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Collagen/PCL Nanofiber Composite for Burn Skin Tissue Engineering

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Abstract

The treatment of burns that have been hampered by limited donors and the high cost of synthesis is answered by the presence of skin tissue engineering. The use of collagen and PCL in skin tissue engineering aims to produce a material that can resemble the properties and functions of genuine human skin. In this study, synthesis of collagen/PCL was done to produce artificial skin tissue using electrospinning. The artificial skin tissue was developed by synthesizing a PCL solution of 20% (w / v) in chloroform and methanol with a ratio of 3: 1, mixed together with a collagen solution with concentration of 8% (w / v) in acetic acid solvent of 80% (v / v) which was then left to stand for 48 hours before the electrospinning process was carried out. There were five variations in the ratio of collagen / PCL volume in this study, namely 20:80, 60:40, 50:50, 60:40, 80:20. The material produced from electrospinning was then characterized using the FTIR test, tensile strength test, surface morphology test, toxicity test, and degradation test. Results of the tests and analysis showed that there were no differences in the functional groups of the five samples formed by the variation of collagen/PCL ratio composition. Differences in volume ratio of collagen and PCL affected the viscosity and conductivity of the solution and thus affected the formed nanofibers and resulted in an increase in the value of Ultimate Tensile Strength (UTS) with an average value that is similar to the original UTS value of human skin. The best results were obtained from the collagen/PCL ratio of 40:60, with fiber size of 861.9 ± 763.7 nm, UTS of 5.82 MPa, live cell percentage of 67.38%. Other than that, results showed that the mass degraded for 16 days is 12.094 (±0.032) %.

Keywords: Burns, Ppolycaprolactone, Collagen, Skin tissue engineering

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

1. Introduction

In 2014 - according to WHO - burns are still among the 7 most common injuries in the world with an estimated death rate of 5% of all injuries. Burns can cause physical complications such as limited joint movement and facial tissue structures deformation. In cases of severe burns, especially in II and III degree burns where damage has reached dermis tissue, burns can damage overall skin tissue resulting in the entry of pathological microorganisms that can disrupt the body's immune system [1]. The development of the skin graft actually existed since the 1870s. In the span of 1910-1960, the skin graft method re-emerged, although there is still much controversy regarding cell viability because skin grafts still originate from the patient's own body (autograft) or a cadaver (homograft). The development of skin grafts continued in the period 1981-1989, with deficiencies such as low mechanical properties, expensive costs, and susceptible to microbial contamination still found [2]. With the advancement in science and technology field, the development of skin graft tissue engineering continues until today.

Collagen is known as the most promising material and has found a variety of applications in tissue engineering because of its excellent biocompatibility and biodegradability [3]. However, collagen has poor mechanical properties so that in its application, collagen is often combined with other ingredients to produce materials with optimal biological and mechanical properties [4]. One of the most used polymers in tissue engineering is polycaprolactone (PCL). PCL has good mechanical properties and easy to fabricate also has an affordable price. In addition, some of the others PCL's properties such as biodegradable, bioresorbable, and also biocompatible made PCL one of the most used synthetic polymers in the tissue engineering field [5]. Advances and developments in scientific technology have had a major influence in the field of tissue engineering, one of them is the presence of nanotechnology. Nanofibers are one of

nanotechnology application which can be used to produce scaffolds made of polymers or natural matrices such as collagen, gelatin, chitosan, etc. [6].

The application of nanofibers with collagen and PCL materials produced using the electrospinning method as a scaffold candidate has been carried out in our previous reference [3]. The difference of this research compared with other research is that this research focuses on volume ratio variation between PCL and Collagen while other research focuses on solvent concentration. The volume ratio exposes several distinct physicochemical properties. The physicochemical characteristic of biomaterials expends major influence over their interaction with cells and hole an important role on the materials' in vivo performance. However, the result still has disadvantages in terms of mechanical strength, which is below the mechanical strength of original skin tissue. In this study, collagen/PCL nanofibers were formed with variations in the composition of collagen and PCL, namely 20:80, 40:60, 50:50, 60:40 and 80:20. By varying the composition ratio of collagen and PCL it is expected to increase the mechanical strength and improve the characteristics of the resulting scaffold.

