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Synthesis and characterization of polycaprolactone for anterior cruciate ligament regeneration

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ABSTRACT

Anterior cruciate ligament (ACL) is the most frequently torn ligament in the knee, and complete healing is unlikely due to lack of vascularization. Current approaches for the treatment of ACL injuries include surgical interventions and grafting, however recent reports show that surgeries have 94% recurrency, and that repaired tissues are biomechanically inferior to the native tissue. These necessitate the need for new strategies for scar-free repair/regeneration of ACL injuries.

Polycaprolactone (PCL) is a biodegradable and biocompatible synthetic polymer, which has been widely used in the connective tissue repair/regeneration attempts. Here, we report on the synthesis of PCL via ring opening polymerization using ε-caprolactone as the monomer, and ammonium heptamolybdate as a catalyst. The synthesized PCL was characterized using Fourier Transform Infrared Spectroscopy (FTIR) and Nuclear Magnetic Resonance (NMR) spectroscopy. It was then processed using electrospinning to form nanofiber-based scaffolds. These scaffolds were characterized in terms of surface as well as mechanical properties, and compared to the properties of commercially available PCL, and of native ACL tissue harvested from sheep. In addition, scaffolds fabricated with synthesized PCL were evaluated regarding their cell attachment capacity using human bone marrow mesenchymal stem cells (hBMSCs).

Our findings demonstrated that the synthesized PCL is similar to its commercially available counterpart in terms of surface morphology and mechanical properties. In addition, fibrous scaffolds generated with electrospinning showed weaker mechanical properties visa vis native ACL tissue in terms of ultimate stress, and elastic modulus. Also, the synthesized PCL can accommodate cell attachment when tested with hBMSCs. Putting together, these observations reveal that the PCL synthesized in this study could be a good candidate as a biomaterial for ligament repair or regeneration.

1. Introduction

Anterior cruciate ligament (ACL) is the most commonly injured ligament in the knee due to aging and rigorous sports-related activities [1]. The vast majority of ACL injuries are reported to occur between 15 and 45 years of age [2], with an annual incidence of >200,000 cases, approximately 100,000 of them requiring reconstruction surgeries [2,3].

The ACL is characterized by its poor vascularity (the nutrients to ACL are dominantly supplied by the synovial fluid), which leads to deprived healing outcomes [4]. Also, the surgical procedures often result with inferior biomechanical properties than those of the native tissue [5,6]. Furthermore, ACL injuries are anticipated to contribute to the initiation of osteoarthritis in the long term [7]. Collectively, these imply that new strategies for ACL treatment are in demand, and this study aims at synthesizing a biodegradable and biocompatible polymer, PCL, testing its surface and mechanical properties, and comparing these properties with the native ACL harvested from sheep.

Tissue engineering is a promising approach to generate structures that can mimic the properties of native tissues. Biomaterial classes such as metals, ceramics, polymers as well as composites of these are useful tools for such structures, and polymers serve as excellent candidates in this regard due to their versatility. Investigations show that PCL, a synthetic polymer, is one of the widely used biomaterial for ligament repair and regeneration due to its strength and elasticity, and that scaffolds/grafts fabricated with PCL can provide long-term support for soft tissues, while also serving as substrates for tissue in-growth and remodeling [8]. Furthermore, its enhanced solubility in organic solvents, processability at low temperatures, as well as non-toxic degradation byproducts make PCL an attractive material choice for tissue engineering [9]. PCL is widely synthesized with enzyme-catalyzed ring opening polymerizations. [10] However, reports demonstrate that scaling up the enzyme-catalyzed polyester synthesis still remains as a challenge. Particularly, enzymes, when used in nonaqueous environment, exhibit relatively low catalytic activities resulting with low reaction rates and

