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Composite of Chitosan-Collagen-*Aloe vera* for Scaffolds Application on Skin Tissue

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Abstract. In case of burns, scaffolds serve as cover for burns and facilitate cells regeneration. Accordingly, this study seeks procedures to synthesize composite scaffolds for burns utilizing collagen-chitosan-*Aloe vera* to find the best concentration of *Aloe vera* as scaffolds application. The synthesis of scaffolds was performed with collagen constituent composition as follow: chitosan (1:1) was dissolved in 0.05M Acetic Acid, then subsequent variations of *Aloe vera* (AV), namely 0% AV; 0.1% AV; 0.15% AV; 0.2% AV; and 0.25% AV were added into collagen-chitosan solution, and freeze dry method was applied. Sample characterization was done by FTIR, tensile strength test, SEM test, cytotoxicity assay and degradation test. Typical absorption bands of collagen in the FTIR test was obtained at 1645.5 cm^{-1} (C = O stretch), chitosan at 1540.28 cm^{-1} (NH stretch) and *Aloe vera* at 3474.41 cm^{-1} (NH stretch). Tensile test data showed the highest number of tensile strength from the 0.1% AV sample at 0.017 MPa. The SEM test revealed a pore size of <50 μm. During cytotoxicity assay, the entire sample is not toxic. Degradation test demonstrated that overall sample was not exhausted within two weeks. In conclusion, sample with 0.2% AV was potential as scaffolds for burns skin tissue.

Introduction

Skin is the largest organ in the human body due to its protective, perceptive, regulatory and aesthetic functions [1]. The research were performed in presenting new breakthrough in medical world related with the existence of artificial tissues and organs. Burke et al. reported the successful use of a physiologically acceptable artificial dermis in the treatment of extensive burn injuries with full thickness component on ten patients [2]. The aim of engineering new biological material is that it can restore or replace a damaged or diseased tissue and organs [3]. The two most important components that decide the fate of tissue engineered construct are cells and artificial extra cellular matrices (ECMs), also known as scaffold or biomaterials that support cellular growth, differentiation, and migration [4]. All the research were focused in the area of regenerative medicine [5]. Scaffolds are needed in facilitating vascularization which yield the closure of wounds with adjustable mechanical strength in order to facilitate tissue formation around implants.

Aloe vera (AV) has been used for years to treat burns [6]. AV serves as anti-inflammatory, skin protector, and also detoxifying agent [7]. This material was chosen as one of the composites due to its ability to modify mechanical forces between chitosan-collagen scaffolds by mixing the material with a natural polymer [8]. Other materials that can be used for the synthesis of scaffolds are collagen and chitosan. Collagen is a material that can be applied to the scaffold and has been used extensively in tissue engineering techniques for superior biocompatibility and biodegradability properties [9]. Chitosan is a natural biopolymer that has biocompatible properties, biodegradable and mechanical strength which can be used as a wound closure so that the substance is able to act as drug delivery and wound healing [8].

A previous research conducted by Rose *et al*. in 2013 []. used the composition of collagen:chitosan (1:1) and the addition of AV concentration (0.1: 0.2: 0.3: 0.4: 0.5 w/v) did not yield maximum results in its mechanical strength in which the AV concentrations of > 0.2% demonstrated poor mechanical strength so that the scaffold was perishable and its pores were non-uniform. The aim of study are to know the synthesis and characterization of composite scaffolds collagen-chitosan- Aloe vera and the best concentration of Aloe vera composite scaffolds collagen-chitosan. The scaffold must have ability to keep up infiltration and vascularisation, in addition the degradibility must also be control, it must have mechanical force which adjusted for facilitate the formation of a network in implant. The best AV concentration required in order to improve scaffold mechanical strength. The results of this research will be proven by several tests including Fourier Transform Infrared (FTIR), Scanning Electron Microscopy (SEM), cytotoxicity test, tensile strength test and degradibility test.

Material and Method

Materials

The equipments used in this study were petri dish of 60 mm^2, beaker glass, magnetic stirrer, measuring cups, spatulas, freeze dryer, aluminium foil, plastic wrap, 24-well cell culture plate, centrifuge, FTIR test equipment (4300 Shimadzu), SEM test tools (Inspect S50. FEI Corp., Japan), tensile test (Autograph Imada HV-500NII), MTT Assay (Elisa reader). The materials used were collagen, chitosan, acetic acid 0.5M, *Aloe vera*, PBS, and distilled water.

