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Composite of PLA-PCL-PMMA-Collagen Scaffold for Meniscus

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Abstract

Meniscus injury is one of the most frequently treated injuries in the world's orthopedic surgery, with an incidence rate (IR) per 1000 people in one year reaches 8,27. Meniscectomy of meniscus and allograft implantation are the most common methods of treatment for meniscus injury. However, meniscectomy causes osteoarthritis, whereas allograft implantation has the potential to transmit disease. Tissue engineering is considered as one of the effective methods of dealing with meniscus injuries. This study is aimed to synthesize scaffold for meniscus with lower toxicity due to chloroform methanol usage. Synthesis of fiber scaffold from PLA-PCLPMMA-Collagen was carried out using a set of electrospinning instruments with voltage 18 kV, flow rate of 0.3 mL/h, and the distance of the syringe to the collector (aluminum foil coated) of 15 cm. Subsequently, scaffold characterization such as Scanning Electron Microscope (SEM), Fourier Transform Infra-Red (FTIR), Degradation Test and Tensile Test were performed. Based on the characteristic results, FTIR shows C-H stretch, C=O stretch, C-H bend, C-O stretch, O-H stretch, and C-O-C stretch with the exception of PLA/Collagen (20:80) found CN stretch, higher uniformity and lower diameter, pore size as collagen increases, high tensile strength, even while PLA / Collagen (60:40) and (100: 0) do not show mass degradation. Based on these findings, based on the tensile strength value and radial tensile strength standards, PLA / Collagen (40:60) is the most ideal variation of meniscus scaffold samples that meet the knee meniscus scaffold degradation rate requirements.

Keywords: Meniscus injury; electrospinning; meniscus; scaffold

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1. Introduction

Meniscus is a fibricartilago bearing attached to the medial (inner side) and lateral/intercondylar (outer side), and peripheral tibial plates with shiny white and crescent shaped. The meniscus has an important role to resist pressure by providing a cushion on the femur and tibia, reducing shaking forces, joint stability, and proprioceptive function. Meniscus also susceptible to lesions/injuries. Meniscus injury is one of the most frequently treated injuries in orthopedic surgery worldwide. This is indicated by the number of surgeries in Europe reaching 400,000 and more than 1,000,000 surgeries in the United States to treat meniscus injuries [1]. In previous studies, acute meniscus injuries were recorded at 100,201 with 12,115,606 people at risk of developing meniscus injuries — overall, the incidence rate (IR) per 1000 people in one year reaches 8.27 — with an indication that the incidence rate increases with increasing age, specifically 40 years and over [2]. Surgical procedure of acute meniscal injury is typically performed through meniscus replacement. However, meniscus replacement through allograft implantation poses a risk of disease transmission, donor availability, high costs, difficulty adjusting to size, and decreased biomechanical strength of the implant due to sterilization and preservation [3-5].

In addition, MAT (Meniscal Allograft Transplant) also has the potential to cause a response or rejection of the patient's body's defense system [4]. As solution, the development of tissue engineering was carried out to replace allograft implants by synthesizing artificial meniscus from synthetic polymers such as PLA, PCL, PMMA, and Collagen [6-7]. Although the mechanical strength of PLA is less than ideal, PLA is able to increase the ability of cell proliferation and osteogenic differentiation [8]. PCL has high ductility with a modulus of elasticity of 0.21-0.44 GPa and is a common synthetic polyester as a soft and hard tissue biomaterial with many advantages, for example: good biocompatibility, inexpensive, and easy to undergo processing [9-10]. In other words, PCL is able to improve the mechanical properties of PLA [9]. In addition to mechanical properties, a mixture of PCL and PCA is able to minimize the inflammatory response and local acidification [9].

