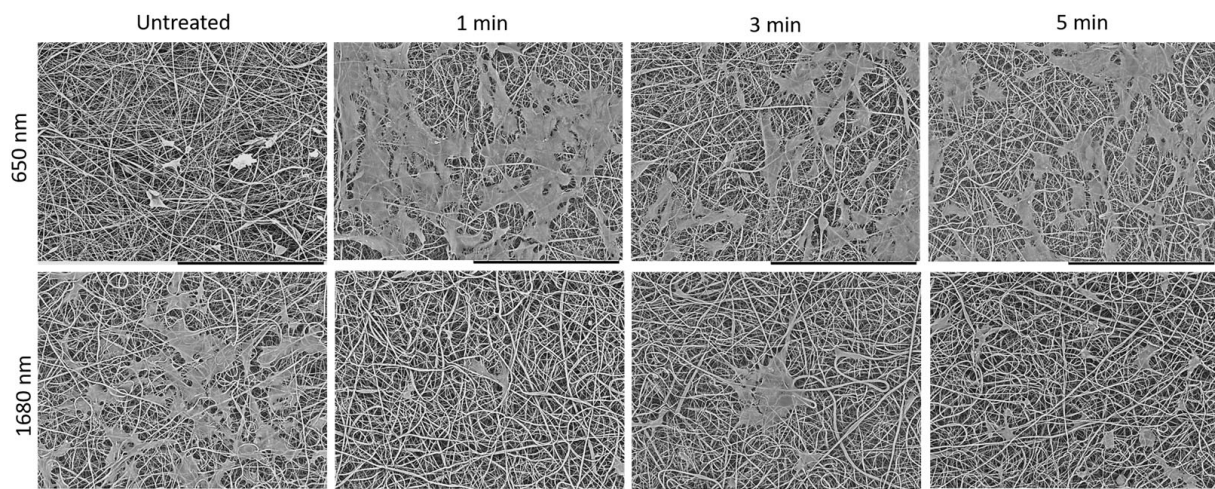


Figure 4. Representative SEM images of the PCL fibres with fibre diameters of 650 nm and 1680 nm that were seeded with ca. 125,000 cells after 2 days of incubation (untreated, 1 min, 3 min, and 5 min plasma treatment). The scale bar indicates 200 µm. Plasma treatment distinctly increased the number of cells attached to the 650 nm PCL specimens (light grey areas), while it decreased the number of cells attached to 1680 nm PCL specimens.

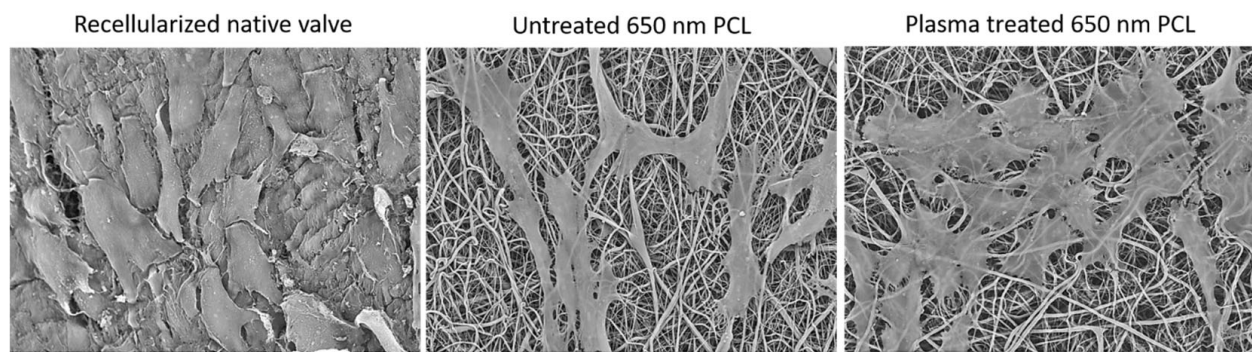


Figure 5. Representative SEM images of the recellularized native porcine heart valve, untreated and 5 min plasma-treated 650 nm PCL seeded with ca. 250,000 cells after 2 days of incubation time. The scale bar indicates 100 µm. Cells seeded on the plasma-treated 650 nm PCL samples showed a closer morphology to that on the native valve, with elongated spread morphology.

