

**FABRICATION OF PCL-COLLAGEN NANOFIBER USING
CHLOROFORM-FORMIC ACID SOLUTION AND ITS APPLICATION
AS WOUND DRESSING CANDIDATE**

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ABSTRACT

In this study, polycaprolactone-collagen nanofiber was prepared with 10% w/v composition using a mixture of chloroform-formic acid. PCL was dissolved in chloroform while collagen was dissolved in formic acid. This research carried out optimization of electrospinning parameters such as flow rate, running time, and collector type to obtain optimum and suitable nanofiber to be applied as wound dressing. The most optimum nanofiber is made with flow rate 0.01 $\mu\text{L}/\text{h}$, running time is 3 hours, and using cylinder collector type. Characterization was performed for five different types of PCL-collagen nanofiber with different treatment, which nanofiber made with cylinder collector, plate collector, addition of citric acid, heating treatment, and nanofiber without the addition of collagen. PCL-collagen nanofiber produces smaller diameter about 200 - 600 nm. Based on the test of mechanical properties, addition of collagen causes its mechanical properties to be lower when compared to addition of crosslinking agents by heating or citric acid. The cytotoxicity test was carried out for PCL, PCL-collagen with addition of citric acid, and PCL-collagen nanofiber treated by heating. PCL was chosen to compare the effect of collagen addition on nanofiber against cell viability. Collagen has an important role for growth, proliferation, and differentiation of cells in tissue engineering. PCL-collagen nanofiber which treated by heating provides better viability of 83.09% while compared to nanofiber with addition of citric acid, because citric acid acidic properties causing the environment around nanofiber have an extreme pH, it may affect the growth of cells and reduce its viability.

Keywords: Nanofiber, PCL, collagen, electrospinning, wound dressing, MTT Assay

INTRODUCTION

Development of technology support researchers to develop fabrication of wound dressing materials which can be applied for wound healing process. Over the last decade, nanofiber membranes have interested many researchers because of its extensive application for medical field, particularly as

wound dressing. The techniques used in fabrication of nanofiber include drawing, template synthesis, phase separation, self-assembly, and electrospinning techniques (Huang *et al.*, 2003). Among these techniques, electrospinning is techniques that most widely studied and used by researchers today, because it provides good nanofiber results to be applied

in tissue engineering, especially as wound dressings.

Synthetic polymeric materials often used for fabrication of nanofiber, but it most have poor biocompatible properties, thus to overcome these constraints used natural polymers which have good biocompatible properties (Huang *et al.*, 2003). Collagen is natural polymer that most widely used in medical applications because its properties resembles human extracellular matrix. Meanwhile, polycaprolactone is synthetic polymer which has an aliphatic polyester group, good mechanical properties, and non-toxic for human skin (Dulnik *et al.*, 2016). Polycaprolactone (PCL) has strong hydrophobic properties, but it can be reduced by the addition of hydrophilic substances such as gelatin or collagen.

Recent studies reported that nanofiber was made of collagen and polycaprolactone provide good materials combination to be applied as wound dressings. The advantages of these two materials are their skin-like properties and human extracellular matrix (Zhang *et al.*, 2005). In this study also aim to developed suitable solvent system and determine the optimum parameters for electrospinning PCL-collagen nanofiber. These researches are using binary solvent system which chloroform for PCL and formic acid for collagen. Generally, the usage of chloroform as a PCL solvent has been widely performed in fabrication of nanofiber which produces diameter of about 3 - 5 μm . Based on Schueren *et al* reported the development of new solvent system for PCL, which is solvent

mixture of chloroform and formic acid can produce nanofiber with diameter about ten times smaller than chloroform only as a PCL solvent. In addition, formic acid may also used as a solvent for collagen besides acetic acid (Schueren *et al.*, 2011).

The application of PCL-collagen nanofiber as wound dressing was performed by in vitro cytotoxicity test using MTT Assay. The results of the test were demonstrated by the number viability of living cells cultured on nanofiber. Therefore, it may hope that fabrication of PCL-collagen nanofiber using chloroform-formic acid can be a solution to produces wound dressings which have biocompatible, biodegradable, and non-toxic properties for human skin.

MATERIALS AND METHODS

2.1 Materials

PCL (Mw = 80.000) was purchased from Sigma-Aldrich, while collagen was obtained from BATAN Jakarta. Analytical grade formic acid were purchased from Merck Co. Huh7 cells, NaOH, Dulbecco's Modified Eagle Medium (DMEM) cell culture medium, Fetal Bovine Serum (FBS), Phosphate Buffered Saline (PBS), MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazazine bromide), dimethyl sulfoxide (DMSO) were obtained from Institute of Tropical Disease Airlangga University.