In addition, PMMA has the advantage of mechanical properties, low toxicity, and can be used in the long run. On the other hand, collagen plays a role in increasing the biodegradability and biocompatibility of the material [11]. The results of the synthesis and characterization of PLA-PCL-PMMA-Collagen fiber scaffold previously shows as an ideal candidate as a knee meniscus scaffold, especially in the variation of Collagen 0.6 gram and PLA 0.4 gram [7]. However, the research conducted still uses hazardous solvents (DMSO) and does not perform FTIR and degradation rates. Based on the discussion, it is necessary to research along with some improvements from previous studies regarding the manufacture of PLA-PCL-PMMA-Collagen fiber scaffold with 0.1g PMMA; PCL 0.3 gram; PLA 1 gram, 0.6g, 0.4g; Collagen of 0g, 0.4g, 0.6g, and 0.8g. This research can observe changes in mechanical strength, biodegradability, and scaffold stability as a function of PLA/Collagen variation.

2. Materials and methods

2.1. Materials

Poly (lactic acid) 2002D (Nature Works), Poly(ε-caprolactone) (Mw = 80,000 g/mol – Sigma Aldrich), poly-methyl methacrylate (Mw = 350,000 g/mol) and collagen type I (BATAN) were used in this work. Chloroform, acetic acid glacial 100%, and methanol were obtained from SAP Chemicals.

2.2. Preparation of Solution

Synthesis of the solution for the electrospinning process is carried out by dissolving PLA, PCL, and PMMA in 10 ml of a chloroform/methanol (3:1) for three hours, respectively. The collagen powder is concurrently dissolved in 10 ml of 80% acetic acid using a magnetic stirrer for three hours. The weight composition of PLA, PCL, and collagen is presented in **Table 1**. Immediately upon dissolving, PLA, PCL, and PMMA solutions are mixed together and stirred for five hours. The mixture of three polymer solutions is put into the collagen solution according to the ratio then stirred for 3 hours. The solution was kept for 48 hours at room temperature until an emulsion was formed. The emulsion is subsequently placed on a 5 mL syringe with a stain-less steel needle of 21G for the electrospinning process [7-11].

2.3. Scaffold manufacture by electrospinning

Synthesis of random fiber scaffold from PLA-PCLPMMA-collagen was performed using a set of electrospinning instruments. The solution was put into a 10 ml syringe. Upon electrospinning, the solution was allowed to stand for several minutes to remove air bubbles that formed during stirring (degassing). The syringe was subsequently mounted on an electrospinning, given a voltage of 18 kV, a flow rate of 0.3 mL/h, and distance of the syringe to the collector of 15 cm. Voltage was applied on needle tip as the anode while the collector was connected to the ground as the cathode.

2.4. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR was used to determine new functional groups that arise through reactions or bonds and processing experienced by PLA-PCL-PMMA-Collagen during synthesis. FTIR test samples were electrospinning samples measuring 10 x 10 mm. The sample was placed on the instrument until a specific absorption spectrum of each functional group was generated. In the FTIR test, there were 4 samples used with different PCL/collagen variations with 3 times repetition on each sample test to ensure validity [12].

2.5. Scanning Electron Microscopy (SEM)

Morphological tests were carried out to determine the surface morphology and porosity of the scaffold fibers formed through the electrospinning process. Morphological tests were performed on each sample (1 × 1 cm) with SEM instrument. Morphological picture of the sample was obtained with an acceleration voltage of 20 kV, a current of 5 μA and magnification of 1000× so that the thickness of the membrane is formed [7]. Furthermore, the image is processed with Image J.

2.6. Tensile test

Tensile strength test was needed to determine the sample ability to hold the force (N) per unit area. Fiber scaffold samples (1 × 5 cm) used amounted to 4 with variations of PCL / Collagen. Each sample had 3 times test repetition. This test produced data in the form of the Ultimate Tensile Strength (UTS) value of each sample with different PLA / Collagen comparisons.

2.7. In Vitro Degradation of Electro-spun Membrane

The degradation test was carried out by measuring and cutting the sample to a same size (1.5 × 0.5 cm), weighing dry weight (Wo), and placed in vial glass that had been given 5 ml Phosphate Buffer Saline (PBS) solution. The samples were then stored in an incubation chamber at 370°C and taken on days 4, 7, 14, and 21, respectively. PBS solutions underwent replacement every 1 week [13]. For weighing, samples that had been soaked over a period of time were removed from vial glass and then rinsed with distilled water and dried.