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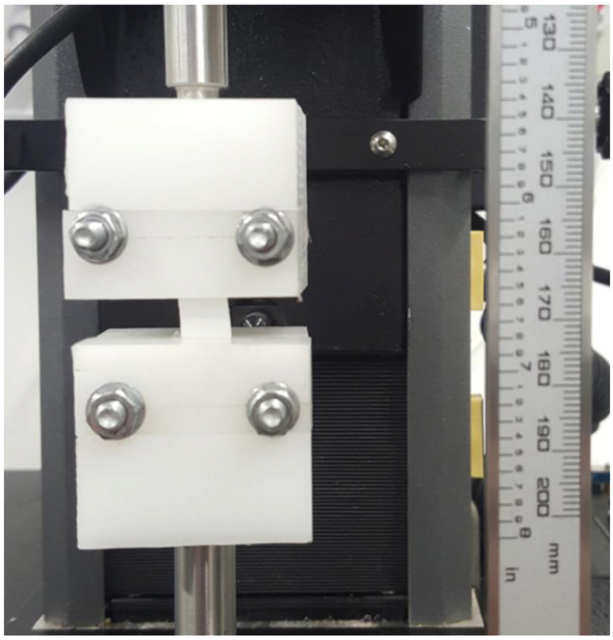

Fig. 1. Custom made setup for testing scaffold mechanical properties.

molecular weights [11] Therefore, alternative approaches for the synthesis of PCL with ring-opening polymerization are under investigation using ammonium heptamolybdate as catalyst [12].

In this study, it is hypothesized that PCL can be synthesized with appropriate chemical structure, and the scaffolds of the same can be produced via electrospinning, with mechanical properties suitable for ACL tissue engineering. In order to test this hypothesis, objectives of this research study are: i) to synthesize a biocompatible material, PCL, via ring opening polymerization of ε-caprolactone using ammonium heptamolybdate as catalyst, ii) to characterize the synthesized PCL with Nuclear Magnetic Resonance (NMR) and Fourier Transform Infrared Spectroscopy (FTIR), iii) to develop a 3D porous PCL scaffold by using electrospinning, iv) to characterize the mechanical properties of both native ACL, and the scaffolds fabricated using synthesized and commercial PCL, and v) to test the biocompatibility of the synthesized PCL scaffolds, partially, via the attachment of hBMSCs.

2. Materials and methods

All materials including ammonium heptamolybdate tetrahydrated (Cat# 09880), anhydrous methanol (Cat# 414816), chloroform (Catg# 34854), ε-caprolactone (Cat# 704067), dichloromethane (DCM, Cat# 676853), NN-dimethyl formamide (DMF, Cat# D158550), and commercial PCL (Cat# 440744) were purchased from Sigma-Aldrich. Heating magnetic stirrer (Cat# F20500011) is a product of Velp Scientifica. All the mechanical tests were performed using Instron (Model 3369). Reflux system is custom made for our study by Labor Ildam Corporation. Vacuum pump (Model: WP6122050) is a product of Millipore. FTIR was purchased from Perkin Elmer (Model Spectrum 100). NMR Spectrometer is a product of Bruker (2′ 300, 400 MHz). The vortex mixer (Model VX14018092) is a product of Fine Vortex Mixer. Syringe pumps (Model NE-300) were from New Era Pump Systems. Scanning electron microscope (Quanta 200) is from FEI. Image J software was available from National Institutes of Health, USA.

Table 1
Major infrared bands of PCL.

Wavenumber (cm^{-1})	Assignment
2949	Asymmetric CH_2 stretching
2865	Symmetric CH_2 stretching
1727	Carbonyl stretching (C═O)
1293	C—O and C—C stretching in the crystalline phase
1240	Asymmetric COC stretching
1190	OC—O stretching
1170	Symmetric COC stretching
1157	C—O and C—C stretching in the amorphous

2.1. PCL synthesis

PCL was synthesized by using ε-caprolactone as the monomer and ammonium heptamolybdate as the catalyst. Ring opening polymerization process was employed based on its efficiency as reported in the literature [12–14]. The molar ratio of monomer:catalyst was chosen as 1:20.000. Briefly, a total of 1.8 mL of ε-caprolactone, 0.001 g ammonium heptamolybdate, and 6 μL distilled water were placed in a 250 mL round bottom flask, which was set up in a reflux system, and processed for 2 h at 155 °C under stirring at 250 rpm.