2.4. Effect of Time-Shifted Seeding of Plasma-Treated 650 nm PCL Specimens on Cell Attachment and Cell Morphology

The time-shifted colonization was studied in particular for potential later use in the clinic. Therefore, PCL was seeded directly with ca. 100,000 MSCs after plasma treatment (0 day), one day later (1 day), and 1 week later (1 week). For control, untreated 650 nm PCL was always used, too. Enhanced cell attachment was seen in plasma-treated samples in comparison to untreated ones. In addition, the covered cell area did not show changes in samples that were seeded one day or one week after plasma treatment (Figure 6A). Time-shifted seeding on plasma-treated 650 nm PCL specimens did not alter cell morphology either. Cells seeded one day or one week later on the plasma-treated samples showed elongated spread morphology similar to those seeded directly after plasma treatment (Figure 6B).

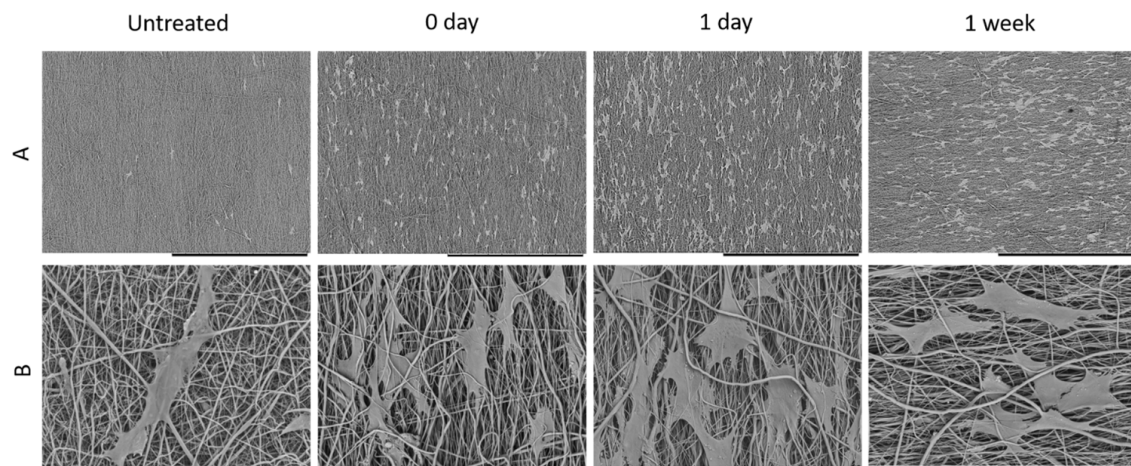


Figure 6. Panel (A) shows representative SEM images of the untreated, 3 min plasma-treated 650 nm PCL seeded with MSCs directly after treatment (0 day), one day later (1 day), and 1 week later (1 week). The scale bar indicates 1 mm. Cell attachment was enhanced significantly on all plasma-treated 650 nm PCL specimens. Panel (B) shows representative SEM images of the untreated, 3 min plasma-treated 650 nm PCL seeded with MSCs directly after treatment (0 day), one day later (1 day), and 1 week later (1 week). The scale bar indicates 100 μm . Cells seeded on all plasma-treated samples showed elongated spread morphology.

2.5. Mechanical Characterization of Untreated and Plasma-Treated PCL Samples

The mechanical properties of the plasma-treated PCL, such as Young's modulus (E_{mod}), the maximum tensile force (F_{max}), elongation at break, and ultimate tensile strength (UTS), were examined under uniaxial loading and compared to the untreated PCL (Figure 7 and Table 2). Based on the statistical analysis of results presented here, the authors believe that plasma treatment did not significantly alter the mechanical properties of PCL samples. Different studied mechanical properties stayed in the desired range after plasma treatment (Table 2).

