Design of Cellulose Acetate-Collagen Nanofiber and its in Vitro Assessment As Wound Dressing Candidate

by Mochamad Zakki Fahmi

DESIGN OF CELLULOSE ACETATE-COLLAGEN NANOFIBER AND ITS IN VITRO ASSESSMENT AS WOUND DRESSING CANDIDATE

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In this study, we developed nanofiber composite membrane of cellulose acetate and collagen via electrospinning. Several variations on electrospinning process such as time, flow rate and collector optimizations have been done. The result showed that the optimum conditions were reached with flow rate at 0.05 µL/h using a drum or cylinder-shaped collector and optimum time for membrane formation for 3 h. The results of the CA-collagen membrane performed good results with the modulus young $1,237 \times 10^{-5}$ GPa. The entire membrane has elongation according to human skin so it can be used as a candidate for wound dressing. The MTT assay reveals that all membrane was non-toxic with viability all over 80%.

1. Introduction

Skin is an external organ of the human body that has a very important role in protecting the inside of the body against the environment that potentially harmful. Skin serves as the first line of defence against the entry of chemicals and microorganisms as well as providing a barrier to prevent fluid loss and regulate body temperature. The appearance of skin lesions will trigger the healing process. Wounds are defined as damage or disturbance of epithelia continuity of the skin or mucosa due to physical or thermal contact. Based on the time in the healing process naturally, it can be categorized into acute and chronic wounds [1]. If the healing process is continuous and normal within the range of 8-12 weeks, then it includes the acute wound category. However, delay on healing processes due to local or systemic factors that occur within months or years include chronic wound categories [2]. Although injuries are categorized as acute or chronic, the basic function of the dressings used in the treatments remains the same, to provide a barrier or protector to prevent bacterial contamination and to absorb exudates [3].

Among the polymers used as wound dressings, cellulose acetate (CA) and polycaprolactone (PCL) perform advantages. PCL is used in the mixture for wound dressing applications because it provides mechanical strength to the dressing, and has been used as a drug carrier in drug delivery system. PCL has good biocompatibility and biodegradation properties so it is potential to be applied in biomedical field [4]. However, PCL also has a very hydrophobic property [5]. Application of electrospinning techniques has succeeded in preparation of nanofiber from CA and has potential for application in the biomedical field. The CA is a biopolymer commonly used for medication purpose due to its high hydrophilicity, good fluid transport and

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water absorption capability. The CA, an acetate esters of cellulose, has been used extensively in the preparation nanofiber by electrospinning that the CA has also some favorable properties, such as good biocompatibility, biodegradation, regenerative properties, high affinity toward other substances, and tensile strength [6]. In addition, CA also has excellent biocompatibility properties with the human body environment [7]. In contrast to PCL, CA have more hydrophilicity properties [8]. The properties of hydrophobicity and hydrophilicity need to be known to examine the wettability of biomaterials as biomedical applications such as wound healing. This is because they will be in contact with blood, water, and other body fluids during their use. The nanofiber membrane with good hydrophilicity and high porosity facilitate wound healing, especially in the early healing phase [9-12].

One of the most clinically effective ingredients used for wound healing and skin regeneration is collagen which is the main protein of the extracellular matrix [10]. The main form of collagen-based wound dressings include: films, gels, sponges and fibers [11]. Collagen provides structural integrity and tensile strength to tissues. Tissue damage after injury requires collagen to repair and restore its structure and function. Collagen has several advantages such as biocompatibility, biodegradability, and low antigenicity [13]. There is no research has been done with the addition of collagen in CA nanofiber composites. The addition of collagen should be considered due to its important role on healing process.

Electrospinning is an interesting technique for synthesizing nanofiber from various biodegradable polymers because of the simplicity of technique and the ability to effectively control the process. The main components of electrospinning consist of high voltage power supply, syringe and syringe pump, and metal collector [14]. Electrospinning is an effective technique because of its flexibility to obtain nanofiber from a wide selection of polymers, the ability to control nanofiber diameters, morphology and fibrous structures, easy modification by adding various solutes or nanomaterials to solutions for electrospinning, possibly obtaining nanofiber with bio-component configurations, and porous [15]. This study tries to develop cellulose acetate-collagen nanofiber membrane (CCM) with electrospinning method in the hope that membrane will enhance its biocompatibility characteristic. Treatment with chemical and physical crosslinking agents was also investigated. The resulted membrane was then characterized and tested in order to obtain valuable information of its biomedical application.