After 2 h of mixing, the mixture was cooled down to room temperature, chloroform was added into the solution dropwise under stirring at 250 rpm, and stirring continued for 1 h. Then, anhydrous methanol was added to the final solution drop-by-drop using a micropipette. During this process, the polymer precipitated, and was filtered under vacuum approximately 10 times to increase conversion efficiency. The polymer precipitate was placed into a petri dish, and dried in a desiccator overnight.

2.2. Electrospinning of PCL

A solution of PCL was prepared by dissolving 0.66 g of PCL in 1.6 mL of organic solvent mixture composed of DCM and DMF at a ratio of 1:1. The solution was mixed overnight at room temperature, transferred to a syringe attached with needle of ~0.9 mm inner diameter, and pumped at a rate of 0.8 mL/h to form fibrous scaffolds. A potential difference of 11 kV was applied during the process, while the distance between the tip of the needle and the collector was kept at 9 cm. A scaffold thickness of approximately 0.5 mm was formed on the aluminum foil.

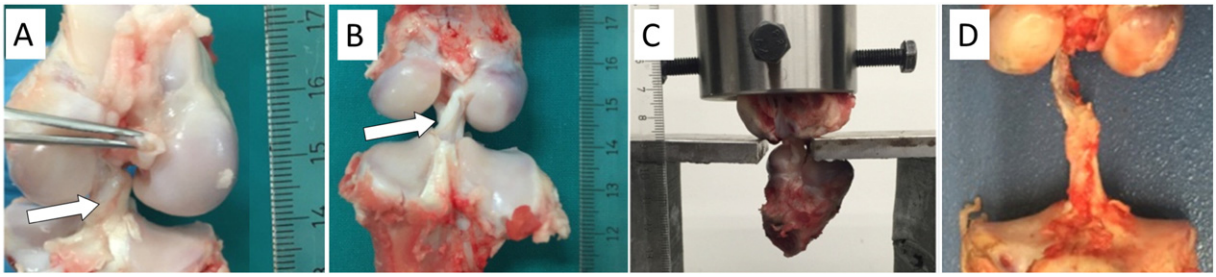

Fig. 2. Anterior (A) and posterior (B) views of ACL tissue, test setup (C), and ruptured ACL (D). Arrows indicate the ACL in the knee (scale unit is cm).

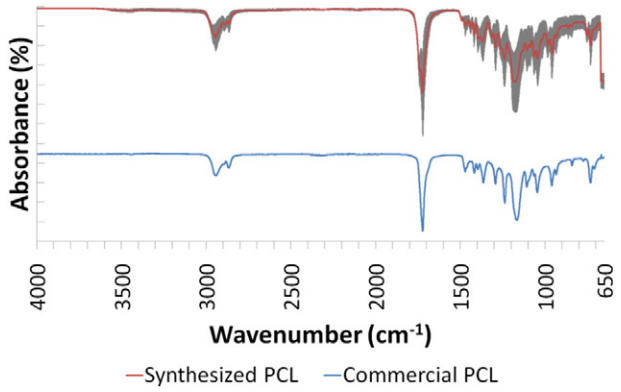

Fig. 3. FTIR Spectrum of synthesized PCL and commercial PCL (shaded area for synthesized PCL depicts standard deviation error bars).

2.3. FTIR and NMR characterization

2.3.1. FTIR

In order to define chemical structure and composition of synthesized PCL, and commercial PCL samples, FTIR Spectrometer was used. Spectra of all samples were recorded in wavenumber range from 4000 to 650 cm^{-1}. The samples were dried overnight in desiccator prior to measurements.

2.3.2. NMR

Molecular structure of synthesized PCL was determined by using Bruker NMR Spectrometer at 300 MHz. 1H spectra was obtained with respect to tetramethlylsilane (TMS) as an internal standard. In the analysis, polymer was dissolved in chloroform-d ($CDCl_3$).

2.4. Mechanical tests

Mechanical properties of the electrospun fibrous scaffolds were determined using Instron. The electrospun scaffolds were tailored to rectangular-shaped specimens (L × W × H, 2 mm × 1 mm × 0.5 mm, n = 3). A uniaxial material testing machine equipped with a 2 kN load cell was run at a constant cross-head speed of 5 mm/min (Fig. 1).