2.2 Methods

2.2.1 Preparation of PCL-collagen polymer solution

In this study, preparation of polymer solutions for nanofiber is by dissolving PCL and collagen separately. PCL was dissolved in chloroform meanwhile collagen was dissolved in formic acid. Both solutions are stirred with magnetic stirrer until homogeneous. After stirring, both of solutions mixed and stirred until the mixtures form homogeneous solution. The polymer solution mixture allowed for a while to remove gas bubbles that arise during the mixing process.

2.2.2 The electrospinning process of PCL-collagen nanofiber

Electrospinning process are using high voltage power supply which set of 15 kV. Collector was installed at 10 cm distance from the tip of syringe. The clamping cable from power supply was clipped to syringe and the other one was connected to collector. Furthermore, PCL-collagen polymer solution injected into syringe and the electrospinning process was carried out with flow rate, running time, and type of collector to be optimized. This optimization aims to get the optimum nanofiber to be applied as wound dressing.

2.2.3 Optimization of electrospinning parameters

In this research, some electrospinning parameters to be optimized which are flow rate, running time, and collector type to obtain optimum and suitable nanofiber to be applied as wound dressing. Variations of flow rate

used were 0.01, 0.05, 0.1, and 0.5 $\mu\text{L/h}$ to obtain optimum flow rate in fabrication of nanofiber. Meanwhile the optimization of running time was done with variations of time 1, 3, 5, and 7 hours. The result of flow rate and running time optimization will be used as reference on optimization of collector type. The collectors used in this research are cylindrical and flat collectors.

2.2.4 Characterization of PCL-collagen nanofibers

At this stage, the characterization performed for five different types of PCL-Collagen nanofiber with different treatment variations. The treatments include PCL-Collagen nanofiber made with cylindrical and flat collectors, addition of citric acid, heating treatment, and without the addition of collagen. These five nanofiber membranes was made based on electrospinning parameters which have been optimized.

2.2.4.1 Scanning Electron Microscope morphological analysis

Determination of PCL-Collagen nanofibers morphology were performed using Scanning Electron Microscope (SEM). The cross section or surface of nanofiber sample will be enlarged at certain magnification by SEM analysis. The morphological test with SEM was performed to find out the surface structure and diameter of PCL-Collagen nanofiber.

2.2.4.2 Mechanical properties analysis

The mechanical properties of nanofibers were analyzed by tensile tests using Autograph. This mechanical properties test was performed to determine mechanical strength of PCL-Collagen nanofiber against the force given from the outside. This tensile test data was used for determining stress, strain, and nanofiber membrane module.

2.2.4.3 Cytotoxicity properties analysis

Prior to in vitro assay with Huh7 cells, PCL-collagen nanofiber was sterilized with UV light for 48 hours. To evaluate cell metabolism in PCL-collagen nanofiber, MTT test for nanofiber membrane was made after 48 hours, the amount of cells that had been seeded is 5.4×10^4 cells/membranes density. MTT test depends on cellular reduction of MTT which have yellow color, by mitochondrial dehydrogenase enzyme from living cells forming blue to purple formazan crystals. The amount of formazan crystals formed comparable with the amount of living cells. The culture medium in well was removed and the incubated PCL-Collagen nanofiber sample was added 300 μ L medium containing MTT solution with 9:1 (DMEM 270 μ L + MTT 30 μ L) ratio, then incubated for 4 h at 37°C and 5% CO₂. After 4 hours, the culture medium was taken from container and

200 μ L of dimethyl sulphoxide (DMSO) was added to dissolve the precipitate formazan crystals formed from the reaction of MTT [5]. Measurement of formazan crystals absorbance was done at 560 nm and 750 nm wavelength with GloMax-Multi Microplate Multimode Reader (Promega).

The absorbance data obtained was used to determine the percentage of living cells (cell viability). If the percentage less than 60%, it is mean toxic and can kill living cells. Measurement results were compared with controls. MTT Assay controls are Huh7 cells which had been seeded in culture medium at well without the addition of nanofiber sample.

RESULTS AND DISCUSSION

3.1 Preparation of PCL-collagen polymer solution

Polymer solution for nanofiber membrane was prepared by dissolving PCL and collagen separately. The total composition of PCL and collagen made is 10% w/v. PCL was dissolved in chloroform meanwhile collagen was dissolved in formic acid. Both solutions are stirred until homogeneous. Furthermore, both of solutions were mixed together and stirred until form homogeneous solution. Then polymer solution mixture was allowed for a while to remove gas bubbles that arise during the mixing process.



Figure 1. Preparation of PCL-collagen solution

3.2 Electrospinning process of PCL-collagen nanofiber

The electrospinning processes are using high voltage power supply which has been set of 15 kV. The collector installed at 10 cm distance from the tip of syringe. The principle of electrospinning is polymer solution in a syringe which has given high electric potential

with a range of several kilovolts, it will affect the surface tension of polymer solution forming conical shaped extension called Taylor Cone at the tip of syringe, and then polymer jet was attracted to the collector forming the continuous fibers. The formed fibers will become thinner due to evaporation of the solvent (Buttafoco *et al.*, 2006).