2. Materials and methods

2.1. Materials

Collagen type I was purchased from National Nuclear Energy Agency of Indonesia (BATAN) (Tangerang, Indonesia). Polycaprolactone (average molecular weight of 80,000 Dalton) was purchased from Sigma-Aldrich. Phosphate-buffered saline (PBS, pH 7,4), acetic acid glacial (100%), chloroform, and methanol were purchase from SAP Chemicals (Surabaya, Indonesia).

2.2. Solution Preparation

Two sets of polymer solution which differed in concentration were prepared. Collagen solution (8%, w/v) was made from collagen type I dissolved in acetic acid (80%, v/v) by stirring the mixture at 300 rpm until the mixture completely blended. In the other hand, PCL (20%) was dissolved in the solvent mixture of chloroform and methanol with ratio 3:1 (v/v) at 300 rpm for 4 h. Both polymer solutions were stirred at room temperature. After both polymer solutions were set, collagen solution was mixed to PCL solution at a various volume ratio, namely 20:80, 40:60, 50:50, 60:40 and 80:20. The solution mixture then was kept for 48 h in order to give time for the collagen to dispersed. After 48 h, an emulsion was obtained and then used for electrospinning process [3].

2.3. Electrospinning Process

Collagen/PCL nanofibers were produced by using electrospinning equipment (Electrospinning HK-7, Genlab) in horizontal mode. In this study, the flat collector (18 x 18 cm) was used to obtained random nanofibers. Before the electrospinning process began, the collector was first wrapped with aluminum foil in order to made it easier to collect the fibers. Collagen/PCL solution was put into the syringe (10 ml) and then installed to the equipment with the distance between the needle and collector was 15 cm. The flow rate was set at 0.3 ml/h and a high voltage which applied to the tip of the needle was fixed at 15 kV. The solution was electro spun for 3 hours at room temperature (260°C) and 48% of humidity [3].

2.4. Characterization of Collagen/PCL Nanofibers

2.4.1. Fourier Transform Infrared (FTIR) Spectroscopy

The functional group in collagen/PCL nanofibers and the interaction between two materials were analyzed by FTIR method using Shimadzu IRTracer-100, which produced waves ranges between 400-4000 cm^{-1}.

2.4.2. Mechanical test

The mechanical test was carried out to measure the mechanical properties of collagen/PCL nanofibers. The dimension of the samples used in this test was 10 mm breadth × 50 mm length, with the thickness were range between 0.17 – 0.98 mm. The maximum load was recorded to be used in further analysis to obtained the Ultimate Tensile Strength (UTS) by the following equation. The materials and methods section should contain sufficient detail so that all procedures can be repeated. It may be divided into headed subsections if several methods are described.

$$\sigma n = \frac{F}{A_1} \quad (1)$$

where σn denotes the value of UTS (Pa), F denotes the maximum load required until the samples reach the break point (N), and A denotes the surface area of the samples (m^2) [7].

2.4.3. Morphological Characterization

The surface morphology of the collagen/PCL nanofibers was studied with scanning electron microscope (Phenom Pro X Desktop), after coating with gold. Further analysis of the images was done using ImageJ software to calculate the mean values of the nanofibers' diameter.

2.4.4. Toxicity Test

The toxicity test was done by MTT assay method. The percentage of cells' viability was used for determining the samples' toxicity. A high number of cell viability percentage means that the samples had low toxicity whereas a low number of cell viability percentage means that samples had high toxicity.

2.4.5. In Vitro Degradability

Collagen/PCL nanofibers were placed in 3 ml of PBS after cut into 5 mm × 10 mm pieces and accurately weighed. The samples were soaked for 4, 8, 12, and 16 days and at each time point samples were dried for 24 h and then weighed again. The weight loss of samples was determined by the following equation:

$$Weight\ loss\ \% = \frac{(W0-Wt)}{W0} x\ 100\ \% \quad (2)$$

where Wo symbolise the weight of the samples at the beginning and Wt symbolize the samples' weight after soaked in PBS [3].

