

Article

Polycaprolactone-Based Fibrous Scaffolds Reinforced with Cellulose Nanocrystals for Anterior Cruciate Ligament Repair

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Abstract: Anterior cruciate ligament (ACL) injuries pose significant challenges, driving the need for innovative repair strategies. Tissue engineering (TE) has emerged as a promising field for ACL injuries. Wet spinning is a filament production technique that enables precise control over filament alignment, diameter, and porosity, making it suitable for developing new scaffolds for ACL injuries. This study develops fibrous scaffolds using wet spinning of polycaprolactone (PCL) reinforced with cellulose nanocrystals (CNC) to enhance mechanical properties. Wet spinning was employed to fabricate fibrous scaffolds, utilizing PCL as the primary polymer due to its favorable biocompatibility and degradability. An automated collector was developed and optimized, which allowed the stretching of the PCL filaments to diameters as low as 30 μm . Several filaments were explored and characterized using SEM, TGA, and mechanical tests. The optimized PCL/CNC filaments were used to develop 3D braided structures that mimic ACL structure. The combination of wet-spun PCL/CNC filaments (with an optimized stirring method) and the braiding procedure allowed for fully biocompatible scaffolds that mimic both the structure and the mechanical properties of native ACL. Cytotoxicity and biocompatibility tests showed cell viability and proliferation values above 99% and 81%, respectively. These findings underscore the potential of CNC-reinforced PCL scaffolds as promising candidates for ACL repair, laying the groundwork for future biomedical applications.

Keywords: anterior cruciate ligament; polycaprolactone; cellulose nanocrystals; wet spinning; tissue engineering



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1. Introduction

The anterior cruciate ligament (ACL) is one of the key ligaments that helps stabilize the knee joint. The ACL connects the femur to the tibia and is essential for stabilizing the knee joint. Its rupture is among the most frequent and debilitating injuries encountered in orthopedics [1]. Tendon and ligament injuries, particularly those involving the ACL, affect millions of people globally each year, imposing significant clinical and economic burdens [2]. Such injuries are often associated with high-impact sports, aging, or traumatic events, leading to impaired mobility, chronic pain, and diminished quality of life. ACL injuries profoundly impact patients' activity levels and quality of life, as recovery often

involves prolonged rehabilitation with varying success rates. Despite advancements in surgical techniques and rehabilitation protocols, outcomes often fail to achieve complete functional recovery or prevent long-term complications, such as re-injury [3]. This requires the exploration of innovative approaches that can provide more effective long-term solutions [4].

Tissue engineering (TE) has emerged as a promising field that overcomes the limitations of traditional treatments by promoting tissue regeneration and mimicking native tissues. TE combines principles of biology, materials science, and engineering to develop biomimetic scaffolds capable of supporting cell growth, proliferation, and differentiation while restoring structural integrity. The goal of TE is to develop scaffolds with mechanical and biological properties that mirror those of natural ligaments [5]. Among the various fabrication techniques, wet spinning has gained attention for its ability to produce three-dimensional (3D) fibrous structures with favorable properties for tissue regeneration. Wet spinning is a manufacturing process that involves extruding a polymeric formulation through a needle into a coagulation bath, where the polymer coagulates into solid fibers. Unlike traditional methods, wet spinning enables precise control over filament alignment, diameter, and porosity, which are critical for replicating the native extracellular matrix (ECM) and achieving tissue integration [6]. In comparison with electrospinning and additive manufacturing, wet spinning does not require high temperatures or voltages, making it suitable for processing highly biocompatible polymers. This method enables the controlled formation of continuous filaments with tunable diameters and superior mechanical properties for large-scale production, making it particularly suitable for scaffolds where high tensile strength and controlled fiber alignment are crucial [7]. Braccini et al. demonstrated that computer-aided wet spinning (CAWS) can fabricate chitosan/hyaluronic acid scaffolds with controlled macroporosity, enhancing cell adhesion and proliferation, underscoring its potential for tissue engineering applications [8]. These findings highlight the importance of advanced fabrication methods in tailoring scaffold properties to meet specific biological and mechanical requirements.

The choice of materials for scaffold production is critical, particularly in the context of sustainability and biocompatibility [9]. There has been a growing emphasis on using biocompatible and biodegradable polymers, driven by environmental concerns and the need for materials that are compatible with human tissues [10]. Natural and synthetic polymers, including collagen, chitosan, silk fibroin, polycaprolactone (PCL), and polylactic acid (PLA), have been widely investigated for their potential in ligament tissue engineering. Collagen and silk fibroin exhibit excellent biocompatibility but often lack the necessary mechanical strength for ACL reconstruction [11,12]. PLA-based scaffolds offer tunable biodegradability, yet their brittle nature can limit long-term mechanical stability [13,14]. An ideal material for ACL repair must balance biocompatibility and mechanical strength, ensuring both cellular integration and sufficient load-bearing capacity. In terms of biodegradability, it should degrade at a controlled rate to provide structural support throughout the healing process while gradually being replaced by regenerated tissue. Polycaprolactone (PCL) has been identified as one of the most promising synthetic polymers for tissue engineering applications, especially for long-term use, due to its slow degradation rate, high mechanical strength, and ease of processing [15–17]. PCL's ability to maintain mechanical stability over extended periods makes it particularly well-suited for applications where prolonged support is necessary, such as in ACL repair, where regeneration and full functionality can take up to 4 months [18]. Additionally, PCL can be combined with other materials or bioactive agents to further enhance its biological properties and facilitate tissue integration.

Calejo et al. investigated PCL-based scaffolds and found that adding hydroxyapatite (HAp) particles enhanced cell proliferation and promoted an osteogenic phenotype in

human adipose-derived stem cells [19]. The study also showed that varying filament diameter by adjusting extraction speed significantly improved the mechanical properties, with higher speeds yielding better performance. Similarly, Gurlek et al. assessed the synthesis of PCL fibers produced by electrospinning, highlighting the material's capacity for cell adhesion and proliferation, although the mechanical properties of the electrospun PCL filaments were somewhat weaker compared to native ACL tissue [20]. These studies underscore the versatility of PCL and its ability to be customized to meet the specific mechanical and biological requirements of ligament repair.

In addition to PCL, cellulose nanocrystals (CNC) have attracted considerable interest as reinforcing agents in polymeric systems due to their high surface area, excellent mechanical properties, and biodegradability [21,22]. CNC, derived from renewable resources, offers a sustainable option for enhancing the mechanical strength of polymer scaffolds without compromising their biocompatibility [6,23–25]. CNC has been shown to improve the tensile strength, elasticity, and structural stability of polymeric scaffolds, making them ideal for load-bearing applications such as ligament repair. Liu et al. conducted a notable study where CNC was incorporated into sodium alginate fibers using a wet-spinning process, resulting in significant improvements in tensile strength and elongation at the break of the filaments [26]. The integration of CNC into PCL scaffolds represents a promising strategy to develop structures with enhanced mechanical properties while maintaining biocompatibility [27,28]. CNC has been widely used as a substance conducive to obtaining filaments with dimensions on the micrometer scale and improved mechanical properties [26,29–31]. Rinoldi et al. emphasized that the wet-spinning technique, despite its limitations in producing filaments primarily at the microscale, offers several advantages, including the ability to control filament orientation and scaffold architecture, which are critical for mimicking the mechanical behavior of ligaments and tendons [32]. Such precise control is vital to replicating the hierarchical organization of native tissues, where filament alignment plays a key role in load distribution and tensile strength.