Figure 2. Electrospinning process of PCL-Collagen nanofiber

3.3 Optimization of electrospinning parameters

3.3.1 Optimization of flow rate

Based on this optimization result obtained the most optimum flow rate was 0.01 $\mu\text{L/h}$. Taylor Cone which produced at this flow rate has perfectly conical shape, the polymer

solution stream is not very fast, and not discard the dop, and produces continuous fibers. At flow rate above 0.01 $\mu\text{L/h}$, more elongated Taylor Cone, not continuous fibers formed, and polymer solutions coming out of the syringe still wet as the flow accelerate with increasing of flow rate, forming hole-shaped at

nanofibers. In the research of Maneji et al states that the increasing flow rate of polymer solution in the electrospinning process

produces larger diameter of nanofibers (Maneji *et al.*, 2014).

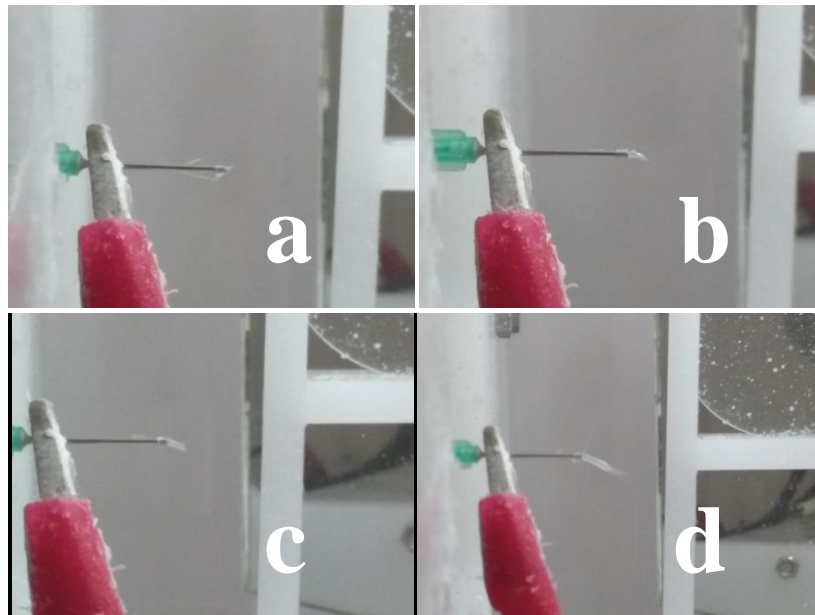


Figure 3. Taylor Cone formation (a) 0.01 $\mu\text{L/h}$, (b) 0.05 $\mu\text{L/h}$, (c) 0.1 $\mu\text{L/h}$, (d) 0.5 $\mu\text{L/h}$

Flow rate in the electrospinning process not always produce continuous fibers, this may occur due to occasional deformation as a result of competition between polymer flow rate coming out of syringe slower to be drawn by electrostatic field generated to the collector. With increasing of flow rate, nanofibers were formed from polymer solution not get enough time to dry before reaching the collector, so it form a large diameter nanofiber (Rajput, 2012).

3.3.2 Optimization of running time

Based on the experimental results, more time of electrospinning process produce thicker nanofibers. This nanofiber thickness

affects the cells to be applied to nanofiber, the nanofibers surface is useful as cells sticking medium and growth. As the nanofiber thickness grows, it is feared that cells can not stick and grow well. Based on this optimization result, running time of electrospinning process for 3 hours was chosen because it is resulting nanofiber which not very thin and thick, and it can be separated from aluminum foil of collector, so it was expected to be used as cell medium to stick and grow. The resulting membrane with electrospinning time duration of 5 and 7 hours were very thick and it easily separated. While the membrane produced with 1 hour electrospinning duration was thinner and difficult to separate from aluminum foil.

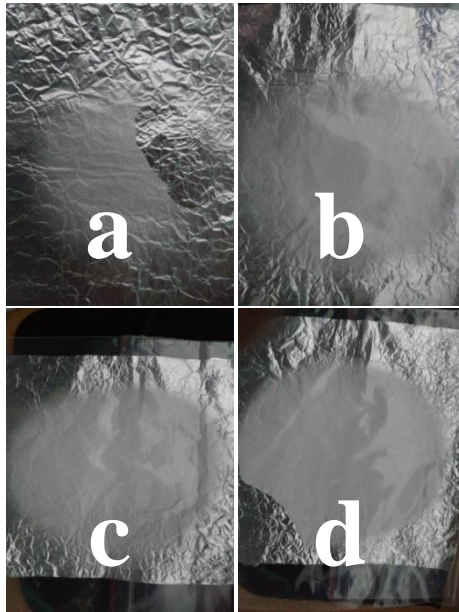


Figure 4. Optimization of running time (a) 1 h, (b) 3 h, (c) 5 h, (d) 7 h

3.3.3 Optimization of collector type

This optimization process was done by using flow rate and running time which has been optimized previously, which 0.01 $\mu\text{L}/\text{h}$ of flow rate and duration of running time for 3 hours. Optimization using flat collector produces uneven and thicker membranes in the center of nanofiber. This may be because during the electrospinning process, the syringe which containing polymer solution was centered only towards the center of flat collector, so it