3. Results and Discussions

3.1. The Characterization of Collagen/PCL Nanofiber

3.1.1. Fourier Transform Infrared Spectroscopy

There are four main bands related to the characteristic band of PCL as aliphatic polyester, which was found in PCL nanofibers samples. The four main bands were C=O stretch at 1722 cm^{-1}, symmetric and asymmetric -CH_2 stretch at 2850 cm^{-1} dan 2920 cm^{-1}, and C-O-O vibrations at 1240 cm^{-1} [8]. Meanwhile, from the collagen sample, O-H stretch band was found at 3279 cm^{-1}. The C-N and C-H stretch were also found at 1244 cm^{-1} and 3072 cm^{-1}, indicates the presence of an amide group as the characteristic of the chemical structure of collagen [9]. Figure 1 shows the IR spectra of all the collagen/PCL nanofibers samples, along with the IR spectra of PCL and collagen alone. Characteristic bands of PCL, such as C=O stretch (1726 – 1722 cm^{-1}), C-O-O (1240 -1238 cm^{-1}) and C-O bands (1105-1103 cm^{-1}) were found in all collagen/ PCL nanofibers samples. In the other hand, characteristic of collagen was shown by the C-N stretch at 1163 – 1182 cm^{-1} and also by N-H bend at 1541 cm^{-1}. Based on Figure 1, IR spectra of collagen/PCL nanofibers were shown more similar to the IR spectra of PCL, indicated that characteristic PCL were more dominant than collagen. This due to the solution concentration used in this study, in which PCL solution (20%, w/v) had a higher concentration than collagen solution (8%, w/v). No new bands were found in the IR spectra of all collagen/PCL nanofibers, mean that collagen and PCL were bonded physically, not chemically [9].

3.1.2. Mechanical strength

The ultimate tensile strength values of each collagen/PCL nanofibers were shown in **Figure 2**, ranged from 0.69 – 5.82 MPa. Sample of collagen/PCL nanofiber with volume ratio 40:60 had the highest value, whereas the lowest value shown by collagen/PCL nanofibers with volume ratio 20:80. PCL as a semi-crystalline polymer was known had better mechanical properties than collagen (a natural polymer), so increasing the volume ratio of PCL should improve the ultimate strength of nanofibers [10]. On the contrary, the result in this study shown random value in each sample, with sample in which volume of PCL was the highest had the lowest ultimate tensile strength of all instead. The diverse result occurred due to differences in the physical form of the samples. There are two main factors – the material's thickness and the given load - that could affect the ultimate tensile strength of materials [7]. The difference in thickness caused variance in the surface area of each sample, and thus affect the values of ultimate tensile strength – as given in Table 1. Differences in physical forms could occur because of the electrospinning method used to fabricate samples. There are several factors in the electrospinning process that could affect the thickness of formed nanofibers, one of them was solution viscosity [6]. As a solution viscosity increased, there was a chance that the stream from needle's tip getting hampered, thus made the fibers could not be formed optimally [11]. Although there have been many studies before that stated that high viscosity in solution could complicate the electrospinning process, a solution with low viscosity also could affect the results of nanofibers. When the viscosity was too low, the solution did not have enough time to be polarized so the fibers could not be formed [6,11]. The results suggested that sample of collagen/PCL nanofibers with the volume ratio 40:60 had the most optimal viscosity, so the fibers could form and had the best mechanical strength of all. With the 5.82 MPa, this number was included in the ranges of the ultimate tensile strength of human skin [12]. Hence, collagen/PCL nanofibers with the volume ratio 40:60 could be potential for skin tissue engineering applications for burns.

3.1.3. Morphological Characterization

In this test, only two samples of collagen/PCL nanofibers, which had the lowest and the highest ultimate tensile strength were analyzed. These two samples were collagen/PCL nanofibers with volume ratio 20:80 and 40:60. The surface morphologies of both nanofibers' samples – as shown in Figure 2 – were smooth, cylindrical, and non-porous. From the analysis done with the ImageJ, the diameter of the collagen/PCL nanofibers with volume ratio 20:80 ranges between 2.7 – 9.5 µm, with average number 5.9 ± 2.3 µm. Whereas the diameter of the collagen/PCL nanofibers with volume ratio 40:60 ranges between 167,8 nm – 2, 4 µm with the average number 401.7 ± 192,8 nm. The result also indicated that sample with volume ratio 20:80 had less formed nanofibers than sample with volume ratio 40:60. The diversity in diameter and the number of formed nanofibers were influenced by many factors, one of them was solution viscosity. The presence of PCL with large enough concentration could increase the solution viscosity so that the diameter of formed nanofibers become larger and wider [8]. Higher viscosity was caused by a large number of chemical bonds which increased along with the solution viscosity [11]. Aside from viscosity, the diameter of the formed nanofibers was also affected by the solution's conductivity. Based on the previous experiments done by the others, the smaller amount of collagen in the solution made the diameter of nanofibers greater [3]. Collagen was polyelectrolyte, a linear polymer which had many functional groups in an ionized state. The presence of polyelectrolyte in solution could increase the solution's conductivity, thus made the morphology of formed nanofibers smoother and had smaller diameter [13-14].

