Study of Bacterial Cellulose-PVA Nanocomposite as a Bone Tissue Scaffold

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Abstract. Bacterial cellulose is a natural polymer that can be produced from various substrates easily found in Indonesia. One of a great potential of bacterial cellulose is an application in bone tissue engineering that has an important role in development of regenerative medicine for osteoporosis and bone fractures, the two common bone problems in Indonesia. Hopefully such bone deceases can be cured by accelerating the regeneration of bone in human body (in vivo method) or growing bone outside human body (in vitro method), in which bacterial cellulose-PVA nanocomposites can be a bone tissue scaffold where cells can attach, proliferate and differentiate. In this study, bacterial cellulose-PVA nanocomposites were synthesized and its potential application as scaffold for bone tissue was evaluated. The synthesis was carried out by injecting PVA into *Gluconacetobacter xylinus* fermentation medium. The PVA concentration was varied as 0, 3, 6, 9, and 12% w/v of fermentation medium. The fermentation culture was stirred with magnetic stirrer for 28 days. The nanocomposite produced was then characterized by FTIR, XRD, DSC, TGA, BET, coating hydroxyapatite and SEM. Some of the sample were subject to an ultrasonic wave treatment and then studied its effect on the composite properties. The prospect of the material application as a bone tissue scaffold was evaluated by immersing the material in Simulated Body Fluid to know whether bioactive hydroxyapatite layer can be formed on its surface.The FTIR test revealed PVA mass fraction in nanocomposit increased from 22% to 48% with more PVA injected into the fermentation medium. The sonication treatment could enhance total surface area of nanocomposite. The SEM photograph confirmed that hydroxyapatite deposition on nanocomposite took place and the XRD test showed strong peaks at 2θ 31.82 and 45.58. These facts indicated that HAp was formed and hence bacterial cellulosa-PVA nanocomposite was a potential material for bone tissue scaffold.

Keywords: Bacterial cellulose; nanocomposite; hydroxyapatite.

Introduction

In the beginning tissue engineering techniques rely on the removal of tissue or organs from various sources. The removal of tissue or organ can be classified into three kinds of autologous (from the patient's own body tissue), allogenic (from another person's body tissue / donor), and xenogenic (from tissues of other species) [1]. Autologous have drawbacks due to the limited amount of tissue that can be taken. Allogenic have issues in

body rejection, disease transfer, and limited amount of tissue. The xenogenic, although the amount was much, but the risk of disease contamination and body rejection also become a bottleneck [2]. Based on these reasons, the development of scaffold framework that can be implanted in the body for supporting cells growth is very interesting.

There are six criteria for an ideal scaffold of tissue engineering: good biocompatibility and bioactivity to support growth of cells, having a high porosity; controlled biodegradable; displaying suitable surface for attachment, proliferation, and differentiation of cells; having suitable mechanical properties with the purpose, and easily processed to various shapes and sizes [3].

One material that has the potential to meet characteristics of the ideal scaffold is cellulose. Cellulose is biocompatible [4], has a fairly high tensile strength, has ability to store water in large quantities, high crystalinity, and easy to shape (mouldability) [5]. Cellulose can be generated from several sources such as animal, plant, bacterial, and chemical reactions. Out of all sources, the cellulose from bacteria or better known as bacterial cellulose having the best characteristics compared to cellulose from other sources in term of the material characteristic and production cost. Bacterial cellulose produced in the form of pure cellulose with no impurities (hemicellulose, lignin, wax, etc.), naturally has a nano-size, high tensile strength and has proven to be biodegradable and biocompatible. Bacterial cellulose also has a cheaper production cost than the fabric scaffold that had been developed previously such as collagen, PHA, PLA, etc. Another important feature is a bacterial cellulose degradation rate is proportional to the rate of cell growth in it [5]. This trait is important to keep the final form of the tissues or organs that are made to conform the shape of the original scaffold.