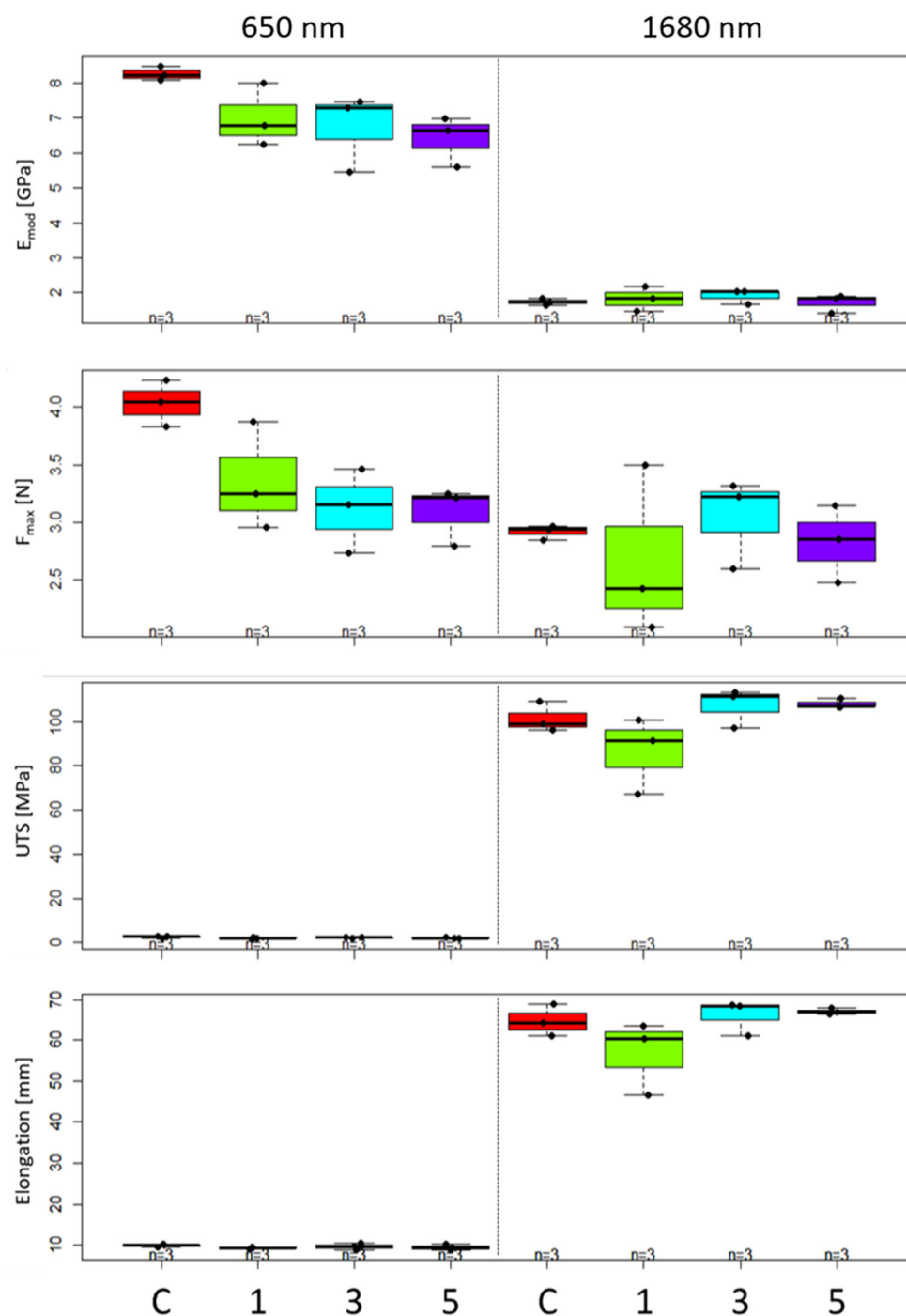


Figure 7. Uniaxial tensile mechanical properties shown as a boxplot for E_{mod} [GPa], F_{max} [N], elongation at break [mm], and UTS [MPa] of untreated as the control (C, shown in red) and treated PCL (1, shown in green for 1 min, 3 shown in blue for 3 min or 5 shown in purple for 5 min plasma treatment) in both fibre diameters (650 and 1680 nm). From the authors' perspective, plasma treatment did not alter the mechanical properties of 650 and 1680 nm PCL specimens.

Table 2. The mechanical properties (E_{mod} , F_{max} , elongation at break, and UTS) are presented as the mean $\pm$ SD. The differences between the mean values of untreated PCL and treated PCL are shown, along with the lower limits of their corresponding one-sided 95% confidence intervals (LL CI 95%) to show non-inferiority. The values of the LL CI 95% show the worst-case scenario. For each parameter $n = 3$. From the authors' perspective, plasma treatment did not alter the mechanical properties of 650 and 1680 nm PCL samples.