3.1.4. Toxicity

Five samples were used in this test using the MTT assay method. The toxicity level was known from the percentage of the cell's viability, in which that high percentage means low toxicity. Samples were toxic if the percentage of cell's viability was below 60% [15]. The result indicated that four out of five samples were not toxic. Sample with 40:60 volume ratio of the collagen/PCL had highest percentage of cell's viability, whereas the lowest percentage was owned by sample with 80:20 volume ratio. Figure 3 showed that from the sample with 40:60 to 80:20 volume ratio of the

collagen/PCL, there was a trend in which the bigger volume ratio of collagen, the lower cell's viability. These results could occur because of the used of acetic acid as collagen's solvent in this study. Acetic acid was included in strong acid group, in which strong acid could cause cells' disturbance and conduct cells' death. Acetic acid could trigger apoptosis, where cells undergo the decreasing of its integrity [16]. However, samples with the lowest volume ratio of collagen (20:80) did not have the highest percentage of cells' viability. This could occur due of several factors when the characterization test was carried out.

The toxicity test using MTT assay was often associated with the number of living cells, although it actually refers to the reduction reaction of tetrazolium dyes that change color when reacting with cell metabolic activity [15]. Reduction levels of tetrazolium dyes could change and also influenced by the physical condition of the culture cell and the number of cells in each well. The ability of cell metabolism also needs to be considered, where there were several culture cells that could metabolize ten times more active than normal condition when reacting with tetrazolium, but there are also cells that undergo decreased metabolism [15].

3.1.5. In Vitro Degradability

The degradation test was carried out for 16 days. The result indicated that sample with 50:50 volume ratio had the slowest degradation rate, $(3.82 \pm 1.90) \times 10^{-6}$ g/hours. The other four samples had a relatively good degradation rate, thus made them suitable for skin tissue engineering for burns. The degradation rate of all samples can be seen in Figure 4. The addition of PCL should decrease the degradation rate of the samples. PCL as a synthetic polymer had a lower degradation rate – 3 up to 4 years to disintegrate - than collagen [17]. In this study, samples with the highest volume ratio of PCL (20:80) had the largest degradation rate instead. This could happen due to the method used in this study [18]. With different methods, different sample shapes will also be produced. Samples made using other methods, such as the separation phase technique, will produce samples in the form of porous scaffolds [17]. In this study, the samples were fabricated by electrospinning method, thus made a difference in the physical form of the samples, which is fiber-shaped (fibrous scaffolds). The difference in structure of the formed fibers caused the degradation mechanism was also different in each sample. Based on the morphology of the formed fibers, samples with 20:80 volume ratio of collagen/PCL had large of a blank area and thus made the samples were hydrophilic. This hydrophilic characteristic promoted the occurrence of mass degradation. Mass degradation also occurred in samples with the highest volume ratio of collagen (80:20), because collagen was hydrophilic natural polymer. However, in the samples with 40:60, 50:50, and 60:40 volume ratio of collagen/PCL, the degradation mechanism which happened was the surface degradation. It developed because of the samples' surface was hydrophilic, as a result due to the existence -CH2 chain in PCL. The semi-crystalline characteristic of PCL could also obstruct the penetration of PBS liquid into the polymer bulk [19]. Degradation was one of the most important factors in tissue engineering. The degradation of the scaffolds should not too fast, so the cells had enough time to undergo the proliferation. In the other hand, slow degradation might also disturb the biological function of the tissue, especially if the degradation of the samples were slower than tissue regeneration itself [20]. Slow degradation could trigger foreign body reaction, and thus developed the undesirable reaction in the human body [21]. Extra Cellular Matrix (ECM) in skin tissue takes approximately 1 month to repair injured tissue. Within 6 weeks, the new skin tissue covering the wound has reached 50% of the strength of the original uninjured tissue and will continue to strengthen over the next 12 months [22]. Overall, the results of the degradation test showed that the five collagen/PCL samples had a degradation rate that was good enough for skin tissue engineering. The five collagen/PCL samples had not completely degraded within 16 days, which means all of the samples could provide enough time for cells to proliferate. The best collagen/PCL nanofiber was 40:60 due to fiber size, tensile strength, biocompatibility status through cell viability percentage and degradation status. All characteristics of collagen/PCL nanofiber with 40:60 volume ratio was in accordance with the standard of biomaterial for skin tissue engineering to support tissue healing.