Furthermore, textile engineering techniques provide the tools necessary to produce filament-based structures that mimic the extracellular matrix (ECM) and promote cell growth and tissue regeneration [33–35]. Braided and woven structures, for instance, have been successfully used to develop ACL scaffolds with mechanical properties comparable to native tissue [36]. These techniques allow for the production of scaffolds with tunable mechanical properties, controlled porosity, and optimal filament orientation, facilitating cell proliferation and migration. The scaffolds produced by these techniques show enhanced mechanical properties by improving load distribution and resistance to deformation. In the specific case of braided structures, they provide increased tensile strength and fracture resistance as the intertwined fibers distribute stress evenly and prevent crack propagation [37]. The ability to precisely control the design of the material, including filament orientation and pore size, is crucial in creating scaffolds that support cell proliferation and maintain structural integrity during tissue regeneration [38]. Combining advanced fabrication methods with material innovations, such as PCL and CNC, holds immense potential for addressing the challenges associated with ACL repair and advancing the field of tissue engineering.

In this study, we developed and evaluated PCL-based fibrous scaffolds reinforced with CNC using the wet-spinning technique. The incorporation of CNC was designed to enhance the mechanical and biological properties of PCL scaffolds, addressing the challenges associated with replicating the strength and structure of native ACL tissue. An automated collector was developed and optimized to ensure filament stretching and diameter reduction. This methodology, which combines a non-destructive fabrication process, an automated collector enabling the proper filament, and the selection of biocompatible

polymers, results in a fully biocompatible system with enhanced mechanical properties. The optimized PCL/CNC filaments were characterized by SEM, TGA, and mechanical tests and used on the development of 3D braided structures that mimic ACL structure. Cytotoxicity of the produced filaments and proliferation tests on the final structures were also performed.

2. Materials and Methods

2.1. Materials

Polycaprolactone (PCL) pellets (molecular weight of 80,000 g/mol) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Cellulose nanocrystals (CNC), used as reinforcing agents, were sourced from CelluForce (Montreal, QC, Canada). Methanol (MeOH), chloroform (CHF), acetone, and *N,N*-Dimethylformamide (DMF) were obtained from Sigma–Aldrich and used as solvents in the preparation of polymer solutions or in the coagulation bath. All reagents and solvents were used as received.

2.2. Preparation of the PCL and PCL/CNC Polymer Solution

For the preparation of PCL solutions, different PCL concentrations (5%, 10%, 15%, 20%, 25%, 30%, and 35% *w/v*) and solvents (CHF, DMF, and acetone) were evaluated. For the dissolution of PCL, the polymer was gradually added to each solvent under constant stirring at 30 °C until a homogeneous solution was achieved. To reinforce the PCL matrix, CNC was incorporated into the optimized PCL solution at concentrations of 1%, 2%, and 3% (*w/v*). CNC was added gradually at room temperature to prevent agglomeration, followed by vigorous stirring for 24 h to ensure even dispersion of the CNC particles within the polymer matrix. The final PCL/CNC solutions were stored until further use in the wet-spinning process.

2.3. Wet-Spinning Process

The wet-spinning process was employed to produce fibrous PCL and PCL/CNC scaffolds using a pump from New Era Pump Systems Inc. Solutions were extruded through different needles of varying diameters (0.41 mm, 0.61 mm, and 0.84 mm) into a coagulation bath (MeOH). Other parameters, such as the flow rate (1 to 9 mL/h), drying temperature (room temperature to 40 °C), and duration (10 min to 2 h), were tested and optimized. An automated collector was developed and optimized to ensure uniform filament stretching and improved data reliability. The optimization process involved a robust structure with adjustable rollers and an electrical system, including a motor and speed regulator, to maintain constant tension. The new automated collector, equipped with adjustable rollers and a motorized tension control system, facilitated the stretching of the filaments during collection, enabling the development of thinner and more uniform filaments (Figure 1a,b).

2.4. Braiding Process

To mimic the fibrous architecture of the ACL, braided structures were fabricated using a Trenz–Export model 16/100 vertical braiding machine (Trenz–Export S.A., Santpedor, Spain), which can accommodate up to 16 bobbins (Figure 1c). Wet-spun PCL/CNC filaments were wound onto bobbins using a Trenz–Export model PR/810 parallel winding machine before the braiding process. An approach using twisted bundles of 10 filaments per bobbin was adopted. The braided structures were produced by interlacing yarns from 16 bobbins in both lengthwise and crosswise directions, forming tubular structures. The machine’s speed was adjusted to control the braiding angle and the structural properties of the resulting scaffolds. Four different tubular structures were produced, varying in core diameters: 0 mm (compact), 2 mm, 4 mm, and 6 mm. The core was temporarily

inserted during braiding to maintain the desired diameter and was subsequently removed. The structures were heat-treated at 40 °C for 30 min to stabilize their shape after the core removal, ensuring that the braided configuration was preserved.