2. Experimental

Materials
Material used in this experiment are cellulose acetate 15 wt% (CA, Sigma Aldrich, Mw : 30 kDa), acetone (Merck), citric acid (Merck). Collagen was obtained from BATAN Jakarta. Analytical grade formic acid were purchased from Merck. HaCaT cell was obtained from Institute of Tropical Disease Airlangga University. Sodium hydroxide, Dulbecco's Modified Eagle Medium (DMEM) cell culture medium, Fetal Bovine Serum (FBS), Phosphate Buffered Saline (PBS), MTT (3-(4,5- dimethyltiazole-2- yl)-2,5- diphenyltetrazrazine bromide), and dimethyl sulfoxide (DMSO) were obtained from Sigma Aldrich.

Electrospinning preparation of cellulose Acetate-collagen membrane
Cellulose acetate solution in acetone (15 wt%) and collagen in formic acid (0.05 wt%) then were blended using a magnetic stirrer to form a homogeneous solution. Then, 0.05 g of citric acid was added into the solution. The physical crosslink is carried out by heating at a temperature of 80 ° C on the already formed nanofiber membrane. Electrospinning equipped with a flat/cylinder collector was operated in a high voltage power supply (12 kV). The cellulose acetate-collagen solution was fed into the syringe with a certain flow rate (0.1, 0.3, 0.5, and 0.7 μL/h).

Meanwhile the optimization of running time was done with variations of time 1, 3, 5, and 7 h.

Characterization of cellulose acetate-collagen membrane (CCM)

Cellulose acetate-collagen membrane with 1x1 cm^2 in size was observed its surface structure by using scanning electron microscope (SEM, Zeiss EVO MA-10). Gold layer was conducted prior to observation. Mechanical properties of nanofibers were analyzed by using universal testing machine, Shimadzu Autograph AG-X (Shimadzu, Japan). Mechanical properties test was performed in order to determine mechanical strength of CCM against the force given from the outside. This tensile test data was used for determining stress, strain, and nanofiber membrane moduli.

Cytotoxicity properties analysis

Toxicity test of CCM was conducted by MTT [3 - (4,5 - dimethylthiazol - 2yl) - 5 (3 - carboxymethoxyphenyl) - 2 - (4 - sulfophenyl) - 2H tetrazolium] assay (Sigma - Aldrich). Prior to in vitro assay with Huh7 cell, CCM was sterilized under UV light. Huh7 cell was seeding at wells with a density of 5.4 x 104, incubate for 24 h at 37°C, 5% in CO$_2$ incubator. The CCM sample was at each well with 0.5x0.5 cm^2 in size and add 200μL medium and incubated for 48 h. A 300μl medium containing MTT (DMEM 270 μL + MTT 30 μL) and incubated for other 4 h was added. The precipitate formed by the MTT result was dissolved by the addition of 200 ml of DMSO. Absorbance measured at 560 nm and 750 nm wavelengths using GloMax-Multi Microplate Multimode Reader (Promega). Measurement results are compared with controls. MTT Assay controls are Huh7 cells which had been seeded in culture medium at wells without the addition of CCM sample. The absorbance data obtained was used to determine the percentage of living cells (cell viability). If the percentage less than 60% it indicates CCM is toxic and can kill living cells.