Bacterial cellulose is an attractive material to study for application in bone tissue engineering scaffold. It is simple to modify its properties by combining with other materials and cheap in the manufacturing costs. In this study bacterial cellulose was blended with polyvinyl alcohol (PVA) by the method of biosynthesis. These composites are expected to mimic the properties of collagen fibres so Hydroxyapatite (HAp) can be formed. The presence of HAp is necessary to initiate the formation of bone in the fracture gap and can eventually fill completely thegap [6]

Experimental

Biosynthesis

Bacterial cellulose-PVA nanocomposite was synthesized by biosynthesis method in which the fermentation medium was modified by the addition of PVA. Nanocomposite is expected to form when PVA is co-crystallized with bacterial cellulose produced by microbes. Culture medium used was Hestrin-Scramms Medium (HS medium) [7] with slight modifications in the original glucose concentration of 2 to 8% and the addition of urea as much as 0.5%. PVA was added to the medium with levels of variation as follows: 0, 3, 6, 9, and 12%.

Sterilization process was given to the medium and then a solution of nata de coco seed containing the bacterial suspension was added as much as 50 mL. Erlenmeyer was placed and stirred for 28 days at scale speed 1 using magnetic stirrer.

Purification and Drying

Nanocomposite formed in the medium was taken out by filtration. The filtered solids washed several times with distilled water. Bacterial cellulose-PVA nanocomposite was freeze dried and then stored in a desiccator.

Composition Analysis

Characterization was conducted to determine the percentage of bacterial cellulose and PVA in the nanocomposite and carried out using Shimadzu FTIR 8400 models. Several assumptions made in the characterization are as follows:

- Components in the nanocomposite are only bacterial cellulose and PVA
- The bacterial cellulose and PVA are only characterized by peaks at 1160 cm^{-1} and 850 cm^{-1}.

Percent mass of cellulose in the nanocomposite is calculated by Eq.(1) [8]

$$y = 268.2x / (1 + 3086\ x) \quad (1)$$

y = percent mass of cellulose in the nanocomposite

x = ratio of the absorption area at a wavelength of 1160 cm^{-1} and 850 cm^{-1} (A_{1160}/A_{850})

Sonication

Ultrasonic waves were applied to nanocomposite with PVA content in the medium 0 and 12%. Method of applying ultrasonic waves proposed by Tischer *et al*[8]. Nanocomposite was taken approximately 0.1 g of dried and then put into 200 mL of distilled water in Erlenmeyer and it was stirred for 15 min. The ultrasonic waves producer was sonochemical reactor set Transonic Elma with a frequency of 40 kHz. Every sample was treated for 30 min with 60% power. Nanocomposite re-dried by freeze drying then characterized.

Thermal Analysis

Thermal characterization (DSC and TGA) was carried out by Simultaneous Thermal Analysis (STA) 499F1 NETZSCH. Sample was placed in the aluminum pan standard in STA device. Temperature range is 25-400^{o}C at an increment rate of 10^{o}C/min. Nitrogen gas flowed at 80 mL/min to maintain the measurement condition.

Pore Characterization of Nanocomposite

This test is using a Nova ® Surface Area Analyser 3200 series. BJH isothermal adsorption measurements using nitrogen gas is used for analysis method. Applied outgassing time is 4 h at 150^{o}C. The data can be obtained from such testing is pore diameter and surface area per mass unit. Analysed samples were nanocomposite with the PVA content 0, 6 and 12 % in the medium

Hydroxyapatite Coating

The assay is carried out to determine whether bacterial cellulose-PVA nanocomposite can be coated with a Hydroxyapatite (HAp) to form a composite bio-ceramics. Nanocomposite samples were immersed in a ±10 ml of Simulated Body Fluid (SBF) for 1 w in a test tube. SBF was replaced with a new one every 24 h. Composite bioceramics will provide bioactivity to stimulate growth and differentiation potential of bone cells into bone cells [9]. If HAp can be formed, thisindicates that the nanocomposite has a great opportunity for further development as a Scaffold in bone tissue.

XRD

This test is done to ensure that the formed layer on nanocomposite is HAp. The scan range is 10-60^{o} with 6^{o}/min scan speed. Applied scan angle is equal to 2θ. HAp is characterized by a peak at an angle of 31.7 and 45.5^{o}.

Scanning Electron Microscopy (SEM)

Nanocomposite surface is viewed using SEM. By looking at the surface of the material, the size of the fibre diameter can clearly be observed. For the nanocomposite samples soaked in SBF for 1 week, the characterization of specific aimed to see whether there is a deposit of HAp in the composite matrix.