The ACL tissues were harvested from sheep knee joints (male, mean age of 9 months) obtained from a local abattoir. Biomechanical properties were measured using a uniaxial material testing machine equipped with a 5 kN load cell. Tibia and femur were fixed using custom-made jaws (Fig. 2) and positioned such that the mechanical axis of the ACL was co-linear with the load axis of the testing device. The specimens were stretched at a constant cross-head speed of 5 mm/min.

2.5. Contact angle measurement

Droplets of 5 μL of distilled water were dispensed onto the scaffolds (n = 3), and images were captured within 5 s. Contact angles were then measured using ImageJ software as described elsewhere [15].

2.6. Cell culture

Human mesenchymal stem cells were provided from Gülhane Military Medical Academy, Ankara, Turkey. Firstly, cells were plated in 75 cm^2 cell culture flasks. Upon confluency, the cells were trypsinized, counted, and approximately 50.000 cells were seeded on each scaffold (n = 3). After 3 days of incubation with fully supplemented medium that contained Dulbecco's modified Eagle's medium plus 10% fetal bovine serum, 1% nonessential amino acids, 1% penicillin/streptomycin, and 1% amphotericin B, the cells were fixed using a mixture of 2% gluteraldehyde and formaldehyde.

2.7. Scanning electron microscopy (SEM)

Scaffolds were fixated in 2% formaldehyde and gluteraldehyde solution, and treated with serial ethanol solutions (50%, 75%, 99%) three times. The specimens were then coated with gold (5 nm thickness) by sputtering and observed under scanning electron microscopy.

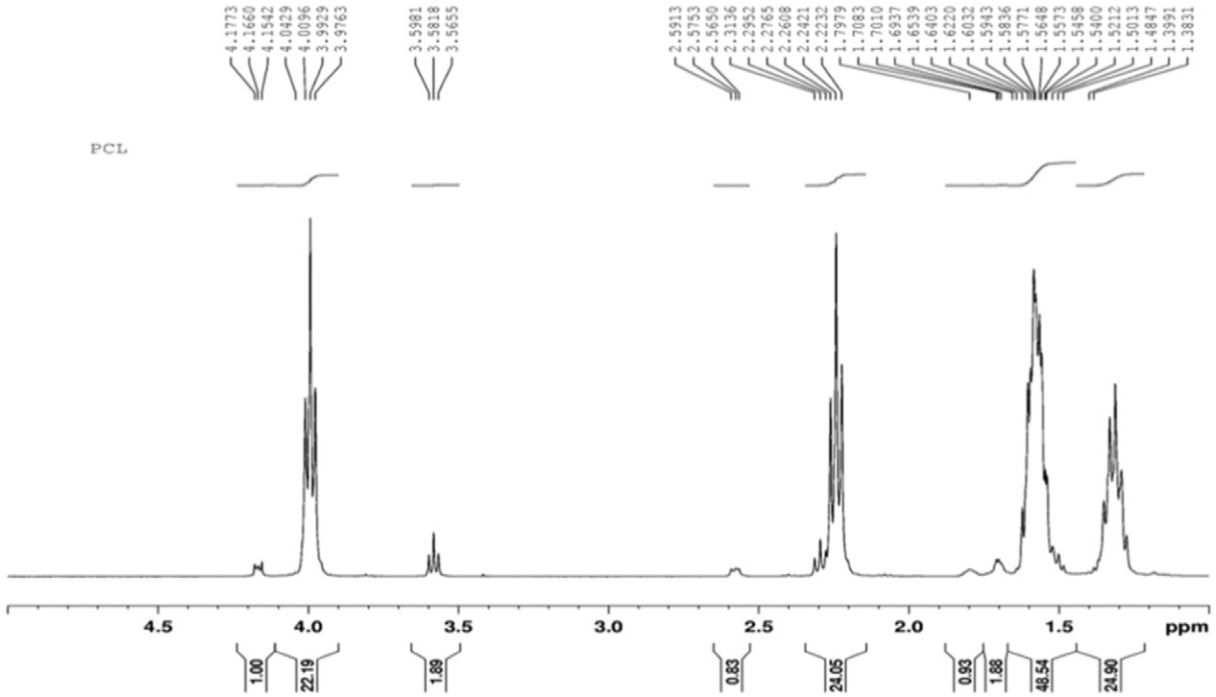

Fig. 4. NMR spectra for synthesized PCL.