Methods

Synthesis of Aloe vera Extract

Mature *Aloe vera* was collected and separated from their skin (±400 grams), so only the clean and clear grains remained. Next, the homogenization was carried out to produce fiberless pulps by using Centrifuge at a speed of 10,000 rpm for 30 minutes at a temperature of 4°C. Results of Centrifuge is then freeze-dried and stored at -20°C [8].

Synthesis Collagen-Chitosan-Aloe vera Scaffold

Collagen and chitosan were dissolved in acetic acid. Collagen (1%) and chitosan (1%) were dissolved in 0.05 M acetic acid at a ratio of 1:1. Aloe vera was later mixed with chitosan-collagen solution with varying amounts, namely 0.1: 0.15: 0.2: 0.25% (w/v). The solution was then poured into a petri dish of 60 mm^2 and 24-well culture plates and stored at -70^oC freezer prior to freeze dry [8]

FTIR Test

FTIR test was used to examine the functional groups formed by using spectroscopy. The procedures performed in preparing samples on this test were mixing/grinding the samples with potassium bromide (KBr) in a ratio of 1:5 and observing the IR spectrum with wave lengths of 400-4000 cm^{-1} and a resolution of 0.7 cm^{-1} [8].

SEM Morphology Test

The addition of *Aloe vera* on collagen-chitosan would certainly affect the surface morphology of the synthesis results. SEM test equipment was used to observe the surface resulting from the scaffold synthesis [10]. Samples also needed to be coated with metal, namely gold [8]. The size of the stage holder is 12 mm or 25 mm. Contact area of the sample was made large in order to ease identification process. The sample was examined in the specimen chamber with size below 200 nm, and the result was captured in tiff image format. The sample preparation procedures for the SEM test were initiated by cutting the samples to a 10 mm size. The sample was then affixed to the double-sided tape conductive. The coating process was performed by using the Quorum Q150RS machine. Subsequently, the sample morphology was observed with a Hitachi TM3000 electron microscope (SEM) with a magnification of 1800-3000X.

Cytotoxicity Test

Cytotoxicity could be used to examine at the proliferation level by immersing cells in scaffold then cells fibroblasts are observed on the day 1,2 and 3. Variation time chosen because by previous studies, the influence of the addition of AV seen in the day 3rd .Living cells are then read on ELISA reader [8]. 100 µl cell are put on each plate with appropriate density and one plate devoid of cells culture as control. Cells are incubated for single night, and added 10 µl solution MTT for each plate then incubated in 4 hours in temperature 37°C. After incubation, apply closure with tin foil and stirring cells with orbital a shaker for 15 minutes then absorption level reading. Cytotoxicity Test or commonly called the MTT- assay is done to see how high the level of the cell life when given scaffold. Cell viability can be calculated using the equation:

$$\% \text{ Living Cell} = \frac{\text{treatment absorbance} + \text{media control absorbance}}{\text{control absorbance} + \text{media control absorbance}} \times 100\% \qquad \text{(Equation 1) [11]}$$

Degradation Test

Degradation test is a required parameter to observe the time taken by degraded scaffolds after new tissue formation [12]. This test was performed by immersing synthesized scaffolds into a solution of PBS pH 7.4 at body temperature (37°C) for 2 weeks [13]. The results of the immersion can be used to obtain biodegradability data according to the following equation:

$$\% \text{ Biodegradability} = \frac{w_0 - w_t}{w_t} \times 100\% \qquad \text{(Equation 2)}$$

w_0 is the initial mass and w_t is the mass after soaking with PBS [10].

Tensile Test

This test is conducted to examine the elasticity of scaffolds upon receiving external tensile force. Preparation was done by forming dog-bone shaped samples with the same thickness (500 mm x 100 mm x 5 mm), and the gage length is set to the length of 15mm [14].

After being cut into their appropriate shapes, the samples were placed on tensile test machine holder and the test speed was set at 5mm/min [8]. The data obtained from the test results were recorded, and then used to obtain the tensile strength based on the following equation:

$$\text{Stress } (Nm^{-2}) = \frac{F\ (N)}{A\ (m^2)} \times 100\% \qquad \text{(Equation 3)}$$

F is the force received by the sample at the break and A is the cross-sectional area of the sample.

