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Effects of Collagen Concentration Variation toward Characteristics of Bacterial Cellulose-Collagen Biocomposites as Candidate of Artificial Dura Mater

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Abstract. Traffic accident has always been the primary cause of head injury. In the field of neurosurgery, it is closely related to the defect of dura mater. This study aims to perform synthesization of artificial dura mater with bacteria cellulose added with various collagen concentration in order to find the right composition. Bacteria cellulose (BC) was synthesized by fertilizing *Acetobacter xylinum* bacteria into coconut water. In addition, BC pellicle membrane was immersed into collagen solution (0.4% w/v, 0.5% w/v, 0.6% w/v and 0.7% w/v). The dried sample was characterized by Fourier Transform Infra Red (FTIR), mechanical test utility, Scanning Electron Microscope (SEM) and swelling test procedure. Based on the result, the biocomposite of bacteria celluose-collagen has a high potential as candidate of artificial dura mater.

Introduction

Traffic accident is the main causes of death. About 60% of deaths were caused by traffic accidents and begin with head injury. It could be stated that the head injury was the leading cause of death. Moreover the rate has been significantly increase to 9 per 100,000 population in a year, especially for young age [5]. In the field of *neurosurgery,* head injury is associated with dura mater defect because it was located in the outermost layer of the brain and closest to the cranial bone. Dural defects have eventually become one of the most common problem experienced by physician and *neurosurgeon.* Open head injury (which is usually caused by traffic accidents), tumor attack, a defect in the *meninges conginetal* or other head disease may lead to dura mater defect [11]

More severe defect cases of dura mater can lead to cerebrospinal fluid leakage (CSF leakage) [5]. CSF leakage is the loss of fluid and pressure from the spinal fluid caused by hole in the dura mater. In the field of cranial surgery, small incision or a cut in the dura mater is also capable of causing CSF leakage. The prevalence of death rate due to cerebrospinal fluid leakage is higher (15-30%). From the facts that have been mentioned above, many complications may follow head injury cases including open brain injury, tumor, and CSF leakage that cause higher urgency of dura mater defect cases. Prevalence rate for this case was significant. For brain tumors, the global incidence rate is estimated at 8-10 cases per 100,000 population per year [14].

To achieve improvements of watertight and avoid potential postoperative complications after surgery, neurosurgeon often closed the dura mater with stitches. In this case, if there is a deficiency of the dura mater, artificial membrane can be attach to prevent the leakage [7]. This condition showed that there is urgency of using dura mater artificial, therefore exploring the best material for dural graft was very beneficial

Bacterial cellulose (BC) is one of the materials synthesized by using *Acetobacter xylinum* fermented with certain media. Bacterial cellulose is a natural polymer that is both biodegradable and has a high mechanical strength. Bacterial cellulose has been developed in a variety of applications of tissue engineering such as artificial dura mater. Previous research has found that bacterial cellulose which has been implanted into the rabbit's skull has good biocompatibility, and capable of repairing dural defects in rabbits. In addition, there are no *CSF (Cerebrrospinal Fluid) leakage,* abscesses, edema and inflammatory response. Those research showed that periosteal new bone was found, and the bacterial cellulose had been fused with the surrounding tissue within a year [2]. However, previous research only conducted in vivo trials of bacterial cellulose without any prior characteristic studies *(in vitro)*.

This research would optimized the properties of BC by in vitro test, another polymer was needed to achieve that. Therefore, collagen was used as composed material of the bacterial cellulose. Collagen was considered as a natural polymer which is capable of balancing the properties of the BC that tend to be crystalline. Collagen was also one of the largest components of the dura mater. It was considered an ideal *scaffold* or matrix for tissue engineering because it was represented the major protein component of the extracellular matrix and provided support to the connective tissue such as skin, tendon, bone, cartilage, blood vessels and ligaments [4].

Previous studies has made a collagen matrix used as an artificial dura mater, then in vivo research was carried out in a white rabbit. It was shown that the collagen was able to blend with the dura mater and successfully trigger regeneration with minimal *foreign body reaction* [1]. According to Zhuravlova *et al.* (2012), the ideal elongation for human dura mater ranging from 7% to 20%. Dural graft material should not swell easily, the surface of material should be flat and smooth, so there will be no body reaction.