Table 2
Mechanical properties of scaffolds and the sheep ligament tissue.

	Thickness (mm)	Width (mm)	Ultimate stress (MPa)	Max load (N)	Elastic modulus (MPa)	Linear stiffness (N/mm)	Ultimate strain (%)
Sigma PCL	0.26	10.01	1.43	3.58	0.005	0.20	259.07
STD	0.03	0.37	0.64	1.30	0.001	0.07	26.17
Synthesized PCL	0.18	13.30	0.38	0.89	0.012	0.95	62.57
STD	0.06	1.09	0.07	0.41	0.002	0.07	29.12
ACL	2.42	6.36	35.71	548.78	2.288	144.97	46.48
STD	0.00	0.40	2.34	41.44	0.568	35.34	14.09

2.8. Statistical analysis

Mechanical properties of the scaffolds and the native ACL were analyzed by using One-Way ANOVA followed by Tukey analysis for differences between the groups, and the contact angles were compared using student's *t*-test. The p levels at which differences between groups are considered statistically significant is taken as 0.05 or less.

3. Results and discussion

3.1. FTIR characterization

Characteristic infrared bands of PCL were defined in Table 1. In this table, the band corresponding to 1727 cm^{-1} is the major transmission peak of PCL, which belongs to carbonyl stretching (C═O) [16,17].

FTIR spectra of synthesized and commercial PCL are seen in Fig. 3. Characteristic infrared bands of PCL at 2943, 2865, 1727, 1292, 1237, and 1167 cm^{-1}, wavelengths, corresponding to asymmetric CH_2 stretching, symmetric CH_2 stretching, carbonyl stretching (C═O), C—O and C—C stretching in the crystalline phase, asymmetric COC stretching and symmetric COC stretching, respectively, can be seen from spectrum [16,17]. Existence of these peaks in the spectrum for synthesized PCL clearly shows conversion of ε-caprolactone to PCL.

3.2. NMR characterization

NMR spectrum of synthesized PCL is given in Fig. 4. This spectrum clearly indicates that properties of the synthesized polymer were consistent with the data of PCL. Chemical shifts (ppm) of PCL seen in Fig. 6 can be described as:

- 4.1 ppm (ethyl group on the oxygen side of the ester bond),
- 3.9–4.0 ppm (α-helical structure, assigned to the protons of CH_2),
- 3.56–3.59 ppm (the presence of hydroxymethyl end group (CH_2OH)),
- 2.56–2.59 ppm ($3CH_3$),
- 2.3 ppm (CH_2CO),
- 1.5–1.7 ppm ($2CH_2$),
- 1.3 ppm (CH_2 which indicates that structure of the PCL polymer still remains).

This molecular structure is specific to PCL and is also consistent with what is reported in literature [10].

3.3. Mechanical tests

Mechanical properties of scaffolds produced with synthesized and commercial PCL as well as those of the sheep ligament tissue are given in Table 2, and Figs. 5 and 6.

Fig. 5A shows that the ultimate stress for native ACL tissue is significantly higher than that for both scaffolds fabricated from commercial and synthesized PCL ($p < 0.05$). There is no difference between the ultimate stress values of the scaffolds ($p > 0.05$). Similarly, the elastic modulus of native ACL tissue is significantly higher than that of both scaffolds (Fig. 5C, $p < 0.05$), while the elastic modulus of scaffolds are similar ($p > 0.05$). The ultimate strain of the scaffold fabricated from commercial PCL is greater than both ACL tissue as well as scaffold fabricated from synthesized PCL (Fig. 5B, $p < 0.05$). ACL tissue and scaffold produced from synthesized PCL have similar ultimate strain values ($p > 0.05$).

The greater ultimate stress and elastic modulus for native ACL tissue as compared to the scaffolds is an expected finding because the native ACL tissue is cellular and contains extracellular matrix (ECM) components contributing to the mechanical properties of the native tissue. It is anticipated that when cells are seeded on these scaffolds, the ECM components produced by the cells will fill the gaps in the scaffold and enhance mechanical properties of the constructs.