Results and Discussion

Collagen - chitosan - Aloe vera composite scaffolds have been made previously by the synthesis of Aloe vera extract. Aloe vera are centrifuged until the separation of ears and fluid happened. Then liquid from of Aloe vera is achieving freeze dried. The freeze dried Aloe vera in the form of gel was put into chitosan-collagen (1 : 1) with solvent 0.05 m acetic acid and variation of aloe vera 0.1; 0.15; 0.2; 0.25 % wt. Solution of collagen-chitosan- Aloe vera are stirred until homogen. Then it is poured on petri dish for the next freeze dry. The result form resembling sponge with colour brownish white and grainy texture on a smooth surface as could be seen in Figure 1.

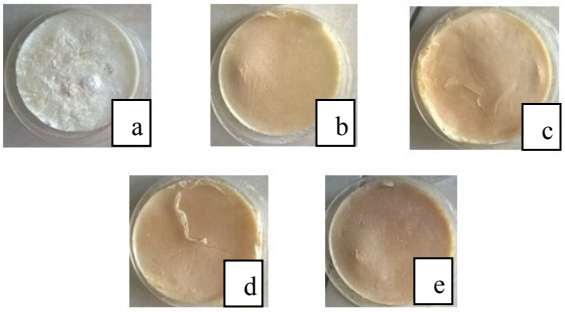

Figure 1. Scaffold samples with AV Variation of (a) 0% (b) 0.1% (c) 0.15% (d) 0.2% (e) 0.25%

Fourier Transform Infra Red (FTIR) Test Result

Scaffolds were characterized to observe the synthesis results. FTIR test results were presented in Figure 2 below:

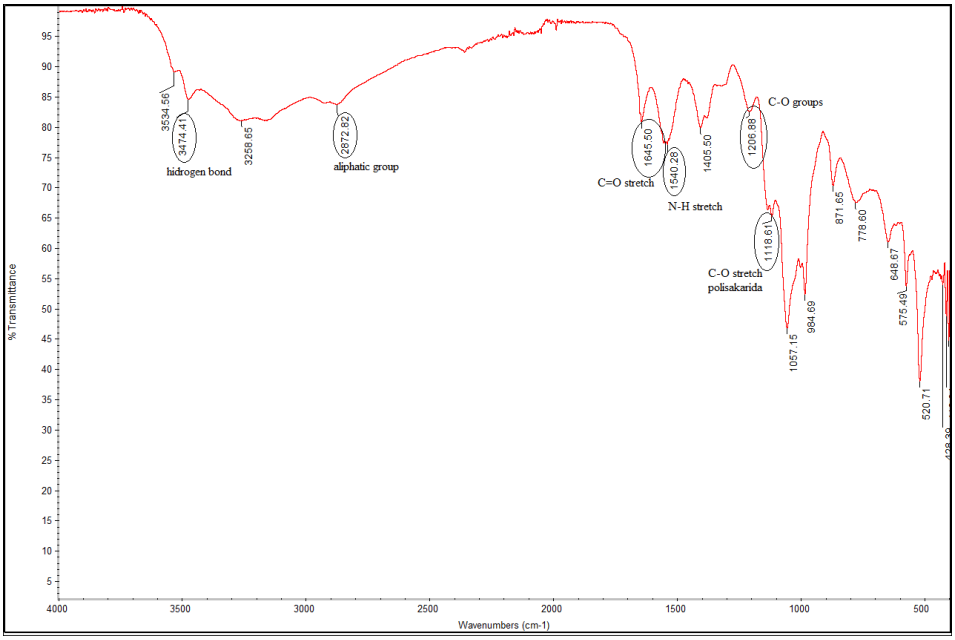

Figure 2. The Scaffolds IR spectrum 0.1% *Aloe vera*

Based on the analysis of FTIR testing, the existence of AV affected the typical absorption bands for each material of the scaffold. The existence of *Aloe vera* (AV) was characterized by the hydrogen bond in the absorption band at 3474.41 cm^{-1} which is a distinctive group of AV due to N-H stretch. Cluster of aliphatic groups was contained in the absorption band at 2872.82 cm^{-1}. Amide group I indicated the presence of collagen on the absorption band at 1645.5 cm^{-1} which is C = O stretch. Collagen also found in numbers absorption 3250,25 cm^{-1} which is a cluster of O - H. It found also group C - O on 1207,39 cm^{-1}. Amida II cluster characterizes chitosan with absorption tape 1539,63 cm^{-1} which is N - H stretch. On absorption tape 2913,98 cm^{-1} is found aliphatic group. The existence of AV affect absorption tape of each material building of scaffolds. Aloe vera characterized in hydrogen bonds on the absorption 3474,41 cm^{-1} that is unique cluster caused by N - H stretch. A cluster of aliphatic group are still visible on AV 0.1 %, but this time the absorption tape is found in 2872,82 cm^{-1}. Collagen and chitosan are still can be read on AV chart. 0.1 % AV is also still found the group C – O which represented the stretching vibration of esther and fenol. In FTIR results with 0.1% AV, there was another typical band at 1118.61 cm^{-1} that was C-O stretch of polysaccharides. All the absorption bands described earlier were still in the range of research conducted by Rose *et al.* in 2013 [8].