To optimize the superior characteristic, BC membrane was then composited with various collagen concentration (0.4% m/v; 0.5% m/v; 0.6% m/v and 0.7% m/v). Characterization performed in this study are FTIR (Fourier Transform Infra Red) to determine the functional groups on the membrane, mechanical testing to determine the mechanical strength, morphology test by using Scanning Electron Microscope (SEM) and a swelling test.

Research Methods

Tools and materials. The equipment used for the manufacture of biocomposite BC-collagen membrane include synthesis and characterization tools. Synthesis tools namely: digital pair of scales, *glass beaker, magnetic bar, magnetic stirrer,* stove, pots, pH meter, micropipette, thermometer, fermentation containers, measuring cups, spatulas, aluminium foil, *oven* and incubator. Test Equipment: SEM (inspect S50, FEI Corp., Japan), *Tensile Test* Imada HV500 NII, FTIR Shimadzu UV 1800, Gemnyco YWC - 010.

Materials used for the manufacture of membrane of biocomposites bacterial cellulose-collagen are *Acetobacter xylinum,* NaOH, deionized water, acetic acid, urea, sucrose, coconut water, collagen type 1 and distilled water.

Bacterial Cellulose Synthesis. The *Acetobacter xylinum* used was cultured in the Laboratory of Microbiology, Faculty of Science and Technology Universitas Airlangga. Synthesis started with bacteria media manufacture of coconut water. 300 ml coconut water that had been filtered, urea 1 gram and 2.5 grams of sucrose were heated until they boiled. The incubation container was sterilized using an autoclave then culture media which had been boiled was placed on the container or fermentation tanks. Once it was cooling enough, some starter bacteria *Acetobacter xylinum* was added, then it was fermented for 1-2 weeks and then washed repeatedly using distilled water and dried the membrane by using an oven temperature of 60 $^{\circ}$ C.

Membranes Manufacture of Bacterial Cellulose-Collagen Biocomposites. Manufacture of collagen-chitosan solution began by dissolving the collagen with variation in the concentration of gram of 0.4% (w/t); 0.5% (w/t); 0.6% (w/t); 0.7% (w/t) (0.4 g in 100 ml of acetic acid, 0.5 grams in 100 ml of acetic acid, 0.6 grams in 100 ml of acetic acid and 0.7 grams in 100 ml of acetic acid) then it was stirred for 30 minutes. After formed a homogeneous solution, the bacterial cellulose membrane which has been preconceived was immersed into the solution for 6 hours. Then, it was kept in the incubator temperature of 34 ^{o} C. The final sample would be Control Sample BC without addition of collagen, Sample A0.4% (w/t); Sample B 0.5% (w/t); Sample C 0.6% (w/t) and Sample D 0.7% (w/t).

Analysis of the Functional Groups *Fourier Transform Infra Red* (FTIR). This test conducted to analyze the functional groups and see if there is a chemical bond or physical bond by review the absorption (infrared radiation) at different wavelengths. Samples were characterized by infrared spectroscopy coming from the laser light reflected by the prism. Samples to be tested were cut small with the size of 0.1-1 mm, then they will be drilled to facilitate the preparation for the membrane-shaped sample. At the time of drilling, granules that were obtained later was placed on KBr. Samples'*infrared* spectrum was tested at wave number 4000-200 cm $^{-1}$

Mechanical Test. Mechanical properties test was done by cutting the membrane in the form of testing standard (ASTM 1822 L). Previously, measurement of membrane thickness had been done with digital micrometer screw or micrometer. The edge of the membrane then attached to the test equipment and towing load was mounted on kgf load units. The maximum weight imposed on the sample was 50N. The membrane was pulled at a certain speed until it was broken. After that, the difference in length and thickness were recorded.