Earlier studies related to mechanical behavior of native ACL tissues generally report results in terms of load and stiffness. Therefore, we also tabulated (Table 2) and plotted (Fig. 6) our results in similar units to better compare our findings with those of previous studies. Our findings reveal that maximum load of native ACL tissue is significantly higher than that of both scaffolds fabricated from commercial and synthesized PCL (Fig. 6A, $p < 0.05$). There is no difference between the maximum load values of the scaffolds ($p > 0.05$). Similarly, the linear

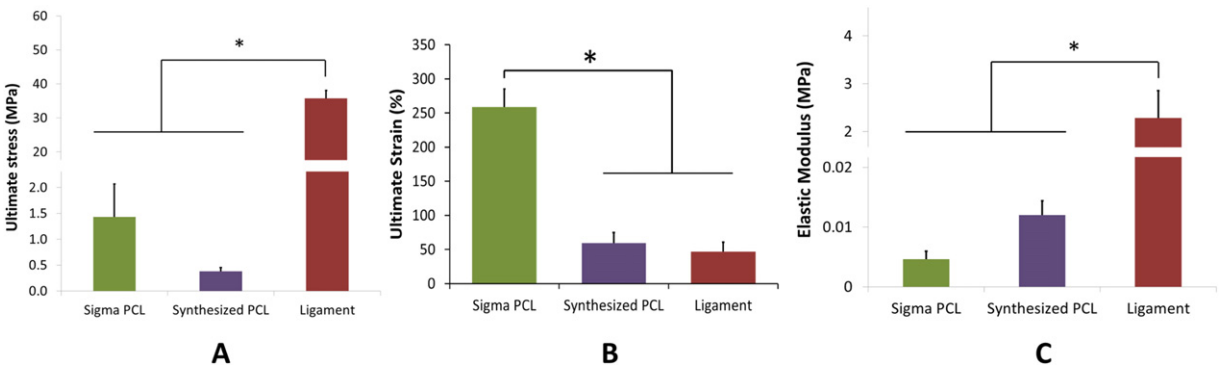

Fig. 5. Ultimate stress (A), ultimate strain (B), and elastic modulus (C) of scaffolds produced with synthesized and commercial PCL as well as native sheep ligament tissue (error bars denote SD, * indicates significant difference at $p < 0.05$).

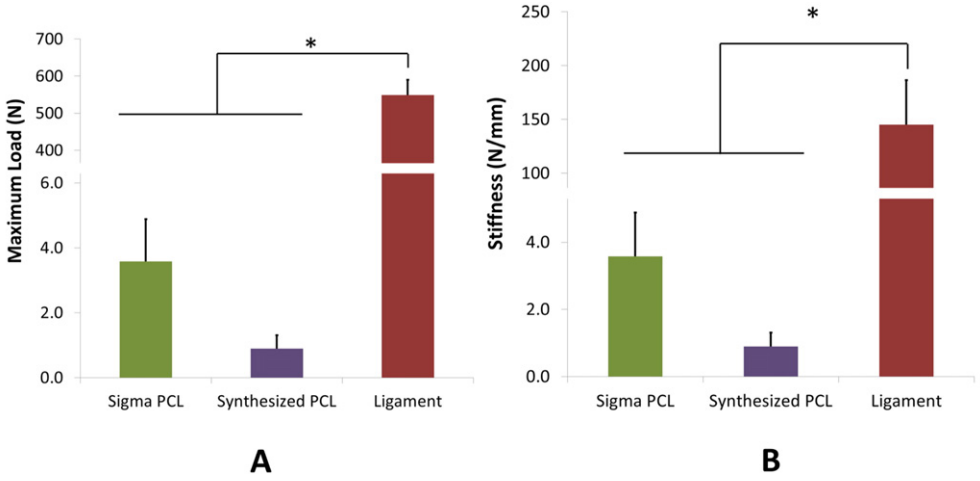

Fig. 6. Maximum load (A), and stiffness (B) of scaffolds produced with synthesized and commercial PCL as well as native sheep ligament tissue (error bars denote SD, * indicates significant difference at $p < 0.05$).

stiffness of native ACL tissue is significantly higher as compared to stiffness values of both scaffolds (Fig. 6B, $p < 0.05$), while the scaffolds are not different from one another ($p > 0.05$).