formed nanofiber distribution in the center only and thicker. While optimization using cylindrical collector produces more evenly distributed nanofiber membrane and thickness. This may be influenced by the nature of its rotating collector, so it formed more evenly distributed nanofiber to all parts of the collector. The cylindrical collector has properties to expand the nanofiber formed due to its influence of rotation (Pra *et al.*, 2017).

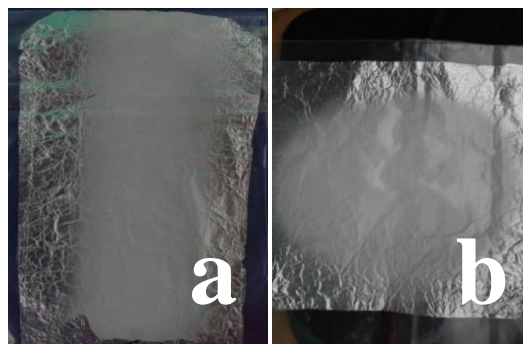


Figure 5. Optimization of collector type (a) cylindrical, (b) flat

3.4 Characterization of PCL-collagen nanofiber

The treatment of nanofiber aims to compare the effectiveness of most optimal PCL-collagen nanofiber used as wound dressing. Five nanofiber was made based on electrospinning parameters which have been optimized previously using 0.01 $\mu\text{L}/\text{h}$ of flow rate, running time for 3 hours, and cylindrical collector usage, except for PCL-collagen nanofiber made by flat collector. PCL-collagen nanofibers were made by flat collector used as comparison for nanofiber which made by cylindrical collector. The addition of citric acid and heating treatment in oven 45°C for 2 hours, serves as crosslinking method to strengthen structure and improve mechanical

properties of nanofibers. While the nanofiber which not added by collagen used as comparison to investigate the effectiveness of membranes with or without addition of collagen.

3.4.1 Scanning Electron Microscope morphological analysis

Scanning Electron Microscope analysis was performed to determine surface morphological and diameter of PCL-collagen nanofiber. The ideal criteria of nanofiber to be applied as wound healing was 50-500 nm of diameter because its size resemble physical structure of human extracellular matrix. In addition, large surface areas support homeostasis during the healing process (Abrigo *et al.*, 2014

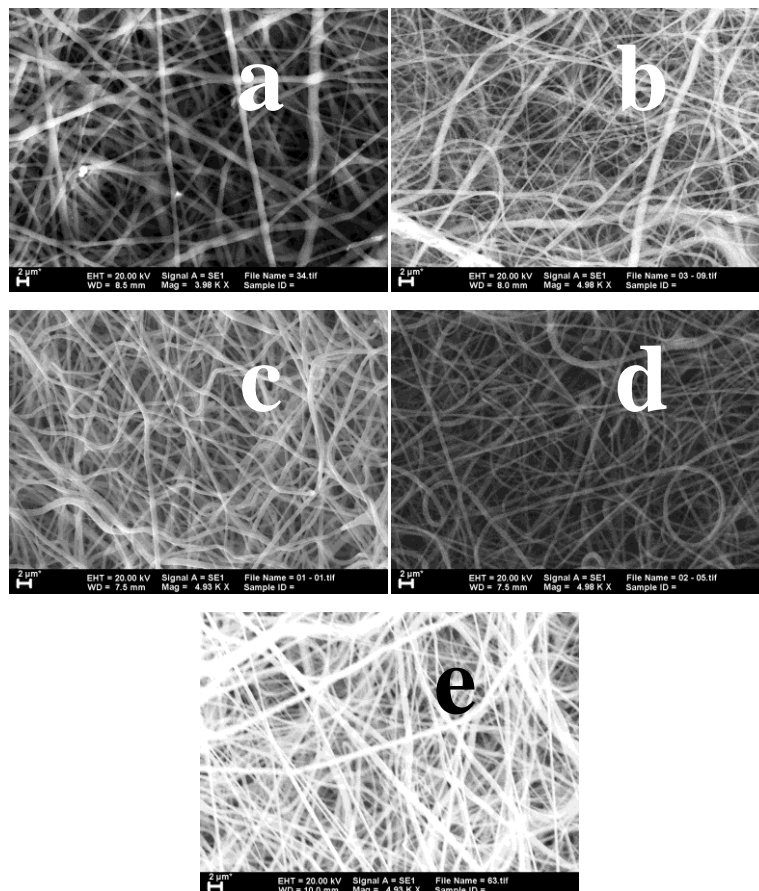


Figure 6. SEM image results of PCL-collagen nanofibers with variation of treatments (a) cylindrical collector, (b) flat collector, (c) addition of citric acid, (d) heating-treated, (e) without addition of collagen