SEM Test. SEM test was performed to determine the morphology of the surface and cross section of bacterial cellulose-collagen biocomposites membrane as a dural substitute. SEM Characterization will capture images *of* the *sample* surface so that the practitioner can easily identify whether variations in sample composition affect the surface and pore structure of the sample or not. In this test, samples were cut with a size of 0.5 mm to 1 cm. The observed sample's morphology was coated first with gold-palladium (80% Au and 20% Pd) and it required conductive *double-sided tape* to stick it. The coating process used ion *sputter* JFC 1100 - machine. After that the sample could be its morphology by using a scanning electron microscope (SEM Inspect S50 Fei Corp. Japan) with a magnification of 5.000x to 40.000x

***Swelling* Test.** Measuring the level of absorption: Membrane was weighed with electronic scales then soaked in distilled water to dry and weighed after 4 hours. Absorption rate (the average value of the six data in each group) were accumulated in the absorption level in accordance with the equation:

$$\% \text{ Absorption} = \frac{\text{Post hydrating mass- Pre hydrating mass}}{\text{Pre hydrating mass}} \text{ x } 100\% \qquad \text{(Equation 1)}$$

Results and Discussion

Analysis of the Functional Groups *Fourier Transform Infra Red* (FTIR)

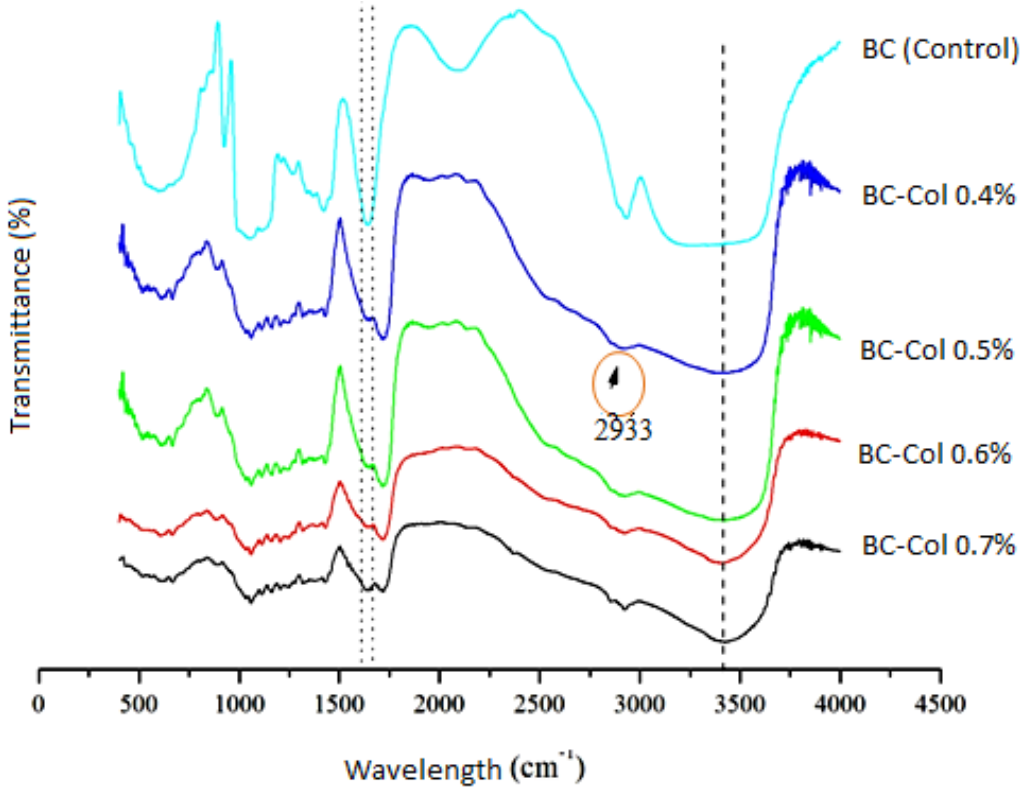

Fig. 1. Comparison of the IR spectra of Samples.

The absorption bands around 3100-3500 cm $^{-1}$ was found in all the IR spectra (Figure 1.). For all samples with collagen treatment, they will lead to peak formation which indicates that collagen binds functional groups that are free in the chemical bonds of bacterial cellulose [10].