Dry samples were re-weighed (Wt) to calculate mass decay with equation 2.3. The number of samples that experienced a degradation test were 4 samples with 3 repetitions each [11]. The standard of testing which used in this research is American Society for Testing and Materials (ASTM). ASTM is classification standards provide the requirements for assigning different materials, services, or systems into various groups which relate to the origin, the physical properties, or the chemical properties of the product itself and helps maintain consistency, reliability, and safety in testing procedures. Standard also can facilitate accurate comparisons, ensures compliance with regulations, and promotes advancements in testing methodologies.

3. Results and Discussions

3.1. Fourier Transform Infra-Red (FTIR)

FTIR test was carried out to determine the functional group of the control variable PLA-PCL-PMMA with other variations using the PLA/collagen ratio. The FTIR method used was the attenuated total reflection (ATR). PLA, PMMA, and PCL have common reflections because they are ester group (O = CO), while Collagen has the characteristics of amide (CN) and amine (NH_2) consisting of Uptake of Amides A, Amides B, Amides I, Amides II, and Amides III. The peak C = O stretch also appeared to be shifted in the wave number as PLA composition increased in the biopolymer. Wave number C = O stretch on PLA / Collagen (2:8) showed a shift to a value of 1724.76 cm^{-1} which was close to the wave number value on PCL. The addition of PLA in the sample variation resulted in an in-crease in the value of the wave number C = O stretch from 1751.79 cm^{-1} in PLA / Collagen (4: 6), 1753.26 cm^{-1} in PLA / Collagen (6: 4), up to 1754, 96 cm^{-1} in PLA / Collagen (10:0). Value shift was caused by the formation of hydrogen bonds from the ester group (C = O) with the hydroxyl group PCL and PMMA [14]. Meanwhile, the presence of collagen in the mixture was identified by the presence of Amide III in the range of 1240 cm^{-1}. Meanwhile, amide III, amide II and amide I as typical characteristics of collagen were not seen in the FTIR results of the PLA/PCL/PMMA/Collagen. The absence of amide I, amide II, and less prominent amide III groups might be caused by decreased intensity due to mixing of materials [15].

3.2. SEM of PLA/PCL/PMMA/Collagen Composites

SEM test was conducted to determine the morphological characteristics of PLA/PCL/PMMA/Collagen electrospinning membranes based on surface structure, pore size, and fiber diameter. The magnification used was 1000x. Data presented in **Table 2** showed decreasing fiber diameter as PLA concentration increased. This is made possible by the presence of collagen which increases the viscosity — an increase in the viscosity of the solution has an effect on increasing the continuity of the chain entanglement and has an impact on increasing the diameter of the fiber [16]. Collagen as an emulsifier initiates the emulsification process by viscosity modification — the medium experiences an increase in viscosity, so that deposits occur while maintaining the dispersed phase deposits. Based on the diameter value, PLA/Collagen (10:0) was rated the best compared to other variations because the average fiber diameter is less than 1000 nm [17].

Large diameter of the fiber produced a large pore size as well. This was indicated by observing PLA/Collagen samples (2:8) having the largest pore size as diameter increases. To determine the optimal scaffold pore, consideration of ability in neovascularization and fibroblast growth was carried out. Neovascularization and Fibroblasts are the two important parameters in tissue regeneration. Scaffolds are able to optimize neovascularization to support the need for transportation of oxygen, nutrients, and disposal of metabolic waste to support the continuity of tissue/engineered organ construction [18]. Meanwhile, the growth of fibroblasts is an important process in healing trauma experienced in surrounding tissues by involving proliferation, freezing of fibrin, extracellular matrix formation (collagen, elastin, laminin, and fibronectin) [19-20]. In scaffolds, the optimal pore size for neovascularization is 5 µm and fibroblast growth is 5-15 µm [21]. PLA/Collagen (6:4) pore size of 5.03 µm is identified to be the closest to the standard range of scaffold pore size.