Based on the SEM image, nanofiber was made from PCL-collagen (Figure 6a-6d) has diameter of about 200-600 nm, while nanofiber which made from PCL only (Figure 6e) has 800-2000 nm of diameter. This suggests that the usage of chloroform-formic acid solvents has an influence on nanofiber morphology. In accordance with research of

Schueren et al which developed new solvent system in the fabrication of PCL nanofiber, in which a solvent mixture of chloroform and formic acid produces nanofiber with diameter of ten times smaller than chloroform only as PCL solvent (Schueren *et al.*, 2011). On the other hand, formic acid may also be used as solvent for collagen besides acetic acid.

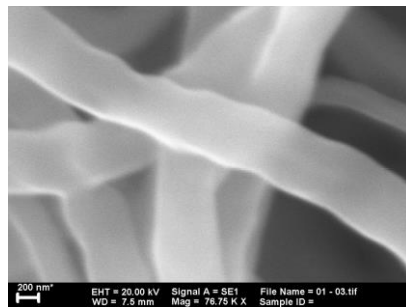


Figure 7. Cross section parts

Figure 6c was the SEM image of PCL-collagen nanofiber with addition of citric acid. Citric acid serves as crosslinking agent to strengthen membrane structure which formed. Figure 6c shows that nanofiber structure has more cross-linked parts. The formation of cross section occurs because of the chemical bond between citric acid, PCL, and collagen. At cross section parts has a level of more O than C atoms, so the chemical bonds formed was made nanofiber becomes more strength. It

supported by SEM EDX data which describes the atomic content of a sample. Based on SEM EDX data shows that C and O atoms content in the cross section parts (Figure 8b) has a ratio of 3.61 (78.12/21.63), meanwhile the non-cross section parts (Figure 8a) has a ratio of 5.92 (85.22/14.39). From these data, the smaller ratio value indicates the presence of more O atoms. Citric acid ($C_6H_8O_7$) was crosslinking agents which have seven O atoms allowing more chemical bonds to be formed.

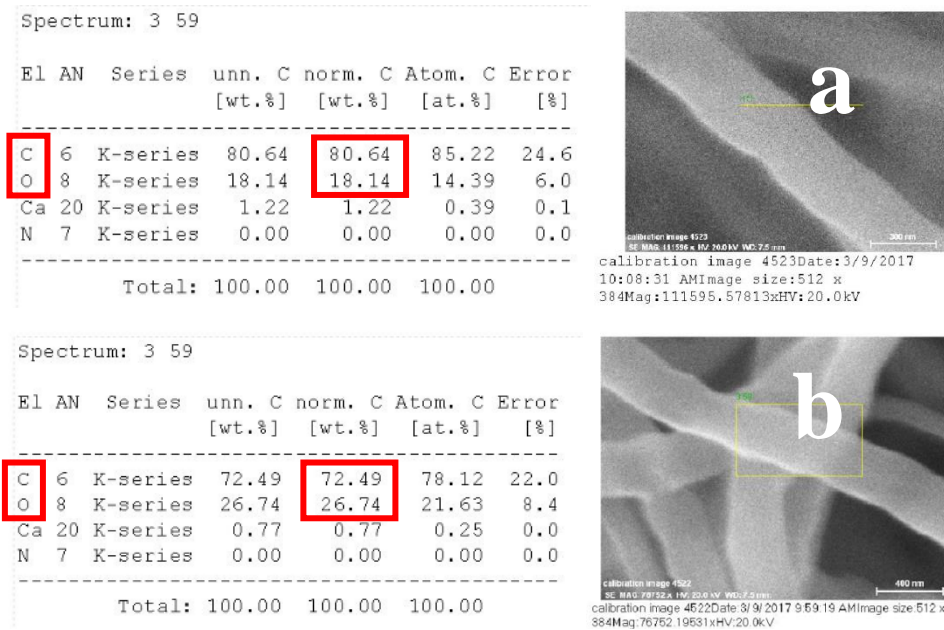


Figure 8. SEM EDX results (a) non cross section parts, (b) cross section parts

Figure 6d was SEM image of PCL-collagen nanofiber treated by heating in a 45°C oven for 2 hours. Based on these images, nanofiber structure has morphology which is no different with the nanofiber been made by cylindrical collector. On the other hand, the presence of heating was feared to damage collagen content because it was a polymer composed of proteins. Collagen has denaturation temperature of 45°C (Nomura *et al.*, 1996). Protein was macromolecule which not resistant to high temperatures, so that its structure becomes denatured. According this damage can reduce the effectiveness of nanofiber in wound healing because nanofiber used as a cell medium to stick and grow.