FTIR also showed that there was a sharp peak which indicates the functional groups OH at wave number 2933 cm^{-1} of the control sample. Those peaks found widened in four samples with addition of collagen concentration. It suggests that hydrogen bonding was occured due to collagen bounded with C-H. From this results were obtained the peak wave number as presented in Table 1.

Table 1. Identification of Functional Groups on Samples.

Sample Type	Peak	Cluster Functions
Control	3340.82 2933.83	NH *stretching*; OH *bending* CH $_2$ *asymmetric stretching*
Sample A (SB-Collagen 0.4%)	3427.62 2920.32 1649.19	NH *stretching;* OH *bending* CH $_2$ *asymmetric stretching* C = O *stretching*
Sample B (SB-Collagen 0.5%)	3414.12 2922.25 1645.33	NH *stretching;* OH *bending* CH $_2$ *asymmetric stretching* C = O *stretching*
Sample C (SB-Collagen 0.6%)	3400.62 2924.18 1651.12	NH *stretching;* OH *bending* CH $_2$ *asymmetric stretching* C = O *stretching*
Samples D (SB-Collagen 0.7%)	3412.19 2924.18 1637.62	NH *stretching;* OH *bending* CH0-$_2$ *asymmetric stretching* C = O *stretching*

According Saska *et al* (2012) the interaction between bacterial cellulose and collagen can be seen by the shifted peak. Figure 1. Showed shifted peak 1649.19 cm^{-1} for sample A, 1651.12 cm^{-1} for sample B and 1645.33 cm^{-1} for sample C and on 1649.19 cm^{-1} for sample D. Four samples showed peak at identical wavenumbers of collagen which is describe functional group of CO *stretching* (Amide I) in the range of 1640 to 1690 cm^{-1}.

Mechanical Test. The analysis of *the mechanical* test results showed the elongation of the membrane, with the addition of collagen was able to increase the value of elongation. High elongation indicated the presence of collagen in the bacterial cellulose fibril. Those fibril produced better flexibility than pure Bacterial Cellulose (BC) or BC control. Thus, it can be used as potential implant material with easier applications for surgery, especially in terms of stitching using a suture thread.

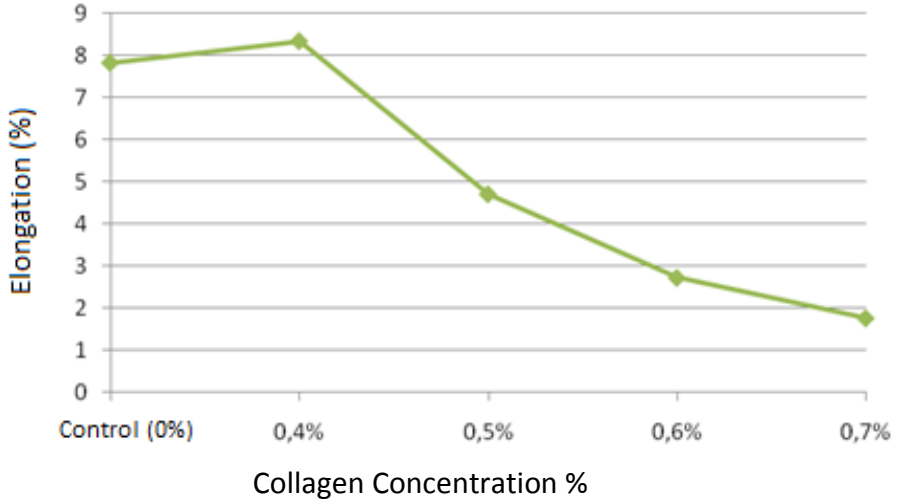

Fig. 2. The relationship between the concentration of collagen Graphics Rated Elongation.

The characteristics of biocomposites are affected by intrinsic properties of each constituent. It was capable to influence arrangement of oriented fiber to volume fraction of a material. Increasing concentrations of collagen which is closely related to the volume fraction would affect the properties of biocomposites.