Results and Discussion

Nanocomposite FTIR Spectra

FTIR spectra of the nanocomposite are presented in Fig.1. OH stretching in the bacterial cellulose is shown by a peak at a wavelength of 3436 cm-1. The peak is slightly shifted from one sample to the other sample as follows: 3% PVA (3436 cm^{-1}), 6% (3400 cm^{-1}), 9% (3350 cm^{-1}), and 12% (3350 cm^{-1}). Fig. 1 shows that the greater the PVA levels in the

nanocomposite will be the broader the OH stretching. This phenomenon is caused by the intermolecular hydrogen bonding between PVA and bacterial cellulose [8]. Peak at a wavelength of about 1733 cm^{-1} is a C = O stretching is not expected to appear. However, the appearance of this peak is probably caused by the presence of unpolymerized vinyl alcohol of the PVA.

Nanocomposite Composition

According to Brown, cellulose and PVA composition can be identified by analyzing the FTIR spectra at wavelengths of 1160 cm^{-1} and 850 cm^{-1}[8]. Isotactic PVA vibrations detected by the peak at a wavelength 850 cm^{-1} [10]. This peak was observed in all composition variations of the nanocomposite and not observed in the spectrum of bacterial cellulose. Table 1 shows that the higher concentration of PVA in the medium is the greater PVA content in the nanocomposite.

Table 1. Nanocomposite composition.

% PVA in medium	A1160/A850	% Cellulose	% PVA
3	2.76	77.79	22.20
6	1.37	70.37	29.62
9	0.97	65.18	34.81
12	0.48	51.95	48.04

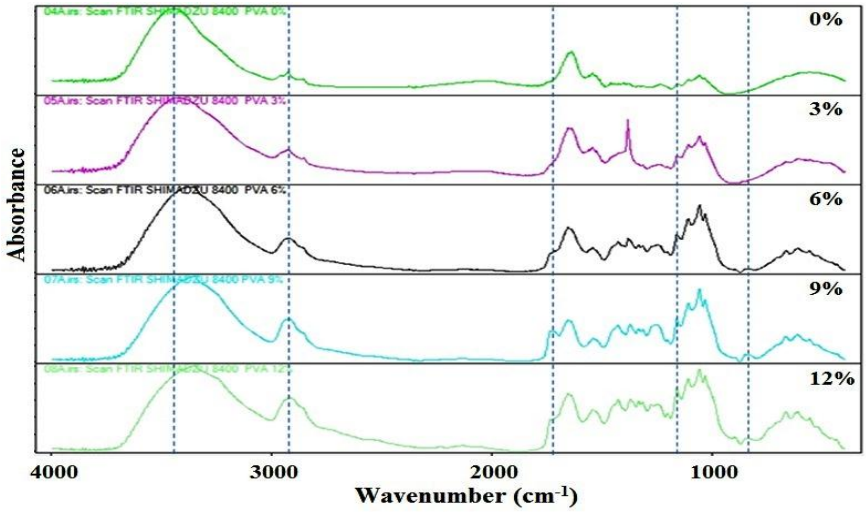

Figure 1. IR Spectrum of Nanocomposite. From up to bottom : 0%,3%,6%,9%,12% PVA in medium.

Thermal Characteristic

Thermal characteristic curves of the nanocomposite are presented in Fig.2 where the Tg, Tm and thermal stability of BC/PVA nanocomposit can be obtained. The melting temperature of PVA will be changed when combined with bacterial cellulose [11]. Fig.

3(a) revealed that BC degradation temperature did not change significantly. BC/PVA nanocomposite is expected to have good thermal stability when it is processed with thermal processing like compressed molding. It is important to make sure the material can be made into various shape with molding process. DSC curves (Fig. 3(b)) show that the pure cellulose (0% PVA) sample has two endothermic peaks and one exothermic peak. The first endothermic peak at 75.3°C is the thermal effect of water evaporation of the sample. The second endothermic peak (350.3°C) shows the sample melting temperature (T_m) [12]. The observed exothermic peak may be due to bacterial cellulose degradation that occurs at 358.3^{o}C. In the sample of pure PVA, melting temperature (T_m) observed at 181.3^{o}C. This result is consistent with observations made by Brown with the result of 184.5^{o}C [8]. Increasing PVA content in the fermentation medium decreases the melting temperature of BC/PVA nanocomposite from 350.3(PVA 0%) to 275^{o}C (PVA 12%). For the nanocomposite sample variation, the peak around the melting temperature (T_m) of Pure PVA disappeared except in samples 9 and 12%. This fact indicates that the PVA and BC was well composited by this biosynthesis method.