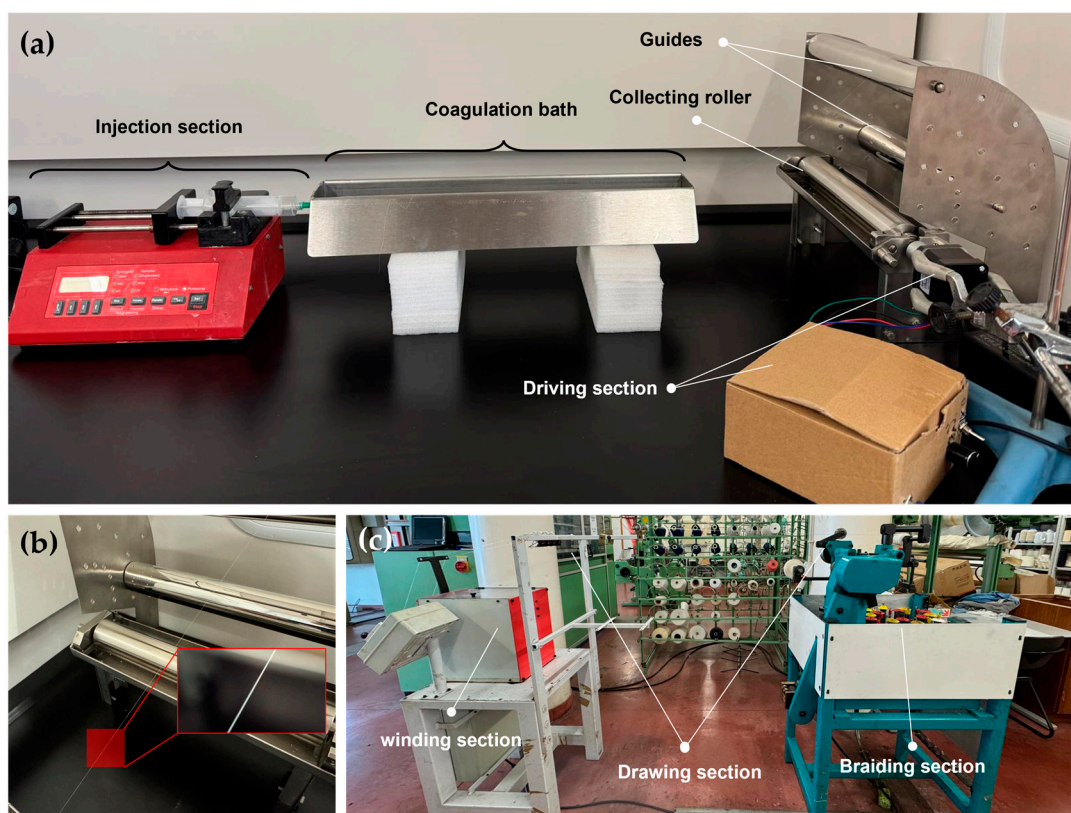


Figure 1. (a) Wet-spinning apparatus; (b) PCL/CNC wet-spun filament; and (c) Trenz–Export model 16/100 vertical braiding machine and PR/810 parallel winding machine.

2.5. Characterization Methods

2.5.1. Microscopy Analysis

Microscopy analysis was performed using a Leica DM750 M Optical Microscope (Leica Microsystems, Wetzlar, Germany) to observe the surface morphology of the wet-spun filaments and braided structures. Microscopy images were used to assess the diameter of the filaments by measuring their width at 100 different points. These measurements were then analyzed to calculate the mean diameter and standard deviation, assuming a Gaussian distribution model for statistical consistency. For more detailed surface analysis, Field Emission Scanning Electron Microscopy (FESEM) was conducted using a Phenom ProX desktop microscope (Thermo Fisher Scientific, Waltham, MA, USA). Samples were mounted on aluminum stubs with conductive carbon tape and analyzed without coating under a voltage of 5 kV. Images were captured at different magnifications to assess filament uniformity, cross-sectional structure, and surface characteristics of the PCL and PCL/CNC filaments, as well as braided structures.

2.5.2. Thermogravimetric Analysis (TGA)

Thermal stability and degradation profiles of the PCL and PCL/CNC filaments were analyzed using a STA 7200 SCANSCI thermogravimetric analyzer (Hitachi High-Tech, Tokyo, Japan). Samples were heated from 30 °C to 600 °C at a rate of 10 °C/min under a nitrogen atmosphere. The weight loss and derivative weight loss curves (DTG) were used to determine the degradation stages and thermal behavior of the filaments.

2.5.3. Mechanical Properties Testing

Tensile tests were conducted to evaluate the mechanical properties of the wet-spun PCL and PCL/CNC filaments. Testing was performed using a Hounsfield Tinius Olsen H100KS machine (Tinius Olsen, Redhill, UK) equipped with a 250 N load cell. Filament samples of 70 mm in length were tested at a strain rate of 25 mm/min following ASTM D5035 [39] guidelines. Each sample was tested in triplicate to ensure reproducibility. Key mechanical parameters, including tensile strength, strain at break, and Young's modulus, were derived from stress–strain curves. The tensile strength corresponds to the maximum stress recorded before the filament fractured. The elongation at break was determined from the total strain at failure. The stress–strain curves were obtained by recording the applied force and the corresponding elongation of the filament until failure. Stress (σ), strain (ϵ), and Young's modulus (E) were calculated using Equations (1)–(3):

$$\sigma = \frac{F}{A} \quad (1)$$

$$\epsilon = \frac{\Delta L}{L_0} \quad (2)$$

$$E = \frac{\Delta \sigma}{\Delta \epsilon} \quad (3)$$

where F is the applied force (N), A is the initial cross-sectional area of the filament (mm^2), ΔL is the change in filament length (mm), L_0 is the initial length of the sample (mm), and $\Delta \sigma$ and $\Delta \epsilon$ are the changes in stress and strain within the linear elastic deformation range.

2.6. Cytotoxicity and Proliferation Evaluation

2.6.1. Samples Preparation

Prior to the assays, the filaments and structures underwent sterilization through a series of washing steps [40]. They were first washed twice with ethanol, followed by two washes with water, two washes with PBS (1×) containing 1% (v/v) penicillin/streptomycin, and two additional washes with water. Each washing step involved 20 min of incubation. After drying, the filaments were sterilized with ultraviolet (UV) radiation for 60 min.

2.6.2. Cell Culture

BJ-5ta cell line (normal human fibroblasts immortalized by overexpression of telomerase) was cultured in four parts of DMEM (powder), containing L-glutamine, D-glucose, sodium bicarbonate, and one part medium 199, supplemented with 5% FBS, 1% (v/v) penicillin/streptomycin solution, and 10 $\mu\text{g/mL}$ of hygromycin.

Cellular subcultures were performed when confluence reached values close to 80–90%. BJ-5ta cells were maintained in 25 cm^2 tissue culture flasks in an incubator at 37 °C in a humidified atmosphere with 5% CO_2 . The cell culture medium was renewed two times per week. For subcultures and plating, the adherent cells were detached with trypsin solution 0.05%, and fresh medium was added in order to neutralize the trypsin. The cell suspension was centrifuged for 5 min at $160 \times g$. The supernatant was discarded, and a fresh medium was added to obtain a new cell suspension. The cell suspension was loaded in a Neubauer chamber, and the concentration of cells was estimated [41].

2.6.3. Cytotoxicity Evaluation

The cytotoxicity of the filaments of PCL with and without CNC was assessed by indirect contact by exposing BJ-5ta cells to conditioned media containing filament film degradation products and leachables.

The filaments were sterilized by UV for 1 h prior to incubation with the culture media. The conditioned media were obtained by incubating the sterilized filaments in 2 mL of medium at 37 °C in a humidified atmosphere of 5% CO₂ for 24 h. Cells were seeded at a density of 10×10^3 cells/100 µL/well on a 96-well tissue culture plate the day before the experiments. The cells were then exposed to the conditioned medium in different percentages (100%, 50%, and 25%) and were further incubated at 37 °C in a humidified atmosphere of 5% CO₂. Cells incubated with dimethyl sulphoxide (DMSO, 30%) were used as control of death, and cells grown with culture medium were used as control of life. At the end of 24 and 48 h of contact, the metabolic viability was assessed by MTS viability assay (Promega, Alexandria, Australia) following the manufacturer's protocol [42]. The reduction in MTS by viable cells was measured with a 96-well plate reader at 570 nm in a microplate reader (Synergy H11, BIOTEK, Winooski, VT, USA). Relative viability was calculated in relation to solvent control and expressed graphically. Each sample was assayed in triplicate.

2.6.4. Cell Proliferation Assays

The ability of filaments composed of PCL and CNC to support cell proliferation was assessed using BJ5-ta fibroblasts and the resazurin-based Xpert Blue Cell Viability Assay (GRISP, Portugal). A cell suspension was prepared at a concentration of 1×10^6 cells/mL, and 5×10^4 cells were seeded onto filaments that had been preconditioned in culture media without FBS for 24 h. The conditioned filaments were placed in a 12-well culture plate, and half of the cells (25 µL of cell suspension) were added to the filaments, followed by a 1 h incubation. Afterward, an additional 25 µL of cell suspension was applied, and the filaments were incubated for 2 more hours [43].