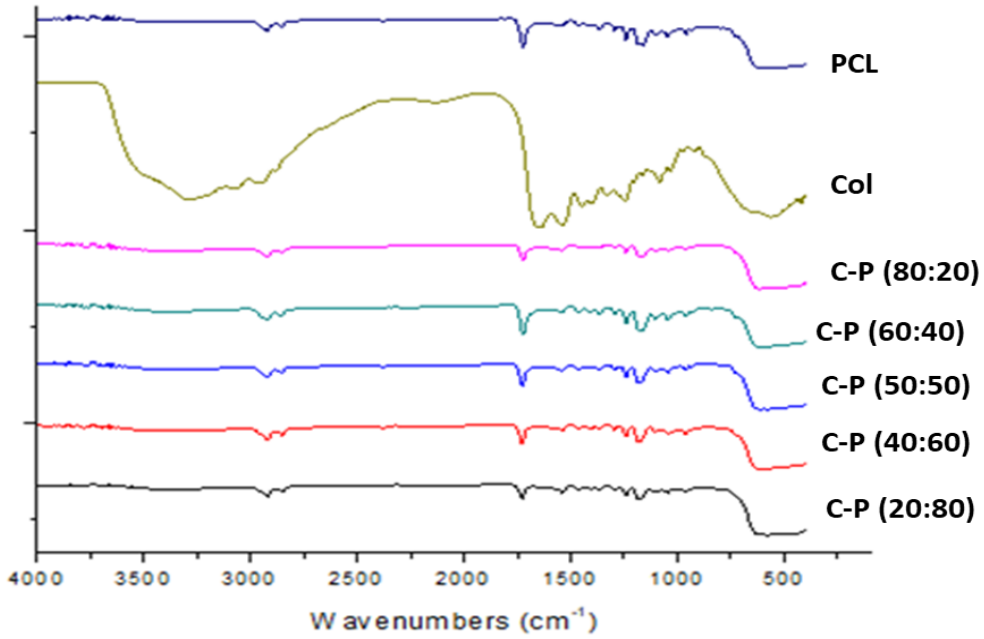
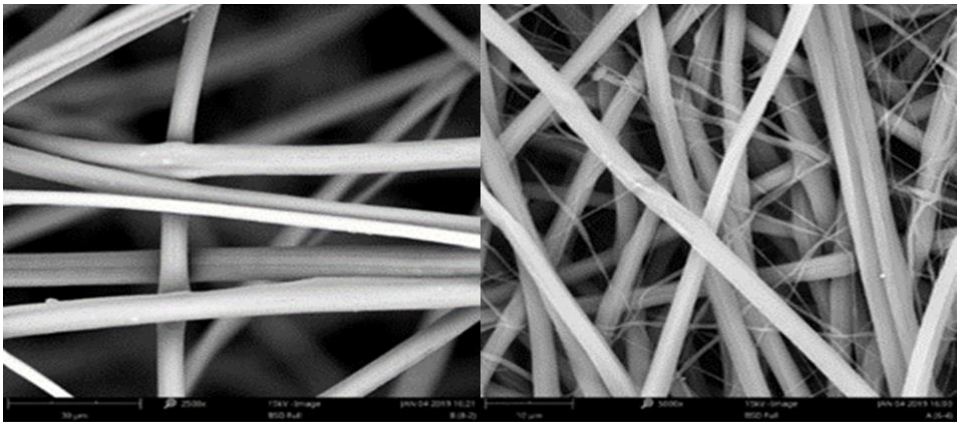


Fig. 1. FTIR spectra of PCL, collagen (Col), and nanofibers collagen/PCL (C-P) with different ratio in volume.

Table 1. Mechanical properties of collagen/PCL nanofibers with different ratio in volume.

Volume Ratio (10 ml)		Load (N)	Thickness (mm)	Surface Area (mm^2)	UTS (MPa)
Col	PCL				
20	80	0.50	0.72	7.2	0.69
40	60	3.90	0.67	6.7	5.82
50	50	3	0.98	9.8	3.06
60	40	3.70	0.80	8	4.63
80	20	0.70	0.17	1.7	4.12



(a) (b)

Fig. 2. SEM micrographs of nanofibers collagen/PCL obtained with different volume ratio: (a) 20:80 and (b) 40:60.