On Fig.6e was morphology of PCL nanofiber without the addition of collagen. From these SEM image result shows morphological structure not much different to

PCL-collagen nanofiber (Figure 6a), as well as the heat-treated nanofiber (Figure 6d). However, this nanofiber structure has larger diameter when compared to collagen-treated nanofiber. Nanofiber was made of PCL (Figure 6e) has diameter of about 800-2000 nm.

3.4.2 Mechanical properties analysis

The mechanical properties of nanofiber were performed by tensile test using Autograph. This mechanical properties test performed to determine the strength of the PCL-collagen nanofiber membrane against the force given from outside. The tensile test data used to determine stress, strain, and nanofibers moduli. According the experimental results, different treatment giving different stress, strain, and modulus young values.



Figure 9. Mechanical properties test using Autograph

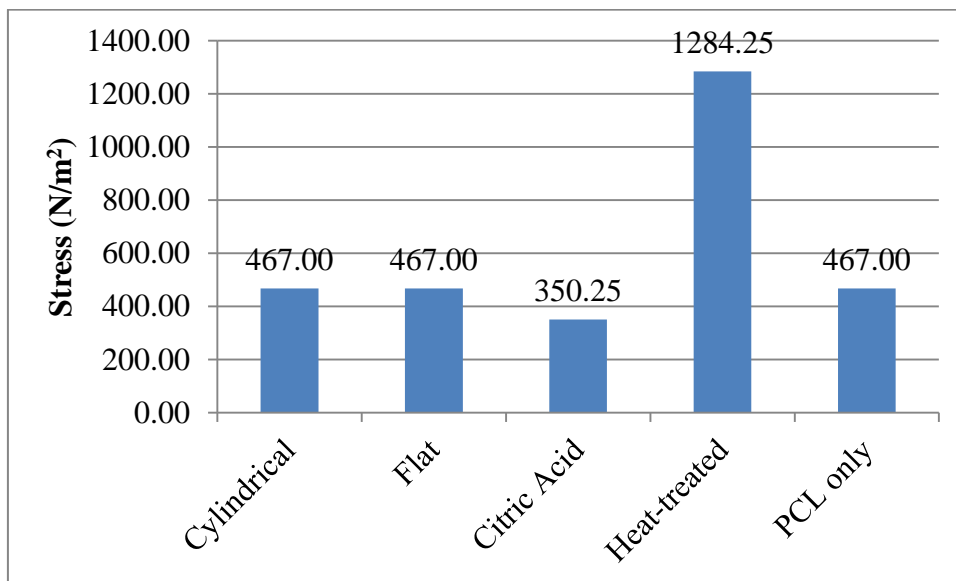


Figure 10. Measurement results of nanofiber stress

Based on the graph in Fig.10, showed that PCL-collagen nanofiber was made by cylindrical and flat collectors and PCL only without addition of collagen has the same stress value of 467 N/m². While the heat-treated PCL-collagen nanofiber was has the greatest stress value of 1284.25 N/m². Contrast to PCL-collagen nanofiber with the addition of citric acid has the lowest stress value of 350.25 N/m². The process of heating and addition of citric acid serves to improve the mechanical properties of nanofibers. Citric acid formed crosslinking parts through chemical bonds

between atoms. Heating was physical crosslinking method which will break the bonds in the membrane (Amalia, 2012). Materials which have more chemical bonds will have more strength mechanical properties, so it tends to have greater value of young modulus. The increasing of young modulus values has a higher stress but smaller strain value (Callister and Rethwisch, 2014). However, based on experimental results, nanofiber with the addition of citric acid did not show these properties.