The MTT reagent used is a tetrazolium salt, which can be broken down into formazan crystals by the succinate tetrazolium reductase system present in the respiration pathway of the active mitochondria in living cells. The intensity of the purple colour that is formed is proportional to the number of living cells.
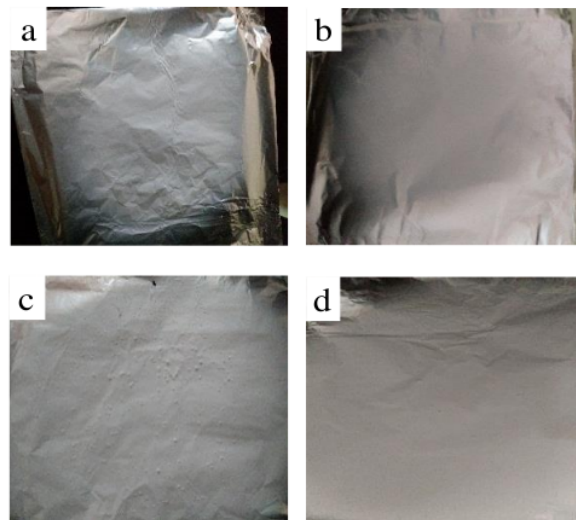
3. Results

Parameter Optimization of Electrospinning

The experimental result showed that 0.05 μl/h is the most optimum flow rate for formation of CCM membrane as shown in Fig. 1. Taylor Cone, which formed at this flow rate has perfectly conical shape and produces continuous fibers. The dope solution attracted and attached on the surface of collector.
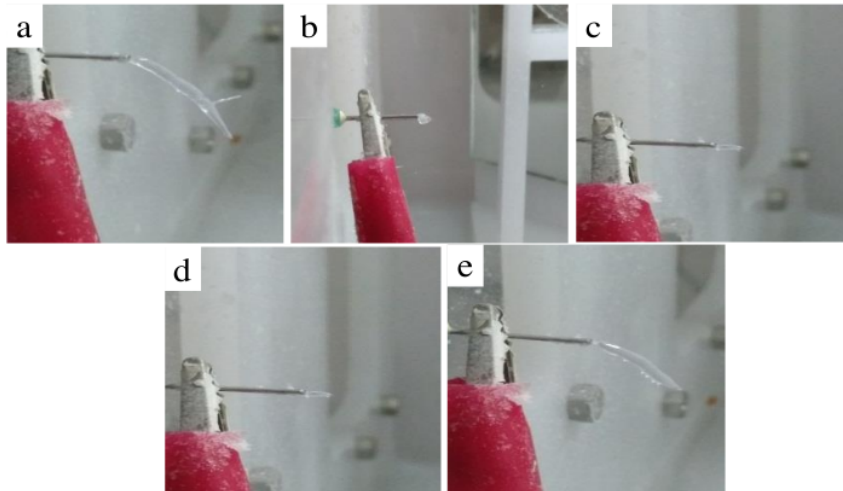
Fig. 1. The shape of taylor cone with flow rate (a) 0.01 µl / h, (b) 0.05 µl / h, (c) 0.1 µl / h, (d) 0.15 µl / h, (E) 0.20 µl / h.

This study also show that longer electrospinning process produce thicker nanofibers (Fig. 2). Meanwhile, variation of running time in the electrospinning process did not give any significant difference in the diameter of the formed nanofiber.

Fig. 2. Variation of running time (a) 1 h, (b) 3 h, (c) 5 h, and (d) 7 h.

The optimization of collector shows that the use of rotating cylinder collectors resulted membrane with uniform thickness on the entire surface. Optimization using flat collector forms uneven and thicker membranes in the center of plate Fig. 3.
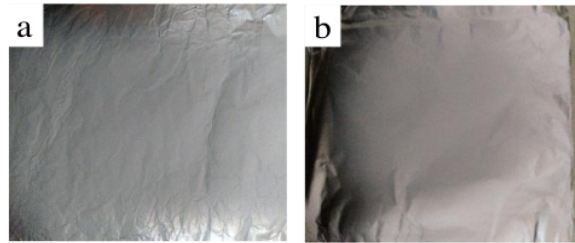
Fig. 3. Collector variation results (a) rotating cylinder and (b) flat.