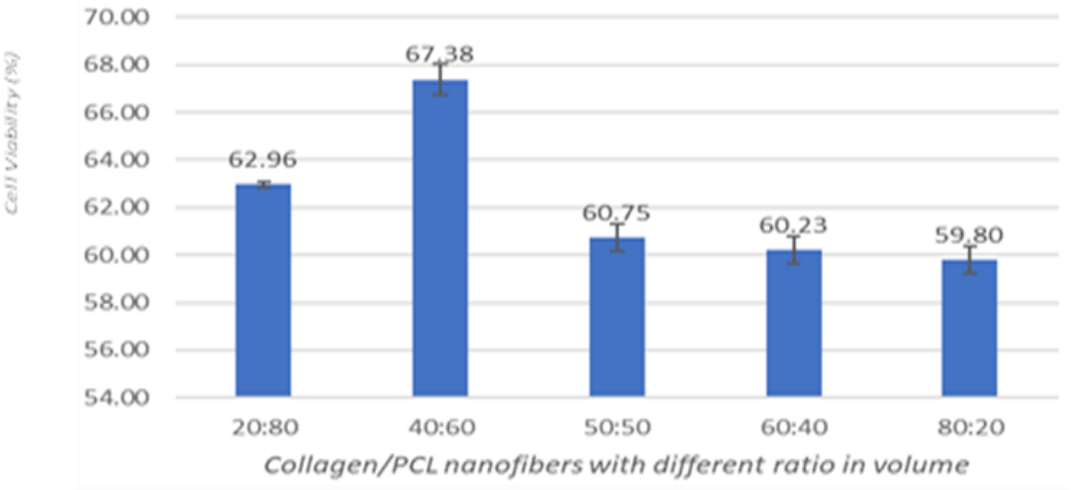


Fig. 3. Cell's viability on nanofibers collagen/PCL with different volume ratio by MTT assay.

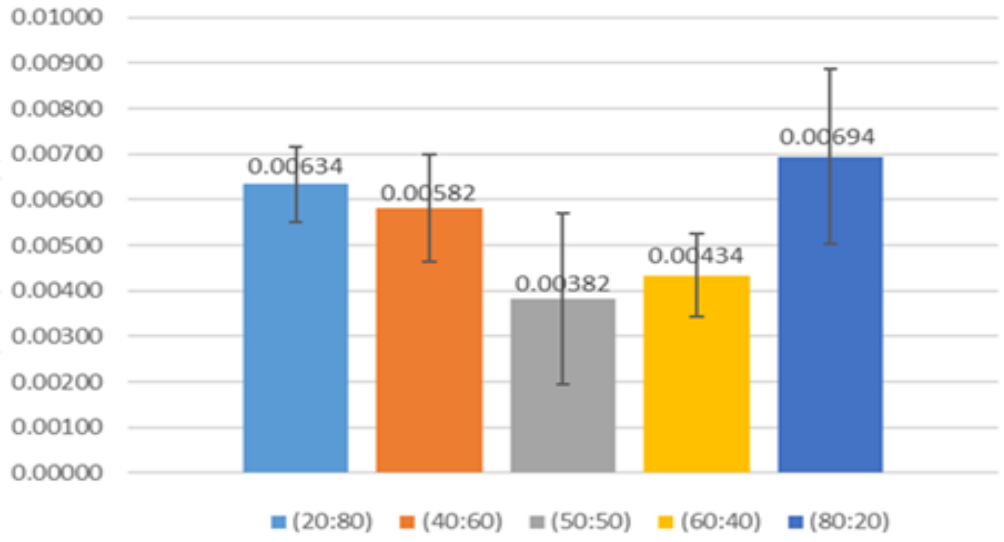


Fig. 4. Degradation rate of nanofibers collagen/PCL with different volume ratio.

4. Conclusions

Differences in volume ratio of collagen and PCL affected the viscosity and conductivity of the solution and thus affected the formed nanofibers. The diameters, the number of formed fibers, and the cell's viability result in MTT assay were several factors which were affected by volume ratio differences. In this study, the result showed that nanofibers with 40:60 volume ratio of collagen/PCL was the most suitable for the skin tissue engineering.

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