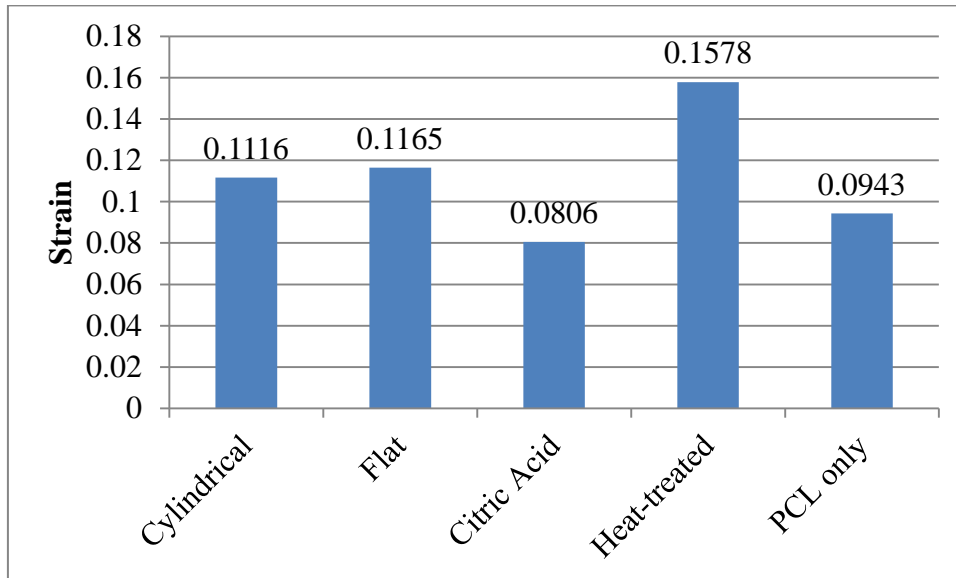


Figure 11. Measurement results of nanofiber strain

Strain value used to determine the elastic properties of a material, which has the ability of an object to return to its original state after being subjected by an external force. PCL-collagen nanofiber heat-treated has a larger strain of 0.1578. This may be the effect of heat dissipation of chemical bonds between atoms in the nanofiber thus increasing membrane strain with the increasing length of nanofiber

(Amalia, 2012). While nanofiber with the addition of citric acid gives the lowest strain value of 0.0806 because the crosslinker agent caused the bond in the membrane to become strongly bound to produce a small strain. Both PCL-collagen nanofiber made by cylindrical and flat collectors has similar strain values, whereas PCL nanofiber without the addition of collagen has a lower strain.

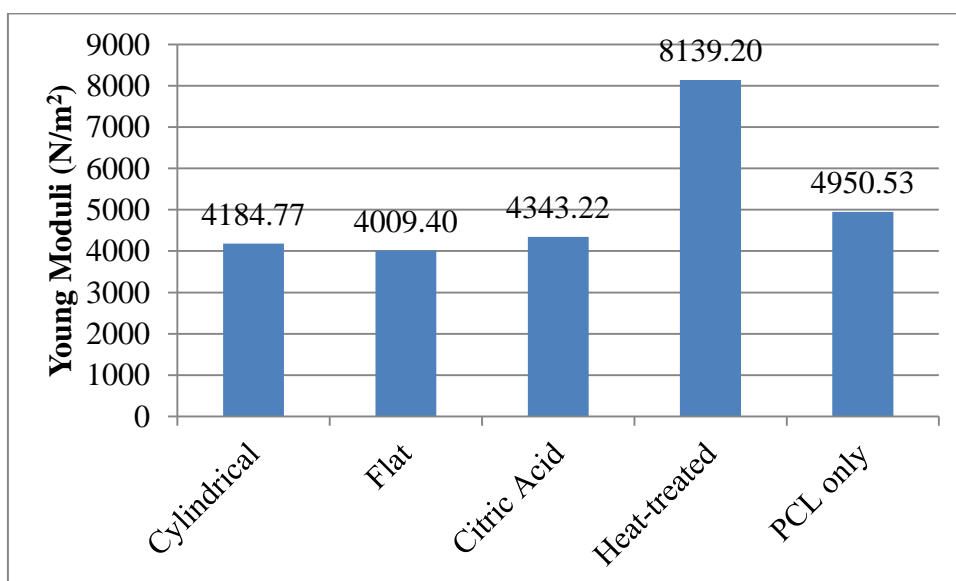


Figure 12. Measurement results of nanofiber young modulus

Young modulus value obtained from the comparison of stress to strain values. PCL was biodegradable synthetic polymer which has good mechanical properties and non-toxic for human skin (Dulnik *et al.*, 2016). PCL can be combined with other polymers to improve polymer stress resistance. Based on the results, the PCL nanofiber without the addition of collagen has a higher young modulus value than PCL-collagen nanofiber, so the addition of collagen may have the effect of decreasing the mechanical properties of membrane and not all polymers have effective to improve mechanical properties of membrane. While, nanofiber which was giving heat-treatment and addition of citric acid as a crosslinking agent has higher young modulus value. Heating and addition of crosslinker agents were method to improve the mechanical properties of a material. Heating was physical crosslinking while citric acid was chemical crosslinking (Amalia, 2012).

3.4.3 Cytotoxicity properties analysis

Prior to in vitro assay with Huh7 cells the PCL-collagen nanofiber membrane was sterilized with UV light. This sterilization process serves as a disinfectant to kill bacteria or microbes that can interfere within cell cultured in nanofibers (Zeybek *et al.*, 2014).

The nanofiber cytotoxicity test was performed with MTT Assay to determine percentage of cell viability. The cells used in this cytotoxicity test were Huh7 cells originating from the liver. Viability is the cell's ability to survive in samples to be tested. MTT Assay depends on yellow cellular MTT

reduction by mitochondrial dehydrogenase enzyme from living cells from blue to purple formazan crystals. Formazan formed was analyzed by GloMax-Multi Microplate Multimode Reader (Promega) to measure the absorbance. The absorbance measurements obtained was compared with the control. The control in MTT Assay was Huh7 cell grown in cultured medium at well without the addition of nanofiber membrane sample. The magnitude of absorbance was comparable to percentage of viability living cells. The darker purple color of the formazan shows the increase of absorbance, indicating that cell viability was higher (Khoswanto *et al.*, 2008). If percentage of measured cell viability was less than 60% then the resulting nanofiber has toxic properties and kills living cells.