Tensile Test

The subsequent test is tensile test. The graph of tensile test results is presented in Figure 3 below:

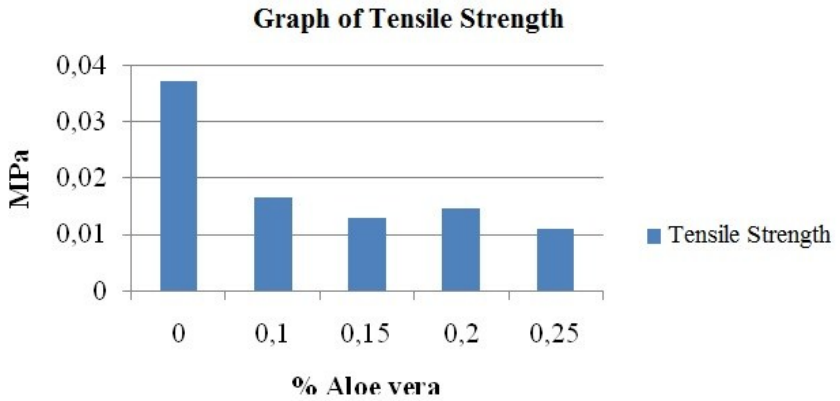

Figure 3. Graph of Tensile Strength Results

The 0% AV sample had the highest value during the tensile test of five samples. This means that these sample able to withstand the load from the outside, higher than four other sample. This might occur due to the bond between collagen and chitosan, namely the interaction of H-bond/ionic among functional groups of -OH, -COOH, and -NH2 in the collagen, as well as -OH and $-NH_2$ groups in the chitosan. Meanwhile, 0.1% AV, 0.15% AV, 0.2% AV, and 0.25% AV samples were more easily broken upon receiving external load, and had a tendency to decrease as the AV content increased on
chemical bonds between collagen and chitosan, wherein the higher the percentage of AV, the lower the tensile strength is [8]. The important thing is that as scaffolds which play role in wound healing, not only the mechanical strength which needed but also the other characteristics such as surface structure, pore size, pore distribution and degradibility.

SEM Morphology Test

The morphology test is used to examine the surface morphology of the sample as could be seen in Figure 4.

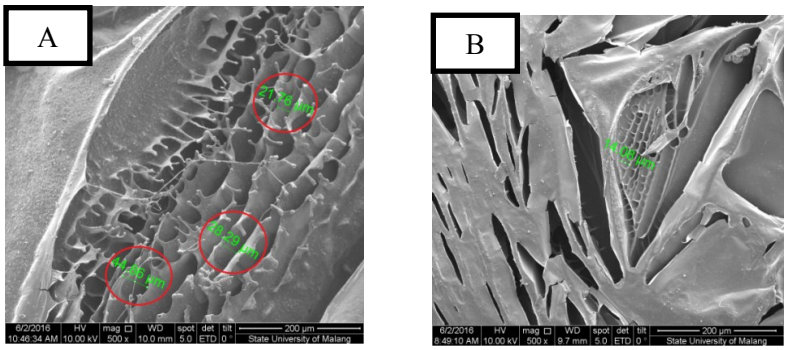

Figure 4. Samples of 0% AV (A) and 0.1% AV (B) (500X magnification)

At 500X magnification, the experiment obtained scaffold pore sizes from sample A at 21.76 µm; 28.29 µm; and 44.86 µm with an average of 31.64 µm. Based on the results of the SEM review, In samples A the pore distribution is easier to observe and in samples B with 0,1 % AV the pore distribution is hard enough to observed. This is related to size of each sample pore itself. Where, aloe vera the cause the decline in size pore, besides it uniformity pore also difficult to gain. The cause of heterogenous pore are because of compound glycoprotein , saccaride, vitamin (B1, B2, B6, choline, folic acids, ascorbic acids, α-tocopherol, β-carotene) [15] and the anti oxidant able to attract growth of cells and influence the scaffolds wall [8]. Based on the results of SEM, sample B with 0.1 % AV has size pore smaller than sample A with 0 %AV, this outcome its not based on human skin size pore in range 20-125 μm [16].This could be caused by freeze dry process, where size pore very influential during the freezing process. The longer of clotting time will produce pore wider [17].