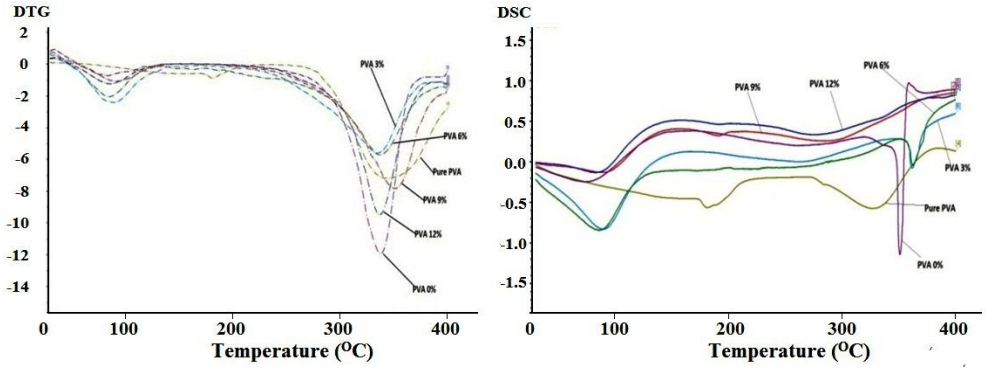

Figure 2.(a)DTG curve (b)DSC curve.

Pore and Surface Area Characterization

Table 2 shows data of pore and surface area characteristics. Sample with 0 and 12% PVA had been treated with sonication and this result in increasing surface area (SA) of the sample and decreasing the average pore diameter (APD). Possibly sonication had caused breaking down the cellulose fiber into sub fiber with smaller diameter. The addition of PVA may close the pores between the cellulose fiber. This case is clear when we compare sample with 0 and 12% PVA in which SA 17.2 nm and APD 29.2 nm decrease to be SA 9.9 nm and APD 20.8 nm. Sonication treatment can increase the surface area a little bit. This phenomenon is likely due to the cellulose fibers are covered and bound by PVA. It

was also possible that sonication caused cavitiesin the nanocomposite and make larger pores as shown by the fact that sonication of 12% PVA has produced a higher average pore diameter.

Table 2. Surface area and pore diameter.

Sample (%mass PVA in medium)	Treatment	Surface Area SA (m^2/gram)	Average Pore Diameter APD (nm)
PVA 0%	-	17.2	29.8
PVA 0%	Sonication	72.7	19.2
PVA 6%	-	6.9	19.2
PVA 12%	-	9.9	20.8
PVA 12%	Sonication	14.3	24

Morphology and Hap Deposition

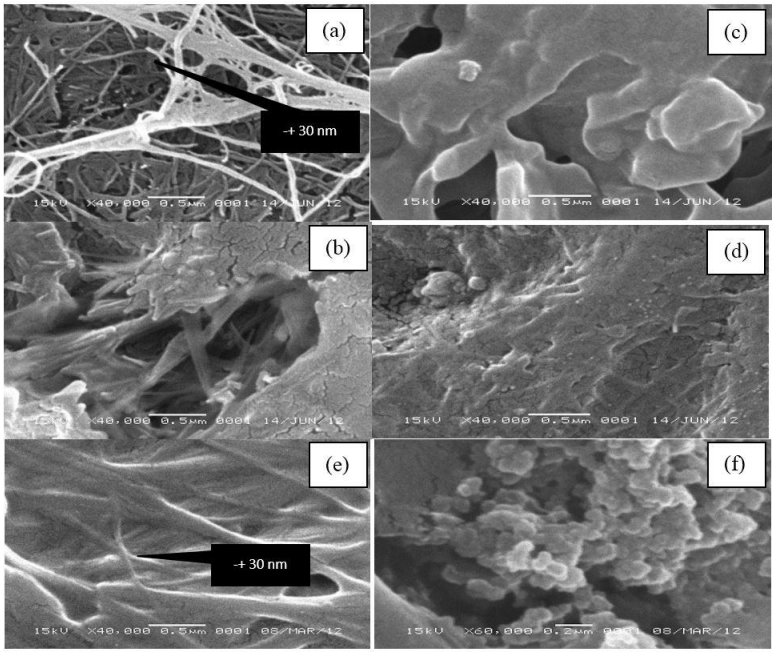

Figure 3: SEM image of nanocomposite.
(a)PVA 0%; (b)PVA 3%; (c)PVA 6%; (d)PVA 9%; (e)PVA12% ; (f)HAp Deposition