Mechanical properties of native ACL from sheep as well as from human were previously reported in the literature. For example, Chandrashekar et al. characterized tensile properties of human male and female ACL tissues (mean age of 36.75 years) and determined maximum load and stiffness values of 1818 ± 699 N (male)/1266 ± 527 N (female), and 308 ± 89 N/mm (male)/199 ± 88 N/mm (female), respectively [18]. Similarly, another group of investigators studied mechanical behavior of ACL tissue harvested from male donors with an age range of 22–35 years, and obtained ultimate load and stiffness values of 2160 ± 57 N and 242 ± 28 N/mm, respectively [19]. As for sheep ACL characterization, Kondo et al. report values around 1500 N and 250 N/mm for maximum load and stiffness, respectively [20]. In another study, Seitz et al. reported a tensile strength of around 1400 N, and stiffness of 225 N/mm for female sheep with a mean age of 2 years. [21].

In our study, the maximum load and stiffness values of native ACL tissue harvested from sheep (male, mean age of 9 months) were found to be 548.78 ± 41.44 N and 144.97 ± 35.34 N/mm. These values are lower than load and stiffness values of human ACL tissues reported in literature [18,19]. Our findings are also lower than the values for sheep ACL. However, it should be noted that the donors in our study had a mean age of 9 months as compared to 2 years old donors employed by Seitz et al. [21] It is known that mechanical properties of native tissue vary depending on age [19], so lower mechanical properties obtained in our study for native sheep ACL tissue is an expected outcome.

The scaffolds fabricated from commercial PCL and PCL synthesized in our study did not show any difference in terms of ultimate stress, maximum load, elastic modulus, and linear stiffness, but ultimate strain (Figs. 5, 6). The synthesized PCL and native ACL tissue yielded comparable ultimate strain values (Fig. 6B, $p > 0.05$), which were significantly lower than commercial PCL (Fig. 5B, $p < 0.05$).

Overall, the PCL synthesized in this study exhibited similar mechanical properties with those of commercially available PCL, which is widely used for tissue engineering applications. As compared to native ligament tissue, scaffolds produced from PCL synthesized here demonstrated weaker mechanical properties. Given the similarity between the synthesized and commercial PCL, it would be fair to recommend the synthesized PCL as a scaffolding material. Although, synthesized PCL has lower mechanical properties than native ACL, we believe that it will be a suitable candidate for ligament tissue engineering, considering that mechanical properties will improve upon cellularization.

3.4. Contact angle

In order to evaluate the hydrophilicity of the scaffolds, the water contact angles on the surface of each scaffold were measured by dropping 5 μL water on the surface of scaffolds (Fig. 7).

Contact angles of scaffolds formed from synthesized PCL and commercial PCL were 113.9 ± 1.8° and 121.4 ± 1.0°, respectively (Fig. 7). Obviously, the contact angle of scaffolds fabricated from synthesized PCL is smaller than that from commercial PCL ($p < 0.05$), indicating that the scaffolds obtained from synthesized PCL are more hydrophilic. The results are in agreement with the values (110°–133° based on mean values) reported by others for commercial PCL obtained from different manufacturers [15,22]. Based on contact angles, it is possible to claim that the PCL synthesized in this study could be effectively used as a scaffolding material for tissue engineering applications.