The cytotoxicity test in this research was performed to PCL nanofiber, PCL-collagen with the addition of citric acid, and PCL-collagen treated with heating. PCL-collagen nanofiber with the addition of citric acid and heat-treated was selected because they have good mechanical properties compared to other treated nanofiber. Both of these nanofiber have a larger young modulus values due to crosslinking effect given on nanofiber. Heating and addition of crosslinking agents such as citric acid was method to improve the mechanical properties of materials. While PCL nanofiber was chosen to compare the effect of collagen addition on nanofiber to cell viability. The nanofiber application in this study will be used as wound dressing which will be in direct contact with

cells in certain tissues, so we need to analyze the membrane cytotoxicity of cell.

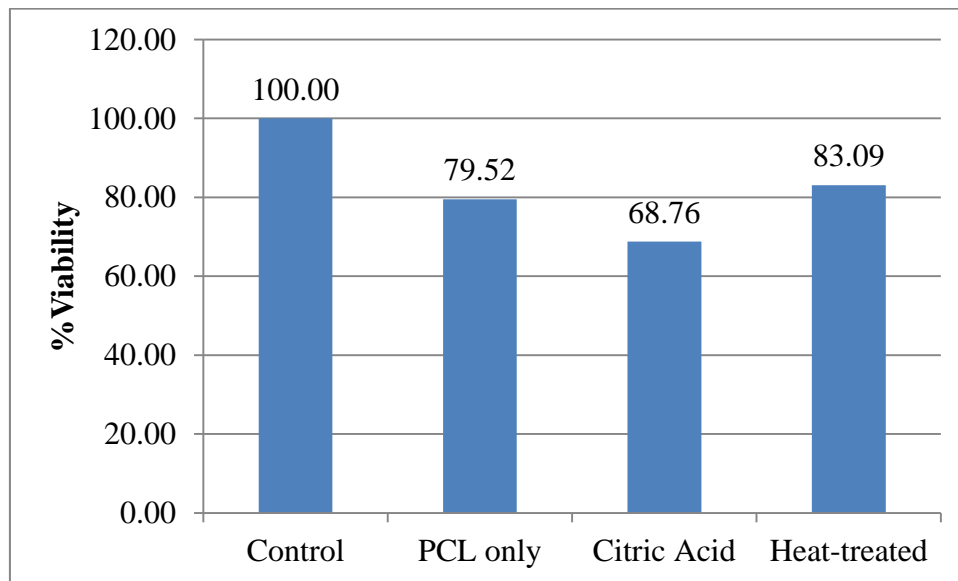


Figure 13. Percentage of cell viability

Based on the above graph data in Fig.13, heat-treated PCL-collagen nanofiber has highest percentage of cell viability of 83.09%, followed by PCL nanofiber with a cell viability percentage of 79.02%, and PCL-collagen nanofiber with addition of citric acid as a crosslinker of 68.76%. From these results, it can be seen that the addition of collagen in PCL-collagen nanofiber provides higher percentage of cell viability than without collagen. Collagen also has an important role for the growth and differentiation of cells in tissue culture (Rho *et al.*, 2006).

PCL-collagen heat-treated nanofiber provides better percentage of cell viability than PCL-collagen nanofiber with the addition of citric acid. Although both treatments were crosslinking methods but provide different activities. The acidic properties of citric acid may caused cells can not survive with extreme pH environments which shows the smallest

percentage of cell viability (Khoswanto *et al.*, 2008). Chemical crosslinking such as the addition of citric acid affects cell adhesion thereby reducing the percentage of viability and cell proliferation (Franco *et al.*, 2012). Thus, PCL-collagen nanofiber with heated treatments was more suitable for wound dressing as the result of MTT Assay in percentage of cell viability of 83.09%. The increasing percentage of cell viability suggests that the nanofiber sample has non-toxic properties.

CONCLUSIONS

The preparation of polymer solution for nanofiber in this research was by dissolving PCL and collagen separately, polycaprolactone in chloroform while collagen in formic acid, then blending both each other. From five treatments provided in fabrication of nanofiber, PCL-collagen nanofiber has

diameter of about 200-600 nm. However, based on tests of mechanical properties, the addition of collagen in these nanofiber causes the membrane mechanical properties to be lower when compared to crosslinking treatments with heated or addition of citric acid. The cytotoxicity test in this study was performed on PCL, PCL-collagen with the addition of citric acid, and heated-treatments PCL-collagen nanofiber. PCL-collagen nanofiber with treatment of heating provides better viability percentage of 83.09% compared to nanofiber added citric acid as crosslinker so it is suitable to be applied as wound dressing, because the influence of citric acid has acidic properties causes the environment around the membrane has an extreme pH that may affect cell growth and reduced its viability.

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