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

Prihartini Widiyanti[1,2,*], Evangelina Tessia Pricilla[1], Gunawan Setia Prihandana[3], Adri Supardi[4]

[1]*Biomedical Engineering Program, Faculty of Science and Technology, Universitas Airlangga, Surabaya 60115, Indonesia*

[2]*Institute of Tropical Disease, Universitas Airlangga, Surabaya 60115, Indonesia*

[3]*Department of Industrial Engineering, Faculty of Advanced Technology and Multidiscipline, Airlangga University, Jl. Dr. Ir. H. Soekarno, Surabaya 60115, Indonesia*

[4]*Department of Physics, Faculty of Science and Technology, Universitas Airlangga, Surabaya 60115, Indone-sia*

**Corresponding Autor's Email: pwidiyanti@fst.unair.ac.id*

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.

INTRODUCTION

Meniscus is a fibricartilago bearing attached to the medial (inner side) and lateral/intercondilar (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 [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 [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 [9] 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 [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 0.1 gram PMMA; PCL 0.3 gram; PLA 1 gram, 0.6 gram, 0.4 gram; Collagen of 0 gram, 0.4 gram, 0.6 gram, and 0.8 gram. This research can observe changes in mechanical strength, biodegradability, and scaffold stability as a function of PLA/Collagen variation

MATERIALS AND METHODS

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.

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 stainless steel needle of 21G for the electrospinning process [7] [11].

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

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.

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].

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 ImageJ.

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.

In Vitro Degradation of Electrospun 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 (W_o), 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 (W_t) 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.

RESULTS AND DISCUSSION

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 (N-H2) consisting of Uptake of Amides A, Amides B, Amides I, Amides II, and Amides III.