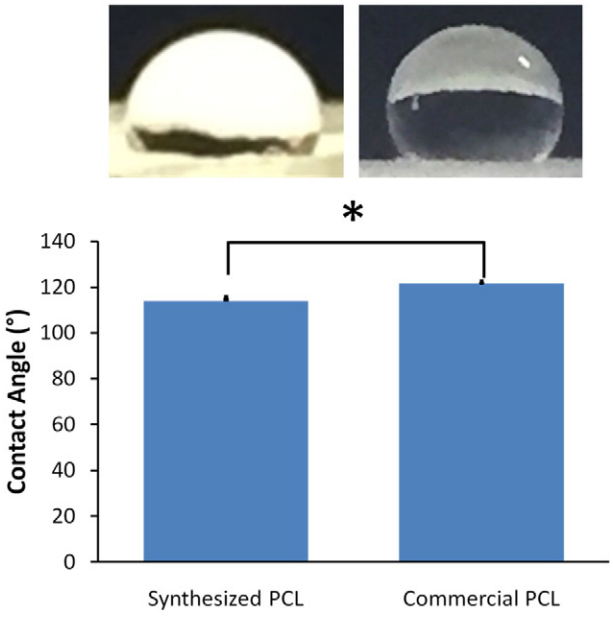

Fig. 7. Contact angles of synthesized and commercial PCL scaffolds.

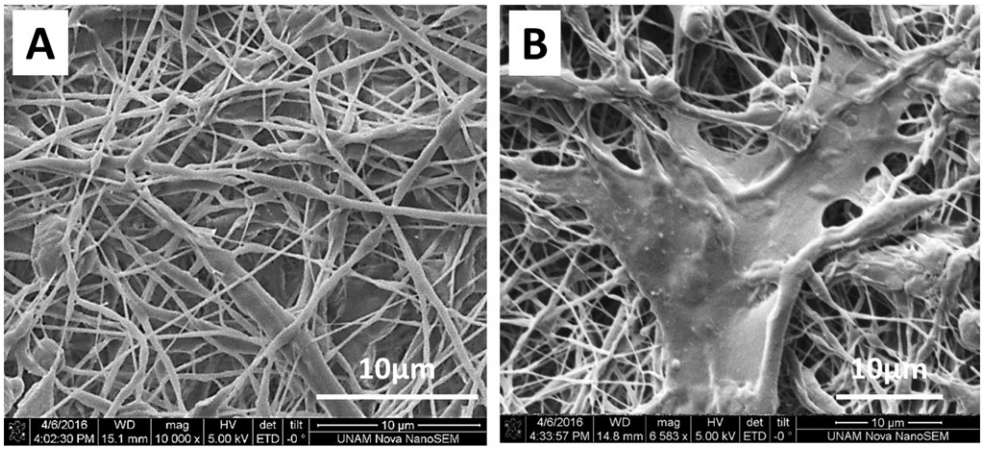

Fig. 8. SEM micrographs of scaffolds produced from synthesized PCL. (A) Cell free, and (B) cellularized scaffolds.

3.5. Assessment of cell attachment and survival

Biocompatibility of the PCL was partially probed by examining the attachment and response of the mesenchymal stem cells (Fig. 8). Attachment of the cells to the fibers was evident within 72 h of seeding. Our observations demonstrated that synthesized PCL scaffold is suitable for mesenchymal stem cell attachment and survival. PCL scaffolds were previously shown to support the attachment, survival, and activity of mesenchymal stem cells derived from bone marrow [23], as well as adipose [24] tissues. Therefore, our synthesized PCL scaffolds could be an appropriate environment for the activity of stem cells towards ligament repair/regeneration as claimed by a recent review for the use of stem cells in tendon/ligament regeneration [25].

4. Conclusions

In this study, we synthesized PCL starting from ε-caprolactone monomer and ammonium heptamolybdate as a catalyst, and investigated mechanical properties of electrospun scaffolds produced from synthesized PCL along with contact angle, and cell attachment and survival. We, then, compared synthesized PCL with a commercial PCL, and native ACL tissue derived from sheep. Overall, scaffolds from synthesized PCL demonstrated similar properties with the commercial PCL. Both fibrous scaffold groups showed inferior mechanical properties to native ACL, with a potential to demonstrate similar characteristics upon cellularization. In addition, human bone marrow derived mesenchymal stem cells adhered and survived on the synthesized PCL scaffolds. Based on our findings, we claim that scaffolds produced from synthesized PCL are good graft material candidates for ligament tissue engineering, but further studies are needed to demonstrate their full capacity to be employed as scaffolds/grafts for ligament regeneration. In the future, we plan to analyze ligament related ECM synthesis, and gene expressions by the cells both quantitatively and qualitatively.

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