Characterization of Nanofiber

SEM results of the nanofiber was performed on Fig. 4 resulted that CCM with electrospinning technique has been formed with the fiber diameter of 200-250 nm. Fig. 4a is CA nanofiber membrane with cylinder collector (CMC) is constructed with a randomly oriented fiber which are not tied each other and inhomogeneous diameter, meanwhile 4b and 4c representing CA-collagen nanofiber membrane with a cylinder (CCC) and flat collector (CCF). In the other side, Fig. 4d and 4e, show CA-collagen nanofiber membrane after heating at 80ºC with cylinder collector (CCH) for 2 h and CA-collagen with addition of citric acid with cylinder collector (CCD). As the Fig. 5 exhibit cross section formation from the addition of citric acid. This cross section indicates the bond between cellulose acetate and citric acid through chemical bonding. SEM EDX analysis as show in Fig. 6 exhibit atomic elements and ratio between cross section compared nun-cross section parts of nanofiber. It shows that the small value of the ratio between atom C and O in the cross section indicates high O levels.
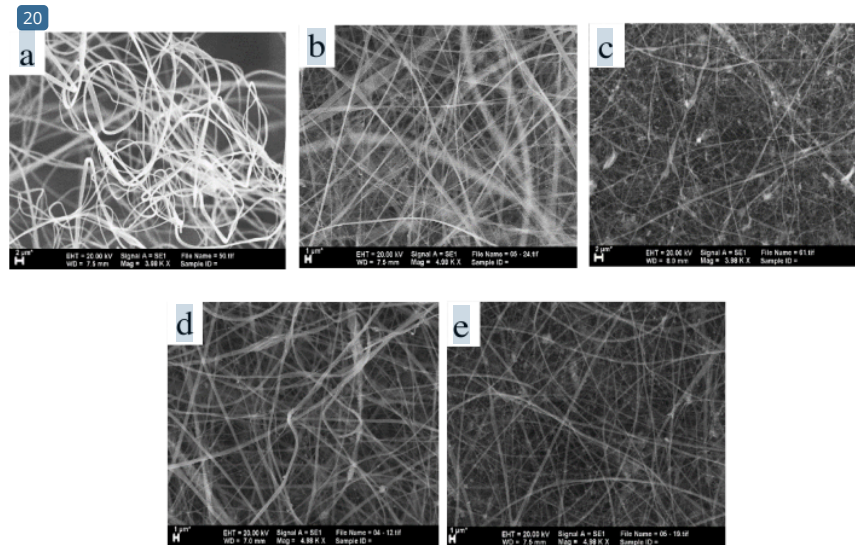

Fig. 4. The SEM test results of nanofiber membrane (a) CA, (b) CA-collagen cylinder collector, (c) CA-collagen flat collector, (d) CA-collagen with crosslink citric acid, and (e) CA-collagen with heating.
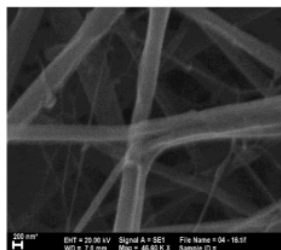
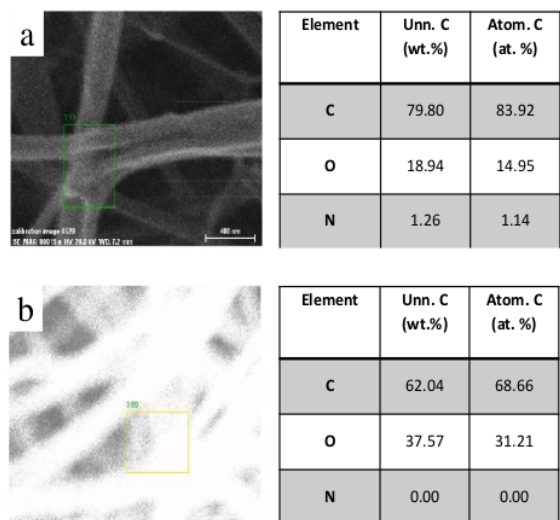*Fig. 5. Cross section formed by the addition of citric acid crosslink agent.*

Element	Unn. C (wt.%)	Atom. C (at. %)
C	79.80	83.92
O	18.94	14.95
N	1.26	1.14

Element	Unn. C (wt.%)	Atom. C (at. %)
C	62.04	68.66
O	37.57	31.21
N	0.00	0.00

Fig. 6. SEM EDX results (a) section of cross section and (b) non cross-section.
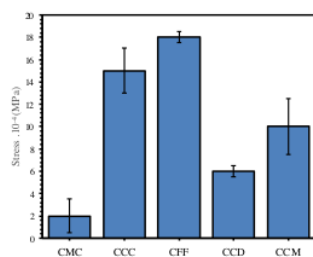

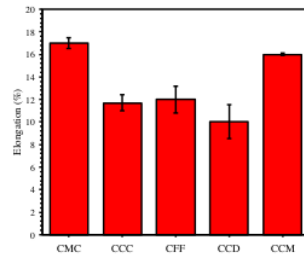*Fig. 7. Stress value on various nanofiber membranes.*

Fig. 8. Elongation measurement results (%) on various nanofiber membranes.