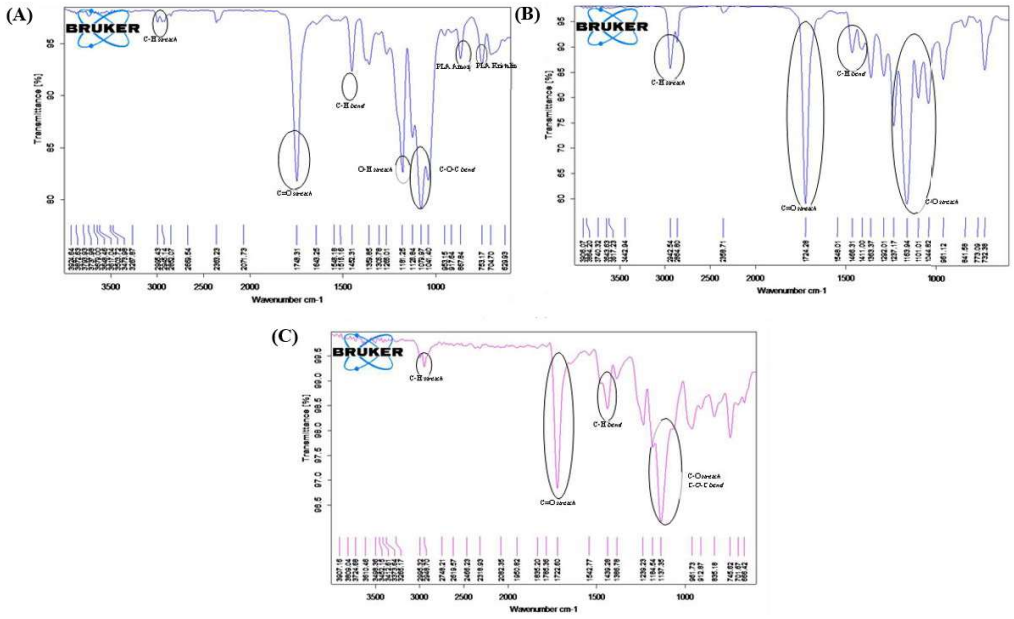

Fig. 1. FTIR of (a) PLA, (b) PCL, (c) PMMA, (d) Collagen

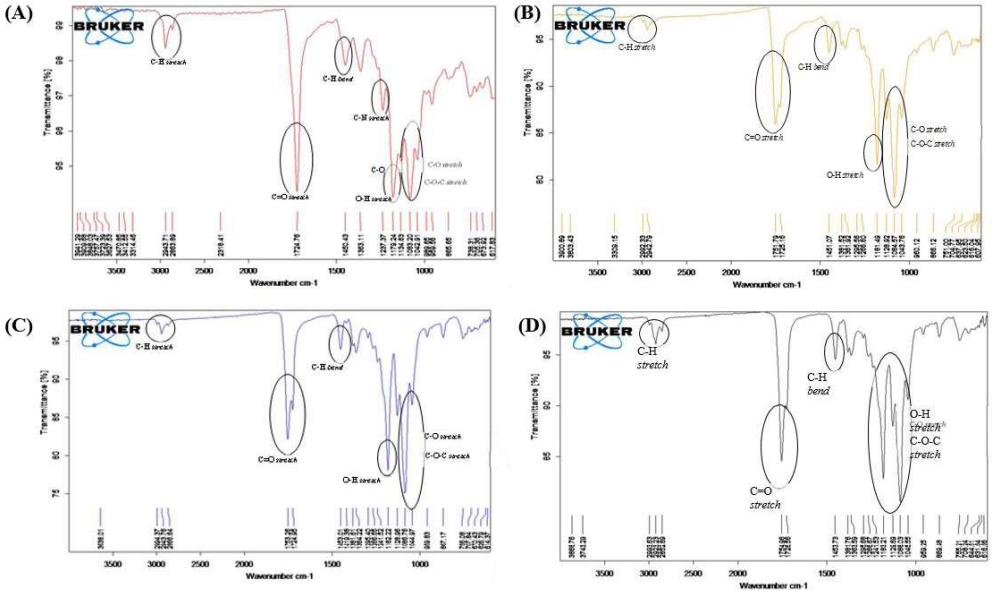

Fig. 2. FTIR of (a) PLA/Collagen (2:8), (b) PLA/Collagen (4:6), (c) PLA/Collagen (6:4), (d) PLA/Collagen (10:0)

The peak C = O stretch in Figure 2 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 / Col-lagen (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 in Figure 2 a. Based on FTIR results, the intensity of amide III was not seen in Figure 2b and 2c. Meanwhile, 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].

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.

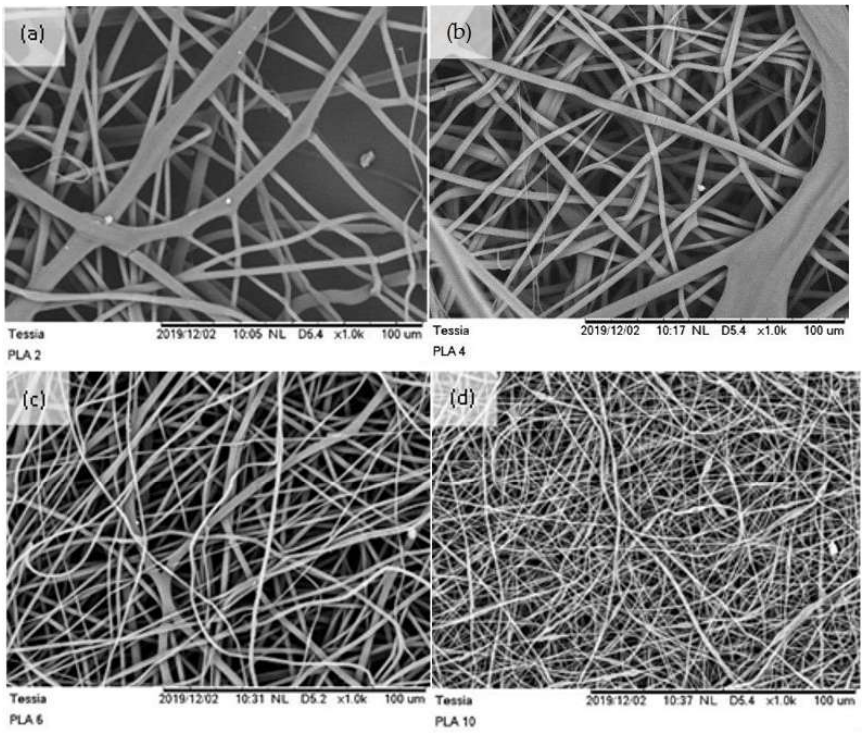

Fig. 3. SEM of (a) PLA/Collagen (2:8) (b) PLA/Collagen (4:6) (c) PLA/Collagen (6:4) (d) PLA/Collagen (10:0)

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

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].