Once seeded, the filaments were gently transferred to new wells, and fresh complete culture media were added. Half of the culture media was refreshed every 48 h. At 24 and 72 h post-seeding, the filaments were moved to new wells, and fresh media containing resazurin were added, in accordance with the manufacturer's instructions. The reduction of resazurin by viable cells was measured using a microplate reader (BioTek Synergy H11, Agilent, Santa Clara, CA, USA) at an excitation/emission (Ex/Em) wavelength of 560/590 nm. Cell proliferation was determined using Equation (4).

$$\frac{\text{Intensity } T_x}{\text{Intensity } T_0} \times 100 - 100, \quad (4)$$

where T_x represents the time points at 48, 72, and 144 h, and T_0 corresponds to the 24 h time point. All samples were prepared in triplicate.

3. Results and Discussion

3.1. Optimization of PCL and PCL/CNC Filaments Produced by Wet-Spinning

The wet-spinning process was optimized by varying key parameters, including polymer concentration, needle diameter, flow rate, and the composition of the coagulation bath, to achieve uniform PCL and PCL/CNC filaments with desirable mechanical properties. Initial tests were conducted with PCL concentrations ranging from 5% to 35% (*w/v*) in different solvents, including CHF, DMF, water, and acetone. Despite testing different solvents, CHF was selected as the optimal solvent due to its superior ability to dissolve PCL and form homogeneous solutions. It was observed that PCL concentrations below 25% were insufficient to produce continuous filaments, while 35% PCL filaments were more irregular, with heterogeneous diameters and distributions. Therefore, concentrations of 25% and 30% PCL yielded more consistent filaments and were used in the condition's optimization.

Flow rates ranging from 1 to 9 mL/h were tested with needles of varying diameters (0.41, 0.61, and 0.84 mm). The optimal needle diameter for 25% PCL was found to be

0.41 mm, while the optimal flow rate was 4 mL/h. Regarding 30% PCL, a needle diameter of 0.61 mm and a flow rate of 6 mL/h resulted in the most uniform filament morphology. These conditions minimized the formation of beads and inconsistencies along the filament length, leading to improved filament homogeneity. The drying process was also evaluated and optimized. It was found that the optimal drying conditions were 30 min at 40 °C.

All solutions were extruded into a MeOH coagulation bath and collected on an automated collector. MeOH was selected as the coagulation bath after testing multiple solvents (water, ethanol), as it provided the most uniform fiber formation. The fast solvent exchange in MeOH allowed for quick polymer solidification, preventing fiber defects and aggregation, which was not achieved with other solvents. The collector was developed and optimized to ensure uniform filament stretching and was based on a structure consisting of a collecting roller positioned below and two adjustable rollers that could be placed at different heights and distances before the collection point (Figure 1a). Moreover, the collecting roller included a motor and speed regulator to maintain constant tension.

The optimization process involved systematically testing different positions of the two adjustable rollers and evaluating their effect on filament stretching. It was observed that the best configuration was achieved by using the two guiding rollers placed in the positions shown on Figure 1a. The developed collector allowed the stretching of PCL filaments, reaching fiber diameters as low as 30 µm. For 25% PCL, a filament diameter of 229.44 ± 7.30 µm was obtained without stretching. With the use of the automated collector, this value decreased to 42.03 ± 3.19 µm, proving the importance of filament stretching. Regarding 30% PCL, diameters of 255.42 ± 7.38 µm and 29.79 ± 1.24 µm were obtained for the filaments without and with stretching, respectively.

The incorporation of CNC was performed on the 25 and 30% (*w/v*) optimized PCL solution. A detailed analysis of the optimization of the mixture between the components under study was conducted, as well as the effect of varying the percentage of CNC (1, 2, and 3% (*w/v*)). The optimized PCL solutions (25 and 30% (*w/v*)) were reinforced with the different percentages of CNC, which were added gradually to prevent agglomeration. The solutions were stirred for 24 h and then wet spun following the same conditions. All filaments were analyzed by microscopic techniques, and the morphology and dimensions of each filament were studied. While pure PCL filaments exhibited a smooth surface, the PCL/CNC filaments demonstrated a more textured morphology due to the presence of CNC. This surface roughness is attributed to the partial exposure of CNC on the filament surface, which can potentially enhance cell attachment in biological applications. The solution parameters, wet-spinning conditions, and the calculated filament diameters are detailed in Table 1.

Table 1. Solution parameters (PCL and CNC percentage), wet-spinning conditions (needle diameter and flow rate), and filament average diameters and standard deviations of the filaments obtained.

Sample	Polymer Percentage (%)	Wet-Spinning Conditions		Mean Diameter (µm)
		Needle Diameter (mm)	Flow Rate (mL/h)	
F1	25% PCL/1% CNC	0.41	4	50.53 ± 1.04
F2	25% PCL/2% CNC			52.67 ± 1.09
F3	25% PCL/3% CNC			55.00 ± 1.32
F4	30% PCL/1% CNC	0.61	6	49.88 ± 1.09
F5	30% PCL/2% CNC			50.26 ± 1.35
F6	30% PCL/3% CNC			53.57 ± 1.64

A mechanical analysis for the six filaments was conducted by performing stress–strain curves. For each filament, 10 replicates were tested. All the stress–strain curves showed a configuration similar to the curve representing a normal tendon when subjected to a mechanical tensile test. The values of maximum tension, maximum stress deformation, breaking point, strain, and Young’s modulus were assessed and are detailed in Table 2.

Table 2. Maximum tension, maximum stress deformation, breaking point, strain, and Young’s modulus of the filaments obtained.

Sample	Tensile Strength (MPa)	Maximum Stress Deformation (%)	Breaking Point (MPa)	Strain (%)	Young’s Modulus (GPa)	R ²
F1	70.28	1130.36	69.86	1129.72	0.18	0.95
F2	131.86	773.28	129.12	786.00	0.19	0.92
F3	201.34	750.30	200.87	762.47	0.21	0.92
F4	80.01	957.29	79.26	858.04	0.17	0.96
F5	169.10	947.16	161.40	956.22	0.22	0.92
F6	277.65	904.40	272.68	913.21	0.27	0.95

After a comprehensive analysis of the results obtained, the maximum stress deformation values stand out as they proved to be remarkably high, highlighting one of the most distinctive characteristics of the PCL polymer, i.e., its elongation capacity. However, it was observed that the incorporation of CNC in the formulations led to a reduction in deformation as a result of the greater rigidity provided to the filaments by the presence of CNC. Even so, the filaments maintained substantial deformation values. According to the literature, healthy tendons and ligaments operate under failure loads between 7% and 40% [32]. It was found that all the optimized formulations in this study significantly exceeded these values.