Fig. 3a or 3e shows that the smallest diameter of a single fiber that can be observed is about ±30 nm. This diameter is larger than that of previous observation 17 nm [8] and 4 nm [13]. The difference in the diameter size was possibly because small fibers might make aggregate and became fiber with larger diameter. Fiber with smaller size like previous observation was not observed in this experiment. This may come from a difference in the synthesis period. Fiber with diameter 4 nm was made after 5 d of culture lifetime the 17

nm was made after 10 d. With longer fermentation time, it is possible that cellulose fiber form aggregate and make a bigger fiber diameter.

The success of HAp coating can be observed from the SEM image in Fig. 3(f). Agglomerated sphere-like particle is HAp. HAp can form when SBF solution which contain calcium and phosphate ion contact with suitable substate. These results were confirmed by XRD test. Fig. 4 shows that the samples are soaked in SBF has distinctive peaks compared to sample not soaked in SBF. Two strongest peaks at 2θ are 31.82 and 45.58. Crystals with a typical peak close to the observations is deficient Calcium Hydroxyapatite crystal (cdHAp)[14]. cdHAp has a typical peak at 31.7 and 45.5^{o}. Formation of cdHAp on bacterial cellulose-PVA nanocomposite is an advantage. In the bone, cdHAp is thermodynamically easier to form than usual HAp due to the presence of hydroxyapatite ions in the body [6]. This nanocomposite may give a good performance in the bone regeneration in the body.

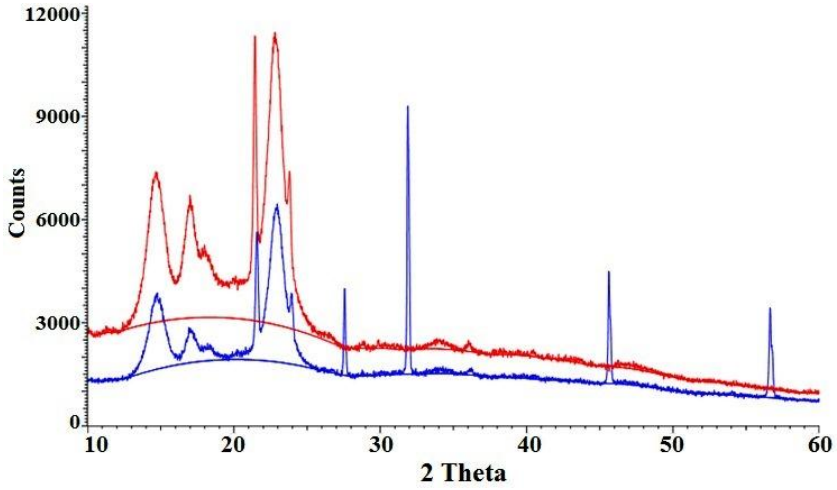

Figure 4 : XRD Spectra (sample : 0% PVA in fermetation medium)
(Red): without HAp deposistion; (Blue): with HAp deposition.

Conclusions

Introducing PVA into the fermentation medium has affect the thermal properties of the nanocomposite produced. Melting temperature (T_m) of the nanocomposite was decreased with increasing PVA content. BC degradation temperature (T_{deg}) did not change significantly by the present of PVA. The thermal test has also shown that the nanocomposite has good thermal stability.

Application of ultrasonic waves in the nanocomposite can increase the surface area per unit mass and increase the amount of pore with bigger size. Scanning electron microscopy

and analysis qualitatively using x-ray diffraction showed that hydroxy apatite successfully deposited in bacterial cellulose-PVA nanocomposite. Then the nanocomposite has a potential to be a bone scaffold.

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