	650 nm	Untreated	1 min	3 min	5 min
E_{mod} [MPa]	Mean $\pm$ SD	8.26 $\pm$ 0.20	7.01 $\pm$ 0.90	6.740 $\pm$ 1.10	6.42 $\pm$ 0.72
	Difference		−1.25	−1.52	−1.85
	LL CI 95%		(−3.06)	(−3.33)	(−3.66)
F_{max} [N]	Mean $\pm$ SD	4.04 $\pm$ 0.20	3.36 $\pm$ 0.47	3.12 $\pm$ 0.37	3.09 $\pm$ 0.25
	Difference		−0.68	−0.92	−0.95
	LL CI 95%		(−1.31)	(−1.56)	(−1.59)
Elongation at break [mm]	Mean $\pm$ SD	9.95 $\pm$ 0.35	9.32 $\pm$ 0.22	9.69 $\pm$ 0.84	9.42 $\pm$ 0.65
	Difference		−0.632	−0.263	−0.526
	LL CI 95%		(−1.70)	(−1.33)	(−1.59)
UTS [MPa]	Mean $\pm$ SD	2.62 $\pm$ 0.49	1.98 $\pm$ 0.44	2.26 $\pm$ 0.33	2.14 $\pm$ 0.36
	Difference		−0.64	−0.35	−0.48
	LL CI 95%		(−1.51)	(−1.22)	(−1.35)
1680 nm					
E_{mod} [MPa]	Mean $\pm$ SD	1.73 $\pm$ 0.10	1.82 $\pm$ 0.35	1.91 $\pm$ 0.22	1.72 $\pm$ 0.27
	Difference		0.09	0.18	−0.008
	LL CI 95%		(−0.35)	(−0.26)	(−0.45)
F_{max} [N]	Mean $\pm$ SD	2.92 $\pm$ 0.06	2.67 $\pm$ 0.74	3.04 $\pm$ 0.39	2.82 $\pm$ 0.33
	Difference		−0.247	0.13	−0.09
	LL CI 95%		(−1.26)	(−0.89)	(−1.11)
Elongation at break [mm]	Mean $\pm$ SD	64.71 $\pm$ 4.02	56.8 $\pm$ 9.05	66.16 $\pm$ 4.54	67.10 $\pm$ 0.82
	Difference		−7.92	1.45	2.4
	LL CI 95%		(−19.45)	(−10.11)	(−9.18)
UTS [MPa]	Mean $\pm$ SD	101.36 $\pm$ 6.85	86.48 $\pm$ 17.35	107.15 $\pm$ 8.78	108.07 $\pm$ 2.25
	Difference		−14.88	5.79	6.71
	LL CI 95%		(−37.01)	(−16.33)	(−15.41)

2.6. Surface Characterization by XPS

Detection of characteristic chemical elements of the functional groups by chemical labelling or chemical shift by X-ray photoelectron spectroscopy (XPS) is an effective and common way for the characterization of functional groups on plasma-treated polymers. Since the electropositive character and lone electron pair of the introduced amino groups are critical for selective chemical synthesis and improved biocompatibility, we qualitatively observed, in particular, the change in the carbon (C1s) and nitrogen (N1s) signals in the XPS spectra (Figures 8 and 9). It can be clearly observed that the carbon signal and its chemical shift in the underlying PCL polymer substrate are affected by the plasma treatment, e.g., the bonds related to carboxyl and carbonyl groups [17,37,38] are reduced and covered by other (amino) groups, which leads to a reduction of the signal (see Figure 8). The appearance of the amino groups at the surface after H₂-N₂ plasma treatment of the PCL can also be seen in the nitrogen signal (Figure 9), where a peak appears after plasma treatment of the PCL, and which indicates the incorporation of N into the polymer surface.

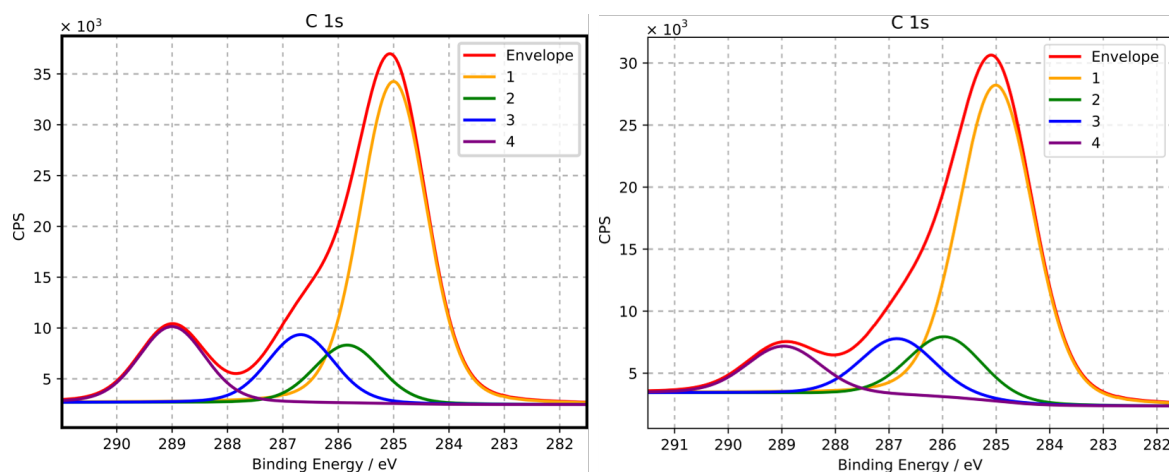


Figure 8. XPS C1s signal for untreated (**left**) and for plasma-treated (**right**) PCL (650 nm).

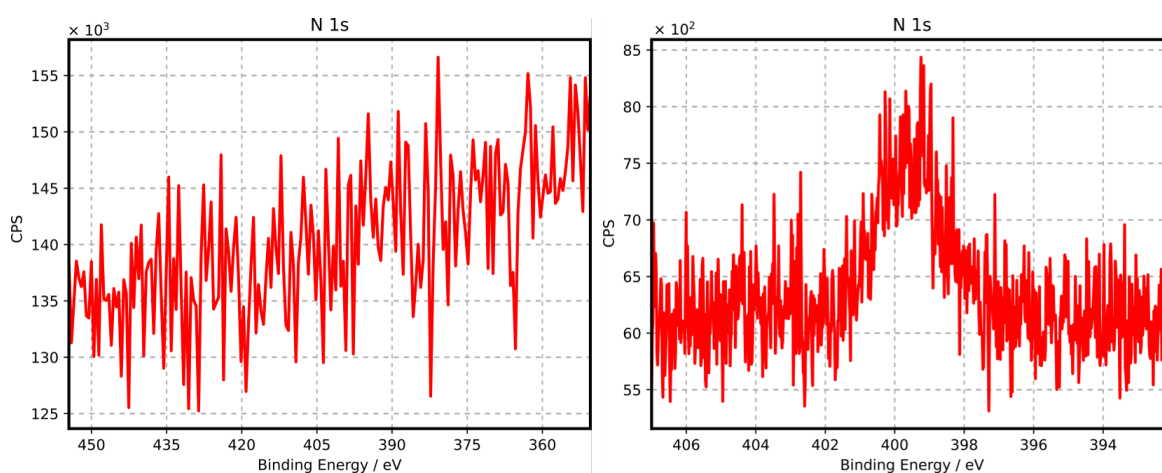


Figure 9. XPS N1s signal for untreated (**left**) and for plasma-treated (**right**) PCL (650 nm).

3. Discussion

Due to the absence of polar functional groups in their side chain and, therefore, their hydrophobic properties, biocompatible polymers used in tissue engineering need surface optimization to improve cell adhesion and growth. Among many different technologies, an environmentally friendly approach, namely plasma surface modification, has been widely used and has shown promising results in the improvement of surface properties in biomedical polymers [described in detail in [6,39]] without damaging the nanofibre structure and affecting their desired bulk properties [39]. For instance, cell adhesion on the PLA surface treated with different plasma gases was improved distinctly as described in a review by Jacobs et al. [6]. Using atmospheric plasma treatment for PLA samples (according to Nakagawa et al. [40] and Teraoka et al. [41]) also showed a drastic decrease in contact angle as well as enhanced cell adhesion and proliferation. In addition to PLA, plasma treatment was also studied in surface modification of PCL, which was used mostly as scaffolds in tissue engineering (e.g., [21]). For instance, Yildirim et al. [42] showed that oxygen plasma treatment significantly decreased the water contact angle and drastically increased cell proliferation rate. Also, by using atmospheric pressure, Dorai and Kushner [43] showed increased PCL wettability through the incorporation of oxygen-containing functional groups.

Similarly, to those studies, our results clearly showed that contact angles in 650 nm PCL specimens were drastically decreased to nearly zero (Figure 2) by increasing plasma

treatment duration, which was followed by enhanced cell adhesion and growth (Figure 3) as well as cell expansion (Figure 5).

Plasma treatment leads to the incorporation of new functional groups, but only on the surface, as confirmed by the XPS measurements (Figures 8 and 9). The rest of the nanofibre structure is supposed to remain intact. This leads to the assumption that plasma treatment should not cause significant mechanical changes [44]. Our results for biomechanical analysis confirm this assumption that plasma treatment does not alter the PCL mechanical properties to an undesirable range of data (Figure 7 and Table 2).