Cytotoxicity Test Result

The next test to observe cell viability was cytotoxicity test, and the results are presented in Table 1 below:

Table 1. Cytotoxicity Test Results

AV Concentration (%)	Living Cell
(A) 0	55%
(B) 0.10	56%
(C) 0.15	72%
(D) 0.20	70%
(E) 0.25	70%

Table 1 shows the percentage of living cells as samples were added in 96 well plate during cytotoxicity test. Sample without Aloe vera (AV) (A) (0% AV) resulted in the lowest cell viability or 55% state that over the 50% of cell viability is still regarded to be non-toxic; therefore, sample A (0% AV) was not toxic because it contained collagen and chitosan that could regenerate new tissue and form granulation tissue [11]. Samples B, C, D, and E had higher percentages of living cells than sample (A) (0% AV), in which the more AV contained in the sample, the higher the percentage of living cells resulted. Danof and McAnalley in Rose et al, 2013 [8] also suggest that the glycoproteins in the AV are capable of stimulating cell proliferation and the growth of human fibroblast tissue; hence, the presence of these substances can help to accelerate the wound healing process. In addition, Davis in Oryanet al, 2010 state that vitamins (B1, B2, B6, Choline, Folic Acid, Ascorbic Acid,α -Tocopherol, β-Carotene) and Essential Amino Acids in AV also help to improve cell viability.

Degradation Test Result

The test is done to explore the length of time needed to decay sample when applied on the human body. PBS is liquid which is similar to the fluids of the body. Observation time of this research are 2 weeks , the observation is made on 7th day and 14th day. Not only mass missing observation, but pH observation is also carried out to make neutral pH of the medium. PBS replacement in each vial were done every week [13]. On the 7th day sample dried in each vial, then calculation of mass is done. Sample is start to degraded, but are still in sheets of scaffolds. On 14th day, some samples experienced vulnerability, and it was difficult to taken from vial and some of scaffolds was very difficult to observe. The last test performed is degradation test whose results are presented in Figure 5 below:

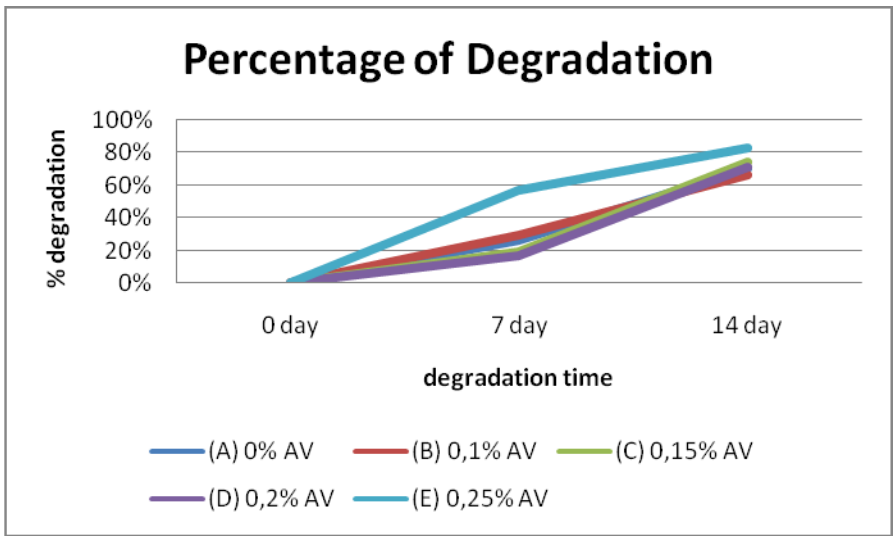

Figure 5.

Degraded masses from the five samples tended to increase alongside the increase of degradation time. Based on these data, the overall sample was not exhausted within 2 weeks. This is because the existence of chitosan content that reaches 50% of the total volume of the collagen