3.3. Tensile Strength of PLA/PCL/PMMA/Collagen Composites

Data obtained from the tensile test were simultaneously analyzed with the thickness of the membrane from the Image J analysis of the SEM results. Measurement of UTS values in scaffold is an important parameter to determine mechanical strength of scaffold when cell proliferation occurs in vitro, in-vivo, and tissue modeling processes. Based on the results of the tensile strength test, the UTS value in PLA/Collagen (60:40) gave the highest value compared to other variations presented in **Table 4**, which is 58,108 MPa. The trend of the value produced seems to increase until it reaches its peak in PLA/Collagen (6:4), then decreases in PLA/Collagen (10:0). Judging from the characteristics of the UTS value, the PLA electrospinning membrane (2.5MPa) is still somewhat greater than Collagen (1.3MPa) [22-23]. Although the UTS PLA value is greater, PLA has poor mechanical and thermal properties as a result of high brittleness and weak crystallization [24-25]. Meanwhile, the magnitude of the degree of elongation from collagen enables the absorption of energy towards the load given to the scaffold [11]. In this case, the addition of Collagen resulted in an increase in the stability of mechanical resistance in the PLA. Microscopically, PLA/Collagen (6:4) has advantages in microscopic characteristics (diameter, arrangement and orientation of fibers) [26]. Judging from the diameter, PLA/Collagen (6: 4) has the lowest value compared to other sample variations that use collagen. Decrease in fiber diameter has an impact on increasing mechanical strength [27]. Through observation of fiber orientation, data that had been analyzed using Orientation J shows PLA / Collagen (8:2) has a random orientation, whereas PLA/Collagen (6:4) has the most uniform orientation compared to other sample variations. Random fiber orientation results in a lack of ability to withstand loads from one direction - in other words, uneven stress distribution [28]. Too high a voltage at a viscosity that is incompatible with the viscosity should be added with a low surface tension of the polymer resulting in an increase in whipping which is able to form small diameter fibers with a low degree of uniformity [29].

The UTS meniscus strength values range from 50-120 MPa [30]. Based on test data, PLA / Collagen samples (6:4) with a UTS value of 58.108 MPa somewhat qualify the mechanical characteristics as a human meniscus scaffold.

3.4. Degradation Rates of PLA/PCL/PMMA/Collagen Composites

The sample used is 1 x 1 cm in size with evaluations carried out at 4, 7, 14, and 21 days through weighing dry weight to determine the difference in mass loss due to degradation. PLA/Collagen (6/4) and PLA/Collagen (10/0) samples did not show mass degradation with respective degradation rates of -5.36×10^{-7} g/d and -1.39×10^{-7} g /h. The PLA/Collagen (4/6) sample had the highest degradation rate value with 1.13×10^{-6} g/h with a degraded mass percentage of 11.95%, followed by the PLA/Collagen (2/8) sample which had a degradation rate value of 1.984×10^{-7} g/h with a degraded mass percentage of 4.11%. From a morphological perspective, degradation occurred in the PLA/Collagen (2:8) and PLA/Collagen (4:6) sample variations due to the large fiber diameter and pore size, as well as the large amount of empty space between the fibers. The looseness of the fibers between each other results in the sample being semi-hydrophilic which allows PBS to enter and cause hydrolysis of the polymer [31]. In addition, collagen, which is amorphous and hydrophilic makes it easier for the scaffold to react with water [32].