Increasing the volume fraction will be increasing the mechanical strength within a certain range [8]. In accordance with this statement, in Figure 2. elongation value was increased to 8.33% in sample A (SB-Collagen 0.4%) compared to the control sample. While in sample B (SB-Collagen 0.5%) elongation value decrease at 4.70%. In sample C (SB-Collagen 0.6%) elongation value back down with a percentage of 2.72% and reached the lowest value in the figure of 1.76% in sample E (SB-Collagen 0.7%). This happened because the addition of collagen concentration has reached the optimum limit hence the sample become more rigid and less elastic**.**

Referring to the paper article of Zhuravlova *et al.* (2012), the 8% elongation is still within the threshold of human dura mater elongation value (7%-20%). Collagen has a complicated structure and it consisted of fibrils that allow the formation of a network that is capable of interacting with other molecules. Collagen is a protein that has a strong tensile strength *(Tensile Strength)* and good elongation strength [9]. According to data from the mechanical test results, it was obtained that best elongation values are showed in the control sample at 7.82% and SB-Collagen sample 0.4% at 8.33%.

SEM Test. Scanning Electron Microscope is a tool used to determine the microstructure morphology of a material. SEM is a test that is usually done to determine the pore size and thickness of a material. According to the samples test result, it was obtained that mechanical strength with elongation of the best values on the control sample and SB-Collagen sample 0.4%. The morphology of both samples will be observed by SEM to analyze their pore size and thickness.

Table 2. (a) Pore size and thickness of samples.

Sample	Pore Size	Thickness
SB	-	305µm
SB-Collagen 0,4%	1,5µm – 3,9 µm	215,6µm
SB-Collagen 0,5%	3,5µm – 4 µm	119,3µm
SB-Collagen 0,6%	1 µm – 5,3 µm	200,7µm
SB-Collagen 0,7%	0,69 µm	276,6µm

It was revealed that a picture of a control sample surface that tends to go undetected of its pore size. As seen in Figure 3 (a) a control sample (bacterial cellulose without treatment) had a smoother surface but the pore size could not be found, because when the magnification was enlarged until 50.000x to see the pore, the voltage of the device (SEM) is also automatically raised to 30 kv so that the sample would have burned.