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

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.

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 ImageJ 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.

Table 4. Tensile Strength of Samples

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

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 (2.5 MPa) is still somewhat greater than Collagen (1.3 MPa) [22,23]. Although the UTS PLA value is greater, PLA has poor mechanical and thermal properties [24] as a result of high brittleness and weak crystallization [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].

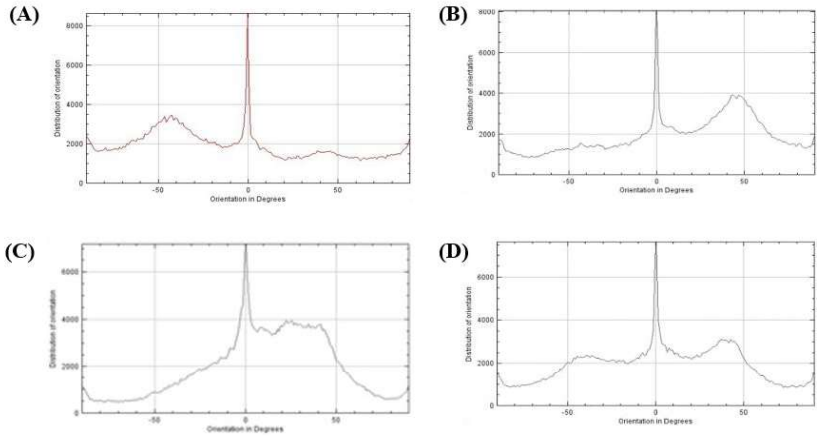

Fig. 4. Fiber Orientations of (a) PLA/Collagen (2:8), (b) PLA/Collagen (4:6), (c) PLA/Collagen (6:4), (d) PLA/Collagen (8:2).

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

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.

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)	0,0042	0,00477	0,0069	0,0061	0,0048	0,0052	0,00420
	0,0034		0,0034	0,0041	0,0045	0,0037	
	0,0043		0,0035	0,0035	0,0035	0,0037	
PLA/Collagen (6/4)	0,0075	0,00450	0,0018	0,0018	0,0015	0,0014	0,00477
	0,0017		0,0043	0,0043	0,0044	0,0044	
	0,0018		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	

Morphologically, the degradation of PLA/Collagen (2:8) and PLA/Collagen (4:6) sample variation was caused by fiber diameter and large pore size, and the amount of empty space between fibers. The loosening of the fiber between one another caused the sample to be semi-hydrophilic which allowed PBS to enter and caused polymer hy-drolysis [31]. In addition, amorphous and hydrophilic collagen made it easy for the scaffold to react with water [11].

In contrast to PLA/Collagen (6:4) and PLA/Collagen (10:0) samples, Figure 3 showed that the density level between fibers was quite high with lower pore sizes compared to other sample variations that inhibited water penetration in the sample. Moreover, the acidic environment which was not too high (pH PBS = ~7.4) yielded in a slow hydrolysis of the ester group (C = O) in PMMA [32]. PCL with its hydrophobic nature due to the presence of a -CH2 repeating chain was also semicrystalline, inhibited the penetration of PBS into the bulk polymer [33]. Another factor was caused by collagen as a hydrophilic polysaccharide which facilitated the absorption of water in the material [11,34].

Timely wise, the PLA / Collagen sample (2:8) took 17 months to fully degrade, while the PLA / Collagen sample (4:6) only took for 5.86 months. Meniscus tissue began to show growth and proliferation in a matter of three months [35]. However, research shows that 30% of the strength of healthy meniscus tissue is still not achieved in the strength of the regenerated meniscus after experiencing it in 2-3 months [36]. Until re-cently, Collagen Meniscus Implant (CMI) is still the best meniscus scaffold with low immunogenicity characteristics and is able to induce tissue, although the estimated time for CMI degradation is still quite long, which is 6-12 months [35]. Based on these con-siderations, PLA / Collagen (4:6) was identified as having the most optimal degradation rate compared to other samples.

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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