In contrast to the PLA/Collagen (6:4) and PLA/Collagen (10:0) samples show that the level of density between the fibers is quite high with a lower pore size compared to other sample variations which inhibits water penetration in the sample. Grumezescu supports this by stating that scaffold with small pore sizes and high porosity experience slower degradation than scaffolds with large pore sizes and low porosity [33]. This is because the small pore size inhibits water penetration to initiate the polymer hydrolysis process [34]. In addition, the acidic environment is not too high (pH PBS = ~7.4) resulting in a slow hydrolysis process of ester groups (C=O) in PMMA. PCL, with its hydrophobic properties due to the presence of $-CH_2$ repeating chains, is also semicrystalline and thus inhibits the penetration of PBS into the bulk polymer [35]. In addition, PLA/Collagen (6/4) and PLA/Collagen (10/0) samples did not show mass degradation with respective degradation rates of -5.36×10^{-7} g/d and -1.39×10^{-7} g /h. The PLA/Collagen (4/6) sample had the highest degradation rate value with 1.13×10^{-6} g/h with a degraded mass percentage of 11.95%, followed by the PLA/Collagen (2/8) sample which had a degradation rate value of 1.984×10^{-7} g/h with a degraded mass percentage of 4.11%. From a morphological perspective, degradation occurred in the PLA/Collagen (2:8) and PLA/Collagen (4:6) sample variations due to the large fiber diameter and pore size, as well as the large amount of empty space between the fibers. The looseness of the fibers between each other results in the sample being semi-hydrophilic which allows PBS to enter and cause hydrolysis of the polymer [31]. In addition, collagen, which is amorphous and hydrophilic makes it easier for the scaffold to react with water [32]. In contrast to the PLA/Collagen (6:4) and PLA/Collagen (10:0) samples show that the level of density between the fibers is quite high with a lower pore size compared to other sample variations which inhibits water penetration in the sample. Grumezescu supports this by stating that scaffold with small pore sizes and high porosity experience slower degradation than scaffolds with large pore sizes and low porosity [33]. This is because the small pore size inhibits water penetration to initiate the polymer hydrolysis process [34]. In addition, the acidic environment is not too high (pH PBS = ~7.4) resulting in a slow hydrolysis process of ester groups (C=O) in PMMA. PCL, with its hydrophobic properties due to the presence of $-CH_2$ repeating chains, is also semicrystalline and thus inhibits the penetration of PBS into the bulk polymer [35]. In addition, collagen as a part of polysaccharides has hydrophilic properties which facilitate the absorption of water in the material [32,36]. The difficulty of hydrolysis of PMMA and PCL due to the hydrophilic nature of collagen allows absorption to occur in the membrane, so that the membrane mass increases in PLA/Collagen (6:4). Apart from collagen, factors that have the potential to increase the final mass can be seen from the sample drying procedure. You et al. carried out a sample drying procedure in an in-vitro degradation test using a vacuum oven at room temperature for 24 hours [37]. It is intended to guarantee that the measured sample does not contain water and affect the results weighing. In a matter of months, the PLA/Collagen (2:8) sample took 17 months to fully degrade, while the PLA/Collagen (4:6) sample only took 5.86 months. Based on Scotti et al. (2013), meniscus tissue begins to show growth and proliferation within three months. However, Albornoz and Forriol (2012) noted that 30% of the strength of healthy meniscus tissue was still not achieved in the strength of the regenerated meniscus after 2-3 months. To date, Collagen Meniscus Implant (CMI) is still the best meniscus scaffold with the characteristics of low immunogenicity and ability to induce tissue, although the estimated time for CMI degradation is still quite long, namely 6–12 months. Based on these considerations, PLA/Collagen (4:6) was identified as having the most optimal degradation rate compared to other samples.