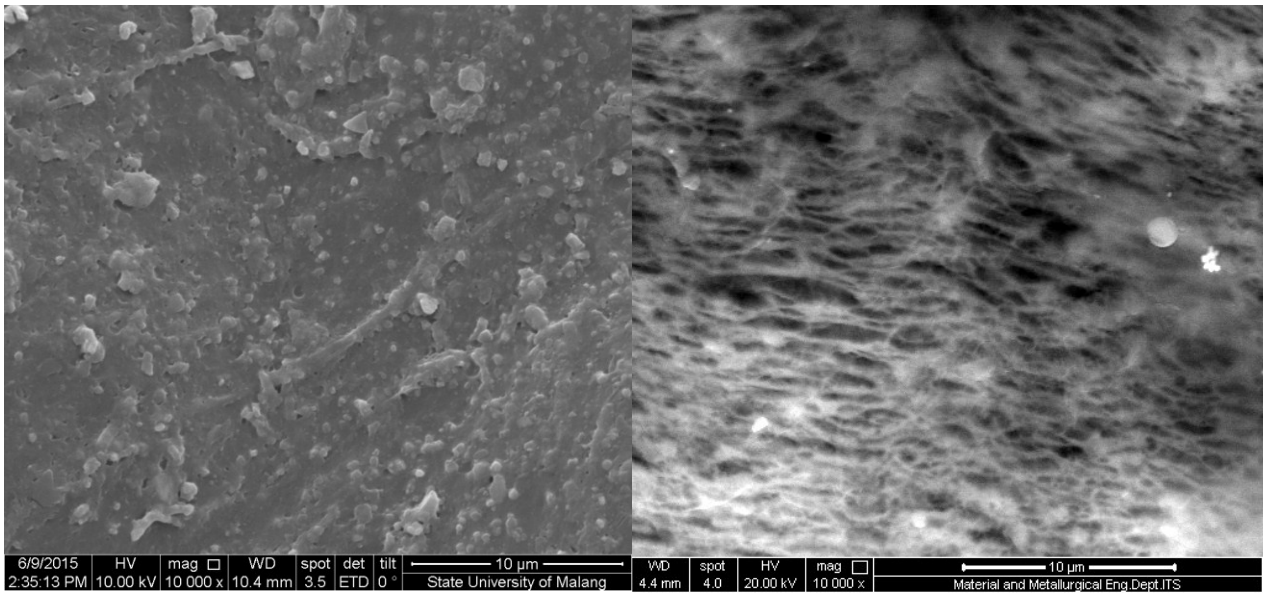

Fig. 3. (a) Sample Surface *(Cross Section Area)* Bacteria Cellulose-Collagen Control with a magnification of 10.000X (b) Surface *(Cross Section* Area) Bacteria Cellulose-Collagen 0.4% with a magnification of 10.000X.

In sample A (bacterial cellulose-collagen 0.4%) as shown in Figure 3 (b), the surface image consisted of yarn fibers that indicated a difference in sample surface between controlled bacterial cellulose and experiment samples. Bacterial cellulose that has been composited with collagen has a rougher surface, more complicated and more porous with pore size of 1.5 µm-3.9 µm compared with the bacterial cellulose control.

Bacterial cellulose-collagen composite has great potential as dura mater implant because it has adequate surface with pores under 22 µm and above 1 µm which allows *attachment* of cells and prevent fluid leakage [11]. Moreover, bacterial cellulose-collagen samples had been proven to be suitable for artificial dura mater (3 µm-10 µm). Those pore properties were able to help the regeneration and the growth of meningeal tissue around the artificial dura mater without leakage [3]. According to Figure 3 (b), it showed that the bacterial cellulose-collagen 0.4% with a magnification of 10.000X showe the structure of collagen that has been lining and filling the bacterial cellulose surface homogeneously.

Swelling Test. Swelling test was done to know the ability of samples to absorb liquid. Swelling test was conducted by soaking five samples into SBF solution (Simulated Body Fluid) for 1 hour to see the absorption properties of each sample. However, it was obtained that the control sample's swelling ratio is 59.86, while the value of sample A is 60.54%, sample B with swelling ratio of 71.63%, sample C with swelling ratio of 74.34% and sample D with swelling ratio of 61.56%. This was revealed that swelling ratios were high for all four treatment samples compared with control. After the addition of collagen, swelling ratio increased around 60% as shown in Table 3.

Table 3. Table Ratio *Swelling.*

Samples	**Swelling Ratio (%)**
Control	59.86
Sample A (SB-Collagen 0.4%)	60.54
Sample B (SB-Collagen 0.5%)	71.63
Sample C (SB-Collagen 0.6%)	74.34
Samples D (SB-Collagen 0.7%)	61.56

As illustrated in Table 3, there was extended swelling ratio compared to control samples. It was affirmed that collagen helps to increase the average value of *swelling* ratio as the presence of collagen is able to reproduce free hydroxyl and carbonyl functional groups [10].

The number of free hydroxyl functional groups will simplify the sample to bind with water which cause an increase in the sample's ability to absorb liquids. In addition, the SEM test results also show that the control sample has less pore than the treatment sample. This is consistent with the results of swelling in Table 3, which shows that the porous material will be able to increase its swelling ratio.

Summary

The elongation value resulted in sample with collagen concentration 0.4% m/v was increased. Then the elongation was then decreased concomitant with the increase in collagen concentration, which are 0.5% m/v, 0.6% m/v and 0.7% m/v subsequently. Meanwhile, based on the morphology test, it is showed that all treatment sample had pores and appropriate thickness compared with control sample. Best composition in terms of mechanical strength was found in sample with composition of bacterial cellulose and collagen 0.4%. Its elongation is 8% which met the criteria of dura mater artificial, the average thickness is 185 μm. As for the pore, the diameter ranged from 1.5 μm - 4.9 μm. Swelling ratio showed that every sample has been increased due to hydrogen bonding within the addition of collagen. However, the swelling ratio need the further study to obtain the optimal value.

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