Despite a little increase in the contact angle in 650 nm PCL specimens one day after plasma treatment (Figure 2B), enhanced properties for cell attachment remained the same after one day or one week (Figure 6). The surface property deterioration, or so-called hydrophobic recovery, can be explained by the surface tendency to minimize its energy and revert to its original structure [12], leading to polar groups re-orientation towards the material bulk, which consequently reduces the interfacial energy in response to the surrounding environment [11]. Although, as it was explained above, enhanced surface hydrophilicity subsequently leads to improved protein adsorption and cell adhesion, there is no linear correlation between these two phenomena [39,45]. Specifically, when chemically diverse materials are studied, no clear trend emerges between water contact angle and cell attachment, suggesting that other factors may affect cell adhesion beyond just surface wettability [45]. Additionally, particular degrees of hydrophilicity can significantly affect how proteins adsorb onto a surface, which probably means that each cell type prefers an optimal water contact angle [46,47].

In contrast to 650 nm PCL specimens, cell attachment was not improved in 1680 nm PCL specimens after plasma treatment (Figure 3; Table 2). Although plasma treatment did not affect its fibre structure (Table 1) and mechanical properties (Figure 7), our results suggested that cell attachment and growth on plasma-treated 1680 nm PCL specimens were distinctly worse in comparison to those of untreated ones (Figure 3; Table 2). A super-hydrophilic surface of 1680 nm PCL specimens, already before plasma treatment and with a contact angle close to zero (Figure 2), can potentially explain this behaviour. In these conditions, further plasma surface modification may shift the surface beyond the optimal hydrophilicity, which consequently leads to reduced cell attachment and proliferation despite unchanged fibre morphology and mechanical properties. Cell interaction with the material surface depends on surface chemical and topographical features like fibre size and its orientation. In addition, cell attachment on surfaces with randomly orientated fibres is multi-directional and gradually changes from a more circular to a more elongated shape on materials with increased fibre diameter [39]. Therefore, cells can sense small size differences by forming different focal contact complexes based on their membrane receptors and the frequency of the available anchor points. Focal complex formation is affected by fibre size as well as their random or aligned orientation [39]. While Chen et al. [48] showed that cell adhesion and growth on PCL fibres with 1051 nm were lower than on fibres with 480 nm diameters, Badami et al. [49] showed that osteoblastic cell density increased by increasing the fibre diameter as described in [39].

A 650 nm PCL in comparison to 1680 nm PCL has close mechanical properties to those of native valves (both aortic and pulmonary) [5] and, therefore, is more favourable for heart valve tissue engineering applications. However, in contrast to 1680 nm, high hydrophobicity in 650 nm PCL can be followed by low cell adhesion and consequently low tissue regeneration rate. Our study showed clearly that plasma surface modification with H_2-N_2 is an assuring approach that can be used to improve human induced mesenchymal stem cell (hiMSC) growth without altering the desired bulk properties and fibre morphology of 650 nm PCL specimens.

4. Materials and Methods

4.1. Electrospun PCL Scaffolds

Commercially available electrospun poly(ϵ -caprolactone) (PCL) nanofibre scaffolds (Nanofibre Solutions, Dublin, OH, USA) were used in this study. These scaffolds were fabricated by electrospinning by the manufacturer and not by the authors, creating a scaffold of disordered nanofibres like the natural extracellular matrix (ECM). The sterile PCL specimens were cut into smaller pieces (0.5 cm $\times$ 1.5 cm) under sterile conditions for seeding experiments. In this study, we used two different fibre thicknesses, 650 nm and 1680 nm. These PCL specimens are identical to those described with their commercial names, 300 nm and 700 nm, in our earlier publications (e.g., [28,29]). The values of 300 nm and 700 nm correspond to the nominal fibre thicknesses provided by the manufacturer and were, therefore, used in our previous studies without independent fibre diameter measurements. Since our recent fibre measurements revealed actual fibre diameters of 650 nm and 1680 nm, respectively, for the same commercially sourced PCL specimens, it was decided to use corrected fibre sizes in the present work to reflect the accurate experimentally measured values.

4.2. Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM, TM-3000 Hitachi, Hitachi High Technologies Corporation, Tokyo, Japan) was used to analyse PCL specimens as well as native porcine heart valve leaflets. For this examination, PCL samples were coated with a 10 nm thick gold/palladium layer by high-vacuum sputtering (Leica EM SCD500, Leica, Wetzlar, Germany). Images were taken at different magnifications ($\times 50$ to $\times 2000$) and were analyzed by ImageJ software (Version 1.54g, National Institutes of Health, Bethesda, MD, USA). The average fibre diameter was calculated from 50 fibres randomly selected from different areas of the sample.

Native porcine heart valve (obtained from a local commercial slaughterhouse) fixed in glutaraldehyde 2.5%. Leaflets were washed with DPBS to remove excess glutaraldehyde. The leaflets were dehydrated with an ascending concentration of ethanol from 30% to 100%. Thereafter, samples were dried according to the protocol for critical-point drying as described by Scherge and Gorb [50]. Finally, a 10 nm thin gold/palladium layer was sputtered on the samples to enhance conductivity.

4.3. X-Ray Photoelectron Spectroscopy (XPS)

X-ray photoelectron spectroscopy (XPS), also known as ESCA (electron spectroscopy for chemical analysis), is a highly surface-sensitive technique used to identify the elemental composition and chemical state of materials. The sample is irradiated with a beam of X-rays (typically Al-K α or Mg-K α) absorbed by surface atoms, causing the ejection of photoelectrons from their core shells. A detector measures the kinetic energy of these ejected electrons. The difference between the known photon energy and the measured kinetic energy yields the binding energy of the electron, which is a characteristic value for each element. XPS only detects electrons that escape without losing energy to collisions, limiting the analysis depth to the top 1–10 nm (about 3–30 atomic layers). Each element (except hydrogen and helium) has a unique set of binding energies, acting like a “fingerprint”. Small shifts in binding energy—known as chemical shifts—may reveal the chemical state (oxide, nitride, etc.) and the specific atoms to which an element is bonded.

4.4. Plasma Treatment

For plasma treatment, an H₂-N₂ high-frequency, low-pressure, low-temperature plasma (bottom-up) was used [51,52]. To avoid the PCL being in direct contact with

the rf-powered electrode and possibly being damaged from ions in the plasma sheath, samples were placed in the plasma chamber on a glass stand at a height of approximately 1 cm. After evacuating the plasma chamber to a base pressure of about 1.1×10^{-2} Pa, the process gases (2:1 ratio $\text{H}_2\text{:N}_2$) were used at a pressure between 13.7 Pa and 14.4 Pa. The samples were then exposed to the plasma with an rf power of 10 W and a duration of 1 min, 3 min, and 5 min.

Prior experiments with other polymer materials (see Supplementary Material) showed better improvement in hydrophilicity after treatments with higher powers (up to 45 W) and longer treatment time (up to 10 min). As the PCL showed thermal damage with higher powers, a value of 10 W was used throughout further experiments. With this setting, a contact angle of 0° could be achieved after just 5 min of treatment. The polymer experiments also proved that treatments at around 14.5–15 Pa are most effective.

4.5. Contact Angle

A goniometer was used to measure the contact angle. The drop test was performed within a few minutes after plasma treatment of the samples. The samples were put on glass slides and weighed down on the edges to keep a flat surface. One drop (around 3 μL) of cell growth medium (DMEM (Dulbecco's Modified Eagle Medium, Thermo Fisher Scientific, Waltham, MA, USA), 10% Fetal Bovine Serum (FBS, Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin/streptomycin (PS, Thermo Fisher Scientific, Waltham, MA, USA) was trickled onto each sample and measured as soon as reaching a steady state (usually within a few seconds). If the drop was completely absorbed, the result was valued with zero degrees ($n = 3$ for each parameter combination).

4.6. Cell Seeding on PCL Specimens

To seed PCL specimens, mesenchymal stem cells (hiMSCs) were used. hiMSCs were derived from human induced pluripotent stem cells (hiPSC) [28]. As described in our previous publication by Lutter et al. [28], human iPSC cell lines ipWT1.1 and ipWT1.3 were generated from primary human fibroblasts derived from skin biopsy of a clinically silent healthy donor and kindly provided by Dr. Lukas Cyganek of the Stem Cell Unit, University Medical Center Göttingen, Germany.

One week before seeding, cells were defrosted and expanded in DMEM, 10% FBS, and 1% PS at 5% CO_2 and 37°C in an incubator (Heracell, Thermo Fisher Scientific, Waltham, USA). For cell seeding, the PCL samples were placed in 12-well plates, and the pre-defined cell number (100,000 cells, 125,000 cells, and 250,000 cells) was carefully dripped onto the surface. After an initial settling time of 30 min, another 2 mL of medium was added to each well, followed by 48 h of incubation. Samples were then washed with Dulbecco's Phosphate-Buffered Saline (PBS, Thermo Fisher Scientific, Waltham, USA) before being fixed with Roti[®] Histofix 10% (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) for at least one hour.

4.7. Recellularization of Decellularized Native Porcine Heart Valve

Decellularization was done as explained by Sierad et al. [53]. To seed the decellularized native porcine heart valve, MSCs were used. For this purpose, a decellularized valved stent soaked in MSC growth medium (consisting of Dulbecco's modified Eagle's medium-high glucose (DMEM-HG) (Thermo Fisher Scientific), 10% FBS, and 1% PS) was incubated in a humidified incubator at 37°C and 5% CO_2 overnight. On the next two days, the MSC cells (about 8×10^6 each day) were extracted from the culture flasks and added directly onto the valve surface. The seeding process was conducted on a cell seeder (Aptus Bioreactors, Clemson, SC, USA) in the incubator. To facilitate cell adhesion onto the leaflet surface, the process started with 3 h of rest and followed by the motion programme of the seeder (2 rpm

at a 30° angle to each side for 1 h, then with 30 min rest, in repetition). During the seeding process and to ensure an equal cell distribution onto leaflets, the stent was rotated regularly. The culture medium was refreshed every three days. Seeding was continued for 21 days. Afterwards, the leaflets were separated from the stent and were washed carefully in warm PBS. To analyse cell growth and attachment, leaflets were first fixed in phosphate-buffered formaldehyde (Roti® Histofix 10%, Roth) for 4 h at 4 °C.

4.8. Cell Morphology Analysis by SEM

The fixed PCL samples were washed two times with PBS and dried with increasing concentrations of ethanol (30%, 50%, 70%, 80%, 90%, 95%, 99.9%,) followed by 1,1,1,3,3,3-hexamethyldisilazan with increasing concentrations (33%, 66%, 100%, 100%, HMDS, Carl Roth GmbH % Co. KG, Karlsruhe, Germany). The sputtering and the microscopy were done as described before (see section for scanning electron microscopy (SEM)).

4.9. Biomechanical Analysis

In this study, different parameters such as maximum tensile force ($F_{\max}$), elongation at $F_{\max}$, and elongation at break, Young's modulus (E_{mod}), and the ultimate tensile strength (UTS) were used to analyse the effect of plasma treatment on the biomechanical properties of PCL specimens. As described before [5], these parameters were measured or calculated through the tensile strength test using a universal testing machine (ZwickRoell Z0.5, Zwick-Roell, Ulm, Germany) with a 0.5 kN load cell with a sensitivity of 2 mV/V. The samples were stretched until complete rupture with a velocity of 5.0 mm/min. Plasma-treated PCL samples were analyzed within a few hours after treatment and were compared with untreated PCL samples.

4.10. Statistical Analysis

The statistical software R (2024) [54] was used to evaluate the data. The data evaluation started with the definition of an appropriate statistical mixed model [55,56]. The model included “variant” and “modality”, as well as their interaction term, as fixed factors. The repetition was regarded as a random factor. The residuals were assumed to be normally distributed and to be heteroscedastic due to the different levels of “variants”. These assumptions are based on a graphical residual analysis. Based on this model, multiple contrast tests [57] were conducted to show the non-inferiority of the non-control “modalities” to the control. The corresponding results are presented as simultaneous, one-sided 95% confidence intervals (instead of p -values).

5. Conclusions

In this study, we investigated the possible effect of H₂-N₂ plasma treatment on PCL surface wettability to see the feasibility of improvement in cell adhesion and proliferation. Our results showed an increase in the hydrophilicity of the 650 nm PCL specimens after plasma treatment, which was followed by a significant enhancement in cell attachment without altering PCL mechanical properties. Additionally, our results with 1680 nm PCL samples clearly showed the complex interaction effect of fibre size and plasma treatment on cell adhesion. In conclusion, our study showed that plasma surface modification with H₂-N₂ is a reassuring approach that can be used to improve hMSC growth without altering the desired bulk properties and fibre morphology of 650 nm PCL specimens.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms27041957/s1>.

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Abbreviations

The following abbreviations are used in this manuscript:

TE	Tissue Engineering
PCL	poly- ϵ -caprolactone
hiMSCs	human induced Mesenchymal Stem Cells
ECM	Extracellular Matrix
PLGA	poly (lactic -co-glycolic acid)
PLA	polylactic acid
HVTE	Heart Valve Tissue Engineering
UTS	Ultimate Tensile Strength
SEM	Scanning Electron Microscopy
PS	Polystyrene
PEEK	Poly(ethylene terephthalate)
PET	Poly(ethylene terephthalate)
PC	Polycarbonate
PE	Polyethylene
PMMA	Poly(methyl methacrylate)
PP	Polypropylene
PVC	Poly(vinyl chloride)
PTFE	Poly(tetrafluoroethylene)

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