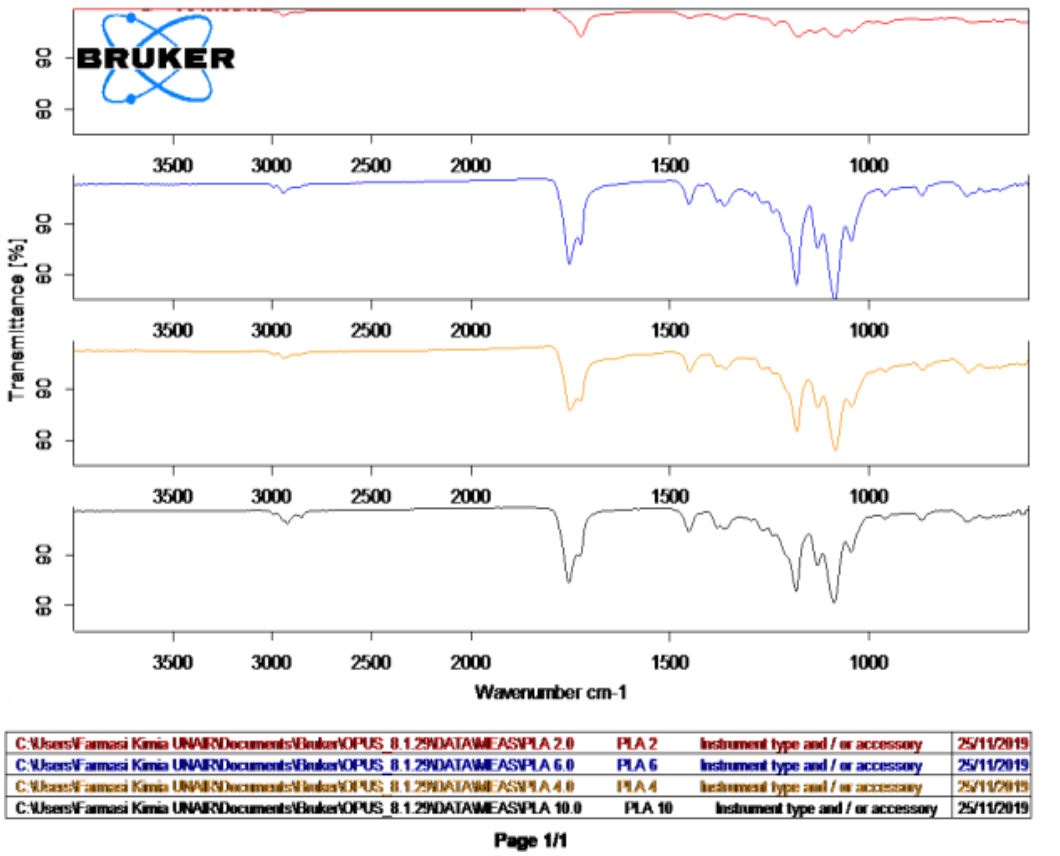

Fig. 1. FTIR of PLA/Collagen (2:8) (a), PLA/Collagen (4:6) (b), PLA/Collagen (6:4) (c), PLA/Collagen (10:0) (d).

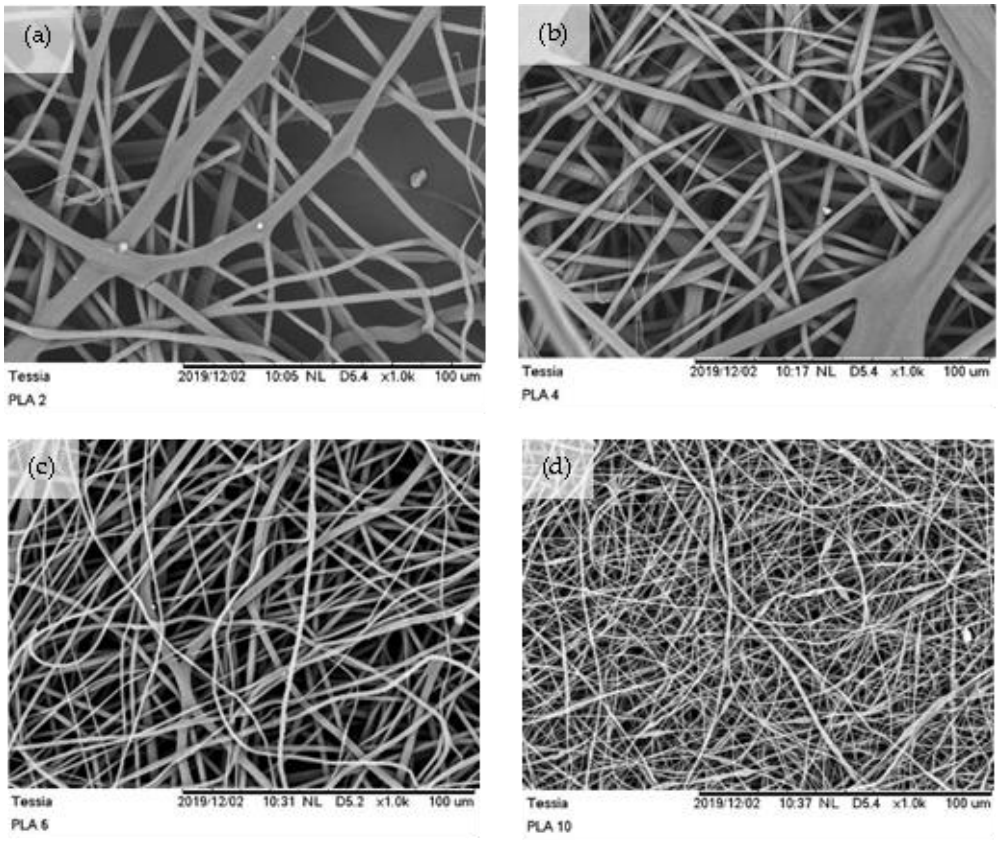

Fig. 2. SEM of (a) PLA/Collagen (2:8) (b) PLA/Collagen (4:6) (c) PLA/Collagen (6:4) (d) PLA/Collagen (10:0).

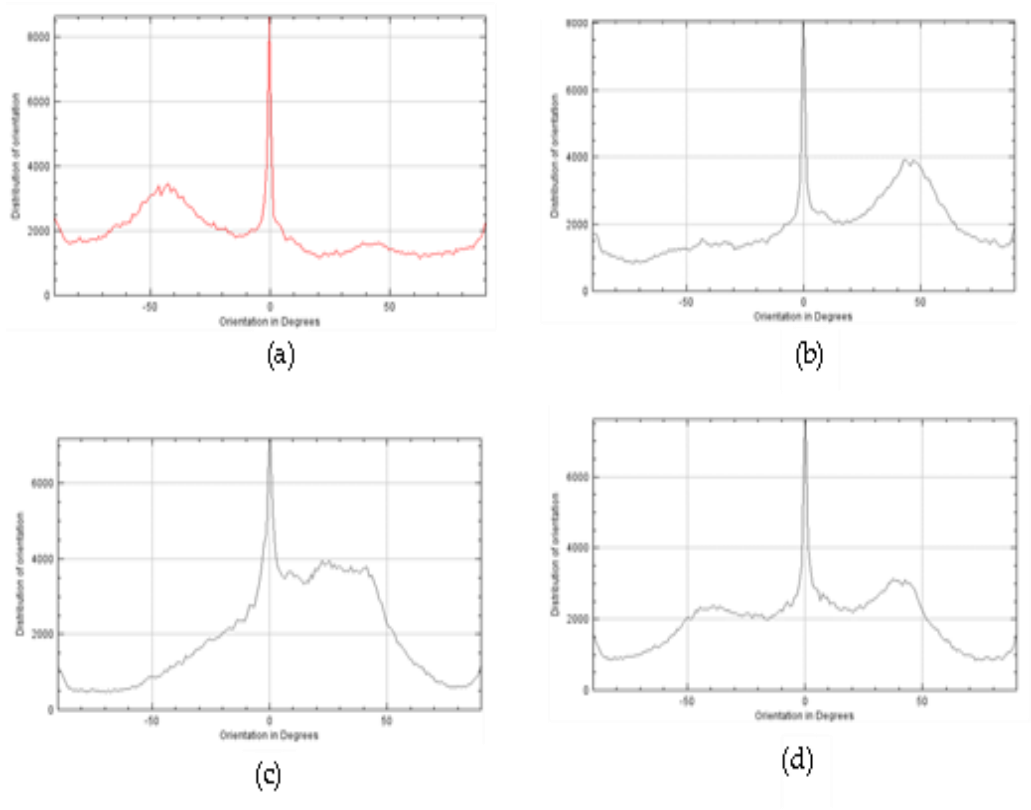

Fig. 3. Fiber Orientations of (a) PLA/Collagen (2:8), (b) PLA/Collagen (4:6), (c) PLA/Collagen (6:4), (d) PLA/Collagen (8:2)

Table 1. Polymer Scaffolds variations.