Further mechanical properties study reveals that CMC has the lowest stress value compare with other membranes due to unlinked fiber structure. Meanwhile the CCD has a stress value of 6.129×10^{-4} MPa, which has a lower value than the membrane with a heating treatment or without treatment (Fig. 7). Fig. 8 shows that the percent value of elongate from the nanofiber membrane: CMC, CCC, CCF, CCH, CCD are 17.08%, 11.56%, 12.13%, 10.48%, 15.83% respectively.

Cytotoxicity assay

CA-collagen membrane with heating gives lower value than CA membrane that is 84,97%. The low degree of viability (%) gives meaning that the membrane with the addition of citric acid are more toxic compared with the other membranes Fig. 9.
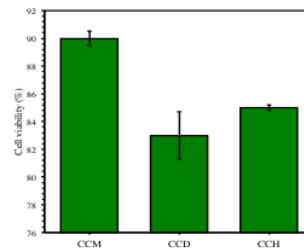

Fig. 9. Cell viability (%) of various membranes.

4. Discussion

Initial, the polymer solution forms a half-spherical surface as a result of surface tension. High voltage is applied between the spinnerets and the metal collector will convert the half-spherical surface of the polymer solution into a cone, called taylor cone [16]. At a flow rate of 0.01 μl / h, the resulting taylor cone dried quickly before the fibers reached the collector. The applied voltage of 12 kV with slower flow rate cause the polymer dope solution dried quickly. More stable taylor cone was formed at higher flow rate (0.05 μl / h). The dope solution attracted and attached on the surface of collector. The higher flow rate causes the balance between the released polymer solutions to the replacement of the polymer solution from within the syringe during jet formation become uncontrolled. The taylor cone to form elongated and the force of gravity to make its shape downward. The flow rate affects the formation of the nanofiber diameter; the minimum flow rate is preferable to maintain a balance between the released polymer solution and the replacement of the solution with the new one during jet formation [17]. Unstable formed taylor cone can be found at flow rate of 0.1; 0.15; 0.2 μl /h as shown in Fig. 1. The experimental result showed that 0.05 μl/h is the best flow rate for formation of CCM membrane.

Formed membrane with operating time 1 h, cannot be peeled from the aluminum foil due to its thin layer. The variation of running time in the electrospinning process did not give any

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significant difference in the diameter of the formed nanofiber [18]. The result of running time variation can be seen in Fig. 2.

It has been found that the use of rotating cylinder collectors resulted membrane with uniform thickness on the entire surface as shown in Fig. 3(a). Uniform thickness only can be found at the center of aluminum foil when flat collector is used (Fig. 3(b)). Fiber with smaller diameter was observed at membrane obtained from cylinder collector. Fiber spread to all direction during rotation of collector, which resulted film with homogenous thickness and smaller diameter. For static collectors, the electrostatic forces give the effect of stretching the fibers transversely to form a fiber density perpendicular between one fiber and the other [19].

As presented in Fig. 4, it can be seen that the CCM with electrospinning technique has been formed with the fiber diameter of 200-250 nm. The CA nanofiber membrane with cylinder collector (CMC) is constructed with a randomly oriented fiber which are not tied each other and inhomogeneous diameter (Fig. 4a). Unlike the case with Fig. (b) and (c) sequentially representing CA-collagen nanofiber membrane with a cylinder (CCC) and flat collector (CCF). The CMC have a relatively short distance between fibers and the fibers diameter distribution is inhomogeneous. On the other hand, between Fig. 4 (b) and (c) gives unequal morphological structures due to the use of different collector. Membrane obtained from cylinder collector has more homogenous on its diameter and comparison in that from flat collector. As seen in Fig. 4c, flat collector produce membrane, which have defect and inhomogeneous fiber diameter. Fig. 4d represents the morphological structure of the CA-collagen nanofiber membrane after heating at 80°C with cylinder collector (CCH) for 2 h. Heating step disrupt the balance of H_2O content in the membrane and lead to dehydration. Exposure to high temperatures results membrane with compressed structure due to denaturation of collagen [20-21].