In general, it can be inferred that the addition of CNC to PCL solutions results in an increase in tensile strength and breaking point, leading to the exclusion of formulations with lower percentages of CNC in all optimized solutions. In addition, the values obtained indicate that increasing the percentage of PCL translates into an increase in the tensile strength and breaking point, pointing to a closer approximation to the main objective when combined with a higher percentage of CNC. The results obtained corroborate the idea that CNC plays a significant role in the mechanical reinforcement of filaments.

Young’s modulus values were similar for all the optimized filaments (0.18–0.27 GPa). The values for Young’s modulus found in the literature for the anterior cruciate ligament (ACL), the patellar ligament (PL), and the tendon of the triple semitendinosus muscle (STT) were found to be 0.195, 0.426, and 0.163 GPa, respectively [44]. This corroborates the potential of the produced filaments to replace ACL, with similar values to Young’s modulus. Particularly, the filament produced with 30% (*w/v*) PCL and 3% (*w/v*) CNC shows a Young’s modulus of 0.27 GPa, which represents a 38% increase when compared to the ACL value.

Overall, the optimized conditions for producing uniform and mechanically robust PCL/CNC filaments involved a 30% (*w/v*) PCL concentration with 3% (*w/v*) CNC, a flow rate of 6 mL/h, and a 0.61 mm needle diameter. These conditions provided a balance between filament uniformity and the desired enhancement in mechanical properties for ACL tissue engineering applications.

3.2. Characterization of PCL/CNC Wet-Spun Filaments

The thermogravimetric analysis (TGA) was conducted to assess the thermal stability and degradation behaviour of the PCL/CNC composite filaments compared to pure PCL

and CNC, which is critical for determining their suitability for ACL tissue engineering applications. The ability of the scaffold to maintain structural integrity under physiological and sterilization conditions is vital for its potential use in biomedical applications. A higher degradation temperature indicates better thermal stability, which is advantageous during manufacturing, sterilization, and implantation. In this analysis, the samples were subjected to a controlled increase in temperature until they reached 600 °C, allowing for the observation of their respective degradation profiles. Figure 2a shows the TGA curves of the samples in question, highlighting the loss of mass as a function of the increase in temperature. Additionally, Figure 2b shows the first-order derivatives of the TGA curves (DTG), which allow a more precise assessment of the degradation stages. The TGA and DTG curves of PCL reveal that this polymer undergoes degradation in a single stage, occurring between 350 °C and 450 °C, resulting in a mass loss of approximately 96%. It is also worth noting that the maximum degradation temperature of PCL is reached at 411 °C, which is consistent with the literature [45]. In the case of CNC, degradation occurs between 250 °C and 380 °C, with a mass loss of approximately 90%, reaching the maximum degradation temperature at 308 °C. Regarding the 30% PCL/3% CNC filament, the TGA curve shows a major degradation process around 400 °C. The degradation of CNC at a lower temperature range (300–350 °C) introduces a secondary degradation peak in the PCL/CNC filaments, which confirms the successful incorporation of CNC into the polymer matrix [46]. The increased thermal stability of the PCL/CNC composite around 400 °C suggests improved interaction between PCL and CNC, which may contribute to enhanced mechanical properties observed in other tests.

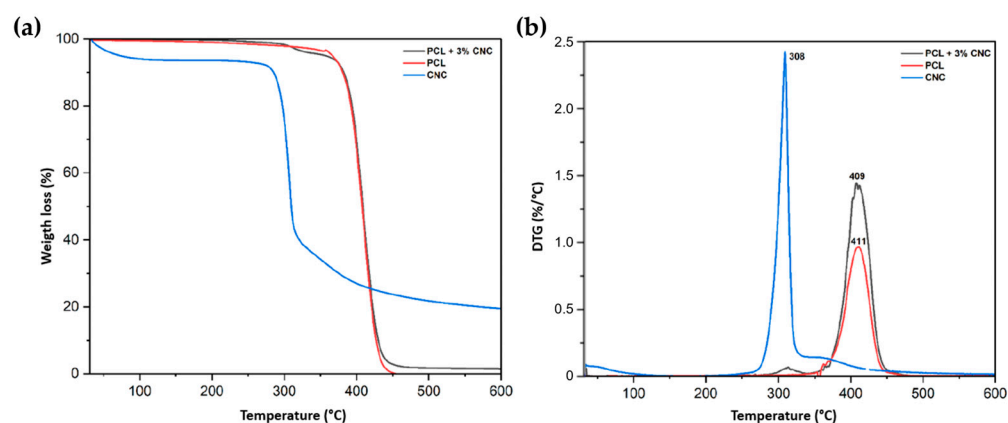


Figure 2. (a) TGA and (b) first-order derivative (DTG) curves of PCL and CNC powder, and 30% PCL/3% CNC filament.

The TGA results demonstrate that the addition of CNC slightly alters the thermal degradation profile of PCL, potentially reinforcing the polymer matrix and enhancing its performance under mechanical stress, as observed in tensile testing. This supports the hypothesis that CNC contributes to the mechanical reinforcement of PCL for ACL repair applications.

Field Emission Scanning Electron Microscopy (FESEM) was used to assess the surface morphology and cross-sectional structure of the PCL and PCL/CNC filaments. The analysis provided insights into the uniformity, surface texture, and integration of CNC within the polymer matrix. Figure 3a,b shows FESEM images of PCL and PCL/CNC filaments. FESEM images of pure PCL filament revealed a smooth homogeneous surface with uniform diameters, indicative of a well-controlled wet-spinning process. In contrast, the incorporation of CNC into the PCL matrix introduced notable changes in surface morphology.

PCL/CNC filaments displayed a textured surface, with slight roughness attributed to the partial exposure of CNC on the surface.

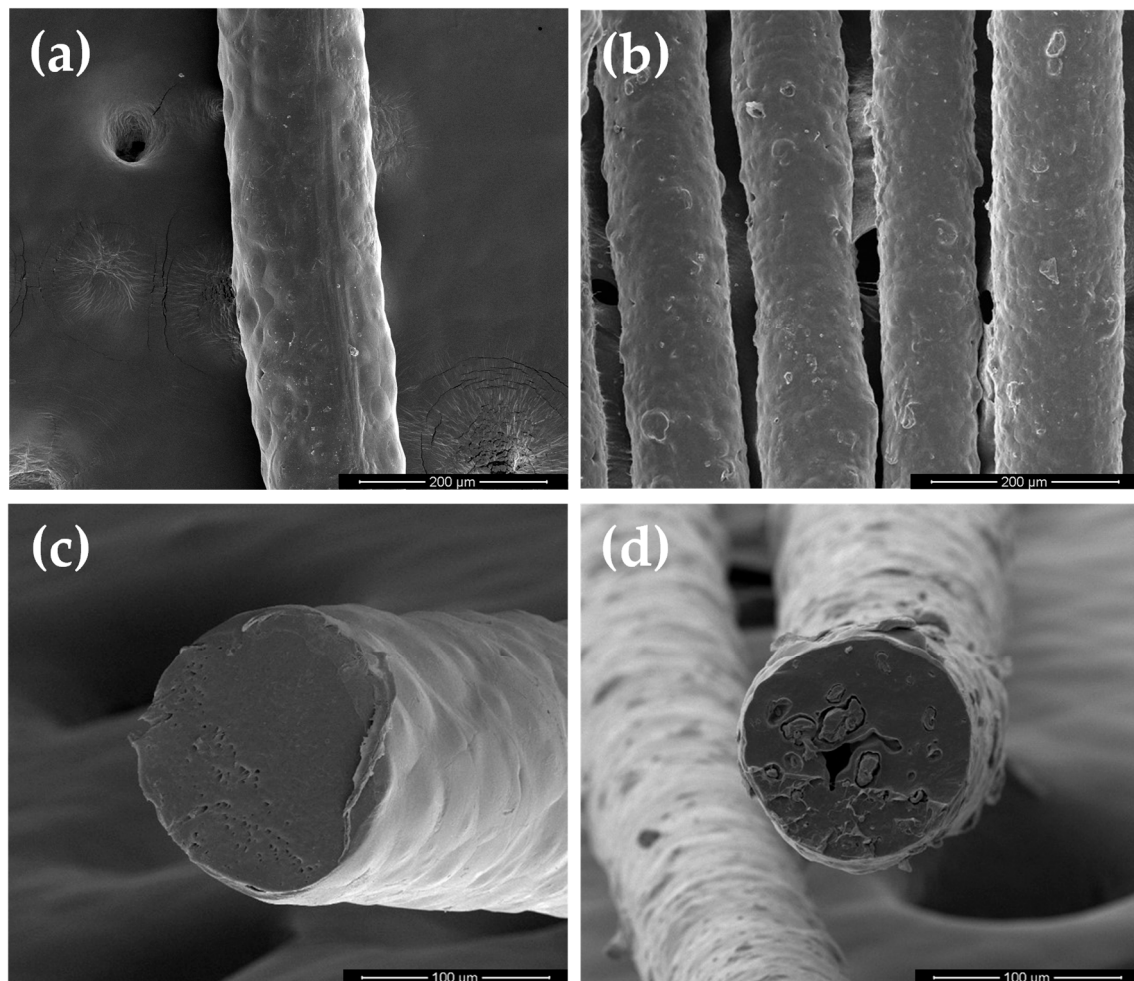


Figure 3. FESEM images at $500\times$ magnification of: (a) PCL filament; (b) PCL/CNC filament; and FESEM images of the cross-section at $1000\times$ magnification of: (c) PCL filament; (d) PCL/CNC filament.

In addition, a comparative analysis of the cross-sections of the filaments was conducted, distinguishing those with and without CNC, as shown in Figure 3c,d. Cross-sectional analysis of the filaments confirmed that the PCL filament exhibited a smooth core structure, while the PCL/CNC filament revealed a more textured core. However, the CNC was well-dispersed within the PCL matrix, with no visible signs of agglomeration. The cross-sections of PCL/CNC filaments displayed a dense core structure, with CNC evenly distributed throughout the polymer matrix. This uniform dispersion of CNC is critical for achieving consistent mechanical reinforcement across the filament [47,48].

Overall, the FESEM analysis demonstrated that the incorporation of CNC into the PCL matrix leads to surface texturing and improved internal structure, which can contribute to enhanced mechanical properties and biocompatibility, which is critical for ACL tissue engineering [32,49]. The surface roughness and textured structure that can be seen on PCL/CNC filament can provide potential sites for enhanced cell attachment and proliferation [50].

Evaluating the potential cytotoxicity of the components that make up a tissue engineering device before it is used in contact with the human body is crucial [18,32]. The device should not display any toxicity towards cells and must not contain toxic degradation

products in order to elicit an appropriate response in a host in a specific application [51]. The cytotoxicity of the 30% PCL filaments with and without 3% CNC was evaluated by exposing the fibroblasts to a culture medium pre-conditioned by contact with the filaments for 24 h.

The results of the indirect contact study after fibroblast incubation for 24 and 48 h with the pre-conditioned culture media are depicted in Figure 4. The data indicated that the addition of CNC did not significantly alter cell viability because the cytotoxicity values obtained for the two types of filaments were similar and did not differ. Moreover, none of the filaments elicits significant cytotoxicity when compared with life control. After 24 h, the cell viability values obtained are all above 90% for PCL filaments and above 91% for PCL/CNC filaments. The cytotoxicity values are even lower after 48 h, with all cell viability values above 95% for PCL filaments and above 99% for PCL/CNC filaments. The reduction in cell viability is most likely caused by the presence of methanol used for coagulation purposes or by some residual chloroform in the filaments' solution.

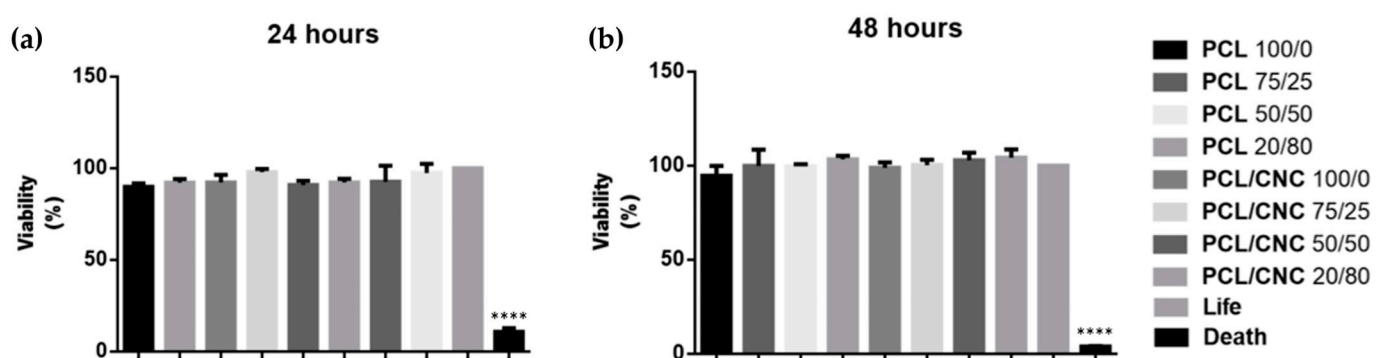


Figure 4. BJ-5ta cell viability was measured by MTS assay at: (a) 24 h and (b) 48 h of culture with undiluted conditioned media and with different dilutions of the conditioned media (1:2–50/50, 1:4–25/75, and 1:5–20/80) preconditioned for 24 h by contact with the PCL and PCL/CNC filaments. The data represent the mean $\pm$ SD of one independent experiment, each with triplicates. Life and death control of the MTS assay at (a) 24 and (b) 48 h of culture. Statistically significant differences were achieved by comparing the results with the control. **** $p \leq 0.0001$ when compared with life control.

3.3. 3D Braided Structures Based on PCL/CNC Wet-Spun Filaments

The organization of the fibrous matrix in tendons and ligaments is crucial for supporting loads and maintaining function. In tissue engineering, creating filament-based structures from biomaterials can mimic this fibrous orientation, aiding in ligament repair. However, a single filament lacks the strength to replicate the natural load-bearing capacity of ligament structures. To address this, a braiding machine was employed to produce more compact and mechanically robust structures from the optimized 30% PCL + 3% CNC filaments.

Initial attempts using a single filament per spool for braiding resulted in frequent breakage, demonstrating that single filaments could not withstand the tension during the process. To improve strength, twisted filament bundles were introduced. After testing configurations with 5 and 8 twisted filaments, the optimal configuration was determined to be 10 twisted filaments per spool. This setup provided sufficient mechanical stability and allowed for continuous production without breakage.

Four different braided structures were produced, each varying in core size to explore their impact on mechanical properties and structure integrity. The structures included a compact design without a core (0 mm core) and three with core diameters of 2 mm, 4 mm, and 6 mm. The use of a core during the braiding process helped maintain the desired structure size, but it was removed after braiding to produce hollow tubular forms. Figure 5

shows the resulting braided structures, labeled as follows: (a) S1—0 mm core, (b) S2—2 mm core, (c) S3—4 mm core, and (d) S4—6 mm core.

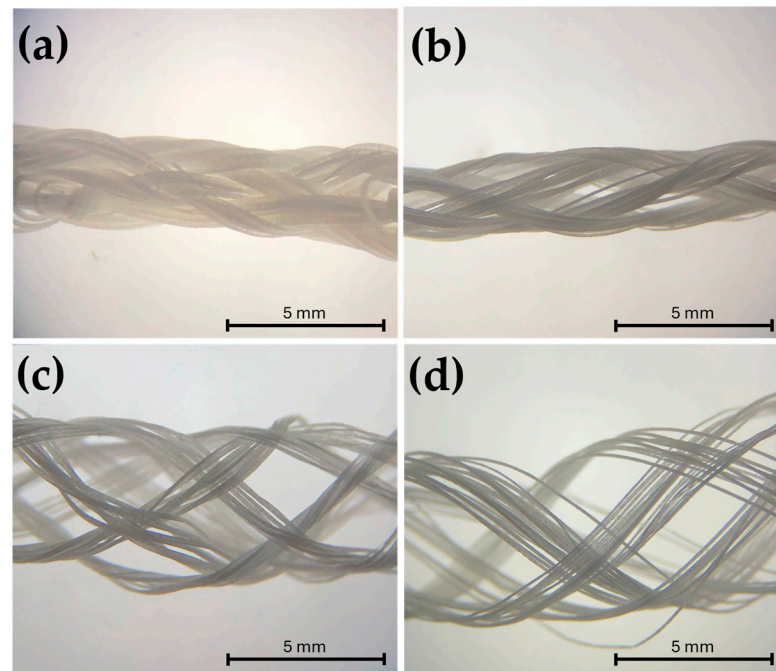


Figure 5. Microscope images of the structures produced with different core sizes: (a) 0 mm; (b) 2 mm; (c) 4 mm; and (d) 6 mm.

To preserve the structural integrity and desired shape, the braided structures were heat-treated at 40 °C for 30 min. This step stabilized the braiding, ensuring that the filaments retained their orientation after the core was removed. Detailed analysis, including microscopy images and measurements of the average diameters with standard deviations, is presented in Figure 5 and Table 3.

Table 3. Average diameter values and the respective standard deviation of the different structures.

Sample	Average Diameter (mm)	Standard Deviation (mm)
(a)	3.394	0.037
(b)	3.805	0.020
(c)	5.992	0.042
(d)	7.162	0.063

Microscopic analysis revealed that larger samples showed wider braid spacing, while smaller samples had more homogeneous structures. The values of average structure diameter were found to be 3.394 mm for S(a), 3.805 for S(b), 5.992 for S(c), and 7.162 for S(d). The natural ACL diameter range is in the same range (4–10 mm) [32,52], which shows the potential application of the four structures on ACL replacement.

For a more detailed analysis of the produced structures, FESEM was used to assess the surface morphology and internal organization of the braided constructs. Figure 6 shows the FESEM images of compare two representative structures: the most compact configuration (S(a)—0 mm core) and the structure with the largest core (S(d)—6 mm core), allowing to evaluate overall filament organization and morphology and for a closer examination of the surface characteristics.

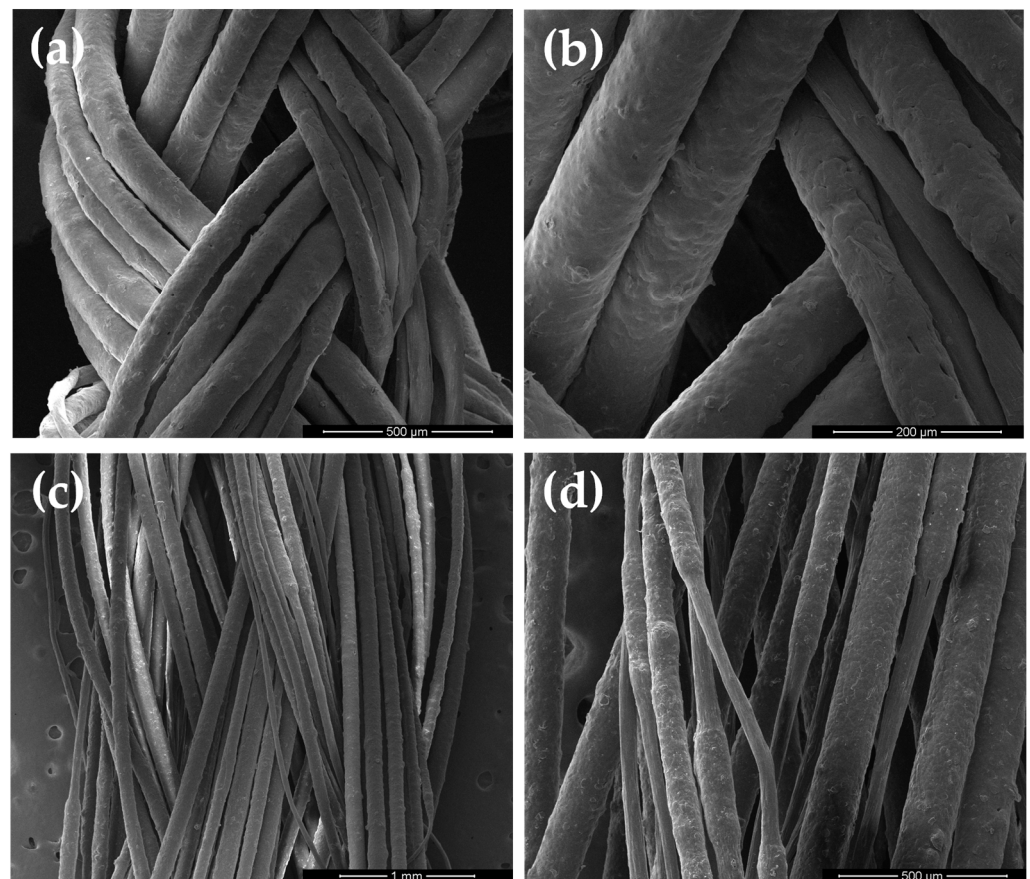


Figure 6. FESEM images of the structure S(a) at: (a) 200 $\times$ magnification; (b) 500 $\times$ magnification and structure S(d) at (c) 80 $\times$ magnification; and (d) 200 $\times$ magnification.

The FESEM analysis revealed notable differences between the two structures. S(a), which was fabricated without a core, exhibited a more uniform arrangement of filaments and a tighter, more cohesive braiding pattern. This uniformity is likely due to the more controlled tension during the braiding process, which was easier to achieve without the use of a core. In contrast, S(d), produced with a 6 mm core, showed a less organized arrangement of filaments with a looser braiding pattern. The larger core size contributed to a less compact overall structure as the filaments were more spread out and less tightly interwoven. The differences in organization and braiding pattern were expected due to the dimensional differences between the two structures.

Cross-sectional analysis, shown in Figure 7, provided further insights into the internal structure of the braided constructs. Both structures displayed compact interior regions, indicating that the braiding process did not compromise the internal morphology of the filaments. The uniform structure observed in both cross-sections suggests that the process of twisting and braiding, even with variations in core size, did not significantly alter the core integrity of the individual filaments.

Overall, the FESEM analysis demonstrated that S(a) achieved a more cohesive and uniform arrangement, making it potentially more suitable for applications where structural integrity and uniformity are critical. A structure such as S(d), while less compact, could be advantageous in scenarios where a larger diameter is necessary, though further optimization in production methods would be needed to improve its uniformity.

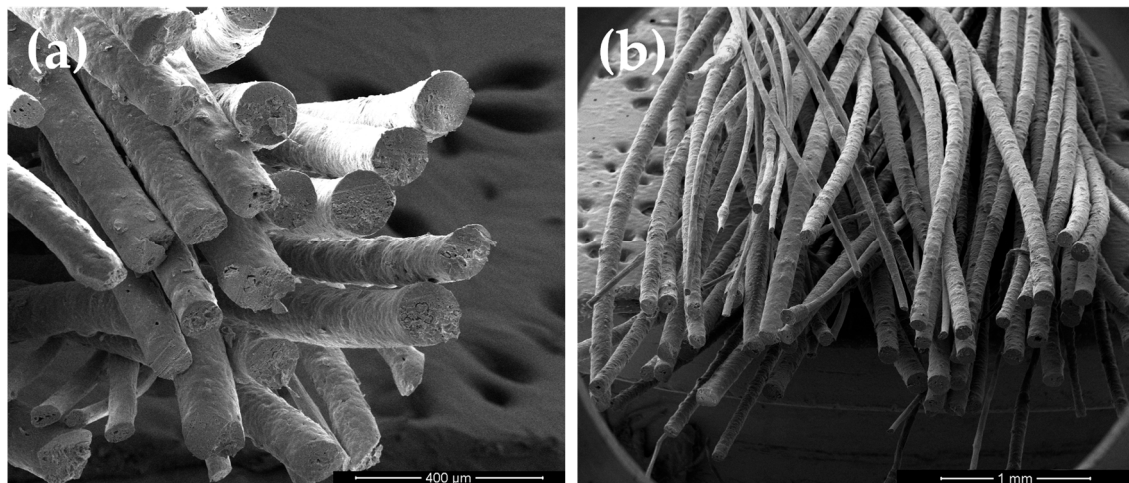


Figure 7. FESEM images of the cross-section of the structures produced: (a) structure S(a) at 250× magnification; (b) structure S(d) at 80× magnification.

To evaluate the ability of PCL/CNC scaffolds to support cell adhesion and proliferation, BJ-5ta foreskin fibroblasts were seeded onto the scaffolds and cultured for 1 and 3 days. Cell proliferation was assessed using the resazurin-based Xpert Blue Cell Viability Assay (GRISP, Portugal), which measures metabolic activity as an indicator of cell proliferation. Scaffolds S(a) and S(b) were evaluated. Table 4 summarizes the proliferation results of BJ-5ta fibroblasts on the PCL/CNC scaffolds after 1 and 3 days.

Table 4. Proliferation results of BJ-5ta fibroblasts on the PCL/CNC scaffolds.

Sample	T0 (Number of Cells 24 h After Seeding)	T2 (72 h)	Proliferation (%)
S(a) (0 mm)	10,194	18,484	81.62 ± 29.06
S(b) (2 mm)	6565	11,918	81.50 ± 12.89

The results showed that both scaffold types supported significant fibroblast growth. After 72 h, the S(a) scaffold exhibited a proliferation rate of $81.62 \pm 29.06\%$, while the S(b) scaffold (2 mm) demonstrated a comparable rate of $81.50 \pm 12.89\%$ despite differences in initial seeding densities. These findings confirm that the PCL/CNC scaffolds provide a favorable environment for fibroblast proliferation, consistent with previous studies emphasizing the biocompatibility of PCL-based materials and the reinforcing effects of CNC [53–55]. Despite structural variations, there were no significant differences in cell numbers between the two scaffold types. Nonetheless, all scaffolds supported cell culture at both time points.

The consistent proliferation rates across scaffold types showcase the versatility of PCL/CNC scaffolds for cell growth. Moreover, these results confirm the findings reported in the literature, indicating that CNC incorporation can enhance the surface properties of PCL, promoting cell adhesion and growth [54].

4. Conclusions

This study successfully developed and characterized PCL-based fibrous scaffolds reinforced with CNC for potential use in ACL repair. The wet-spinning process, combined with a precise control of parameters such as polymer concentration and flow rate, allowed for the production of uniform and mechanically stable filaments. An automated collector was developed and optimized, which allowed for the stretching of the PCL filaments to

diameters as low as 30 μm . The optimized filaments were produced with a 30% (*w/v*) PCL concentration and 3% (*w/v*) CNC, a flow rate of 6 mL/h, and a 0.61 mm needle diameter. The produced filaments showed an average diameter of 53.57 μm and demonstrated good mechanical properties, achieving a balance between tensile strength and flexibility (Young's modulus of 0.27 GPa), making it a suitable candidate for tissue engineering applications. The cytotoxicity results indicated that the 30% PCL/3% CNC formulation did not elicit any adverse effects on fibroblast viability, supporting its potential for safe use in biomedical applications.

The study also explored the development of braided 3D structures from the optimized filaments, aiming to replicate the fibrous organization of natural ligaments. The use of twisted filament bundles and varying core sizes allowed for the creation of structures with different mechanical and dimensional properties without changing the core integrity of the individual filaments. Proliferation tests demonstrated an 81% success rate, indicating a promising level of cellular compatibility and suggesting that the fabricated structures have significant potential for applications in tissue engineering.

The 30% PCL/3% CNC filaments and their derived braided structures show promise as scaffolds for ACL repair. The combination of PCL, a biodegradable and biocompatible polymer, with CNC, a reinforcing agent with similar properties, enables the development of a fully biodegradable system with enhanced mechanical performance. By employing an optimized wet-spinning process and a braiding technique, this approach integrates ultra-thin filaments into 3D structures with superior tensile strength and Young's modulus, closely matching the mechanical properties of native ACL tissue. Future work will focus on refining the production process for 3D structures, particularly improving the uniformity and mechanical performance of larger-diameter scaffolds and conducting *in vivo* studies to further validate their efficacy for ACL tissue engineering.

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