Sample Variation	PLA	PCL	Collagen	PMMA
A	1 g	0,3 g	0 g	0,1 g
B	0,6 g	0,3 g	0,4 g	0,1 g
C	0,4 g	0,3 g	0,6 g	0,1 g
D	0,2 g	0,3 g	0,8 g	0,1 g

Table 2. Diameter of samples.

Variations	Range of Diameter (nm)	Average of Diameter (nm)
PLA/Collagen (2:8)	1830 – 25700	1586,38 2567,72
PLA/Collagen (4:6)	1510 – 4890	2213,44
PLA/Collagen (6:4)	1279 – 3180	1282,85
PLA/Collagen (10:0)	568 – 1060	466,53

Table 3. Pore sizes.

Variations	Range of Pore Size (µm)	Average of Pore Size (µm)
PLA/Collagen (2:8)	2,8 – 17,2	6,65
PLA/Collagen (4/6)	1,45 – 8,61	3,87
PLA/Collagen (6:4)	2,87 – 7,88	5,03
PLA/Collagen (10:0)	0,764 – 2,58	1,24

Table 4. Tensile Strength of Samples.

Concentration (%)		Force (N)	Thickness (mm)	Area (mm^2)	UTS (MPA)
PLA	Collagen				
20	80	0,5	0,0064	0,064	7,813
40	60	0,9	0,0080	0,080	11,250
60	40	4,3	0,0074	0,074	58,108
100	0	2,1	0,0110	0,110	19

Table 5. Degradation Rates.

Variations	Initial Weight (Day -0) (g)	Average of Initial Weight (g)	Sample Mass in PBS (g)				Average of Terminal Weight (g)
			4 Days	7 Days	14 Days	21 Days	
PLA/Collagen (2/8)	0,0022	0,00243	0,0018	0,0017	0,0017	0,0018	0,00233
	0,0037		0,0034	0,0035	0,0035	0,0035	
	0.0014		0,0014	0,0015	0,0015	0,0017	
PLA/Collagen (4/6)	0,0067	0,00477	0,0069	0,0061	0,0048	0,0052	0,00420
	0,0042		0,0034	0,0041	0,0045	0,0037	
	0,0034		0,0035	0,0035	0,0035	0,0037	
PLA/Collagen (6/4)	0,0017	0,00450	0,0018	0,0018	0,0015	0,0014	0,00477
	0,0043		0,0043	0,0043	0,0044	0,0044	
	0,0075		0,0077	0,0077	0,0095	0,0085	
PLA/Collagen (10/0)	0,0019	0,0018	0,0026	0,0026	0,0019	0,0019	0,00187
	0,0017		0,0017	0,0017	0,0017	0,0019	
	0,0018		0,0016	0,0016	0,0018	0,0018	

4. Conclusions

PLA-PCL-PMMA-Collagen knee meniscus scaffold showed the following proper-ties: the tensile strength value of the sample was within the standard range of tensile strength of the human meniscus, the morphological of the sample can be categorized as nanofiber with optimum porosity as a medium of neovascularization and fibroblast growth with thickness values below the standard thickness of the human meniscus membrane, the FTIR results did not show the presence of the Amide III group, other than PLA/Collagen (2:8), and the result of degradation of PLA / Collagen samples (4:6) was stated to meet the degradation requirements of the meniscus scaffold. Based on the tests conducted, each variation of the sample showed its superiority in each test. However, as a whole, PLA/Collagen (40:60) was the most ideal variation of meniscus scaffold samples in terms of tensile strength values that meet the knee meniscus scaf-fold degradation rate requirements and tensile strength values according to radial ten-sile strength standards, but this variation requires further studies related to morpho-logical characteristics and functional groups.

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