Morphological structure of CA-collagen with addition of citric acid with cylinder collector (CCD) can be seen in Fig. 4e. Nanofiber is connected each other with citric acid as crosslink agents. The cross section image formed from the addition of citric acid can be seen in Fig. 5. Citric acid connects one cellulose acetate with others via chemical bonding. Both of citric acid and cellulose acetate contain C and O atoms. In the cross section the content of O atom is greater than in the non-cross section. It is possible that in addition to the cellulose acetate itself the presence of the O atom is from citric acid, which also has much O on its structure. The number of O atoms in the cross section compared with the non-cross section obtained from to use SEM-EDX instrument, as in Fig. 6. The percentage ratio of C and O at cross section (Fig. 6a) is 2.19. While the ratio of percentage C and O in the non-cross section (Fig. 6b) is 5.61. It shows that the small value of the ratio between atom C and O in the cross section indicates high O levels due to chemical bonds between citric acid with cellulose acetate.

It's seen in Fig. 7, the CMC has the lowest stress value due to unlinked fiber structure. The highest stress value is obtained from CCF, but the membrane has an inhomogeneous thickness. CA-collagen membrane collected from cylinder collector has a high stress value and the membrane has a homogenous thickness. Heating process has damaged the collagen structure, which decreases the mechanical properties of the membrane. CCD has a stress value of 6.129×10^{-4} MPa, which has a lower value than the membrane with a heating treatment or without treatment.

Fig. 8 shows that the percent value of elongate from the nanofiber membrane: CMC, CCC, CCF, CCH, CCD are 17.08%, 11.56%, 12.13%, 10.48%, 15.83% respectively. The experiment data are in agreement with Maganaris and Paul (1999) that the mechanical properties of human skin have a percent elongation between 1-25%, so then obtained membrane can be applied as wound dressing. It's found that the modulus young value of CA-Collagen (flat collector) > CA-collagen (cylinder collector) > CA-collagen (heating treatment) > CA-collagen-citric acid > CA. The ratio between stress and strain will give the value of young modulus which is in the range of hooke law is still valid [22].

From the results of the MTT cytotoxicity test, the CA nanofiber membrane provides the lowest cell toxicity value indicated by the high percentage cell viability obtained. Viability (%) is a value that indicates the presence of living cells. In this study, the viability value was determined from the absorbance of the treatment group compared with the control group. CA-collagen membrane with heating gives lower value than CA membrane that is 84,97%, while membrane nanofiber CA-collagen with addition of citric acid show the degree of viability (%) lowest equal to

71,28%. The low degree of viability (%) gives meaning that the membrane with the addition of citric acid are more toxic compared with the other membranes. The viability (%) result can be seen in Fig. 9.

5. Conclusions

Electrospinning is an effective technique in preparation of nanofiber membrane. The CCM is optimally made at a flow rate of 0.05 μl / h with a drum or cylinder collector for 3 h running. The characterization results show morphologically the diameter of nanofiber fiber measuring 200-250 nm. The tensile test gives results that the CA-collagen membrane with the cylinder collector gives a high yield. While the 80 ° C heating treatment did not give better results because the collagen has been denatured. Citric acid crosslinked the cellulose acetate via chemical bonding that can be observed through SEM instruments. The percent value of elongate from the nanofiber is in agreement with the mechanical properties of human skin, so then obtained membrane can be applied as wound dressing. The amount of modulus young values obtained from the largest was the CCF, CCC, CCD, CCH, and CMC. The MTT toxicity test shows that the entire membrane is not toxic with percentage viability of CMC, CCH, CCD, are 89.96%, 84.97%, and 71.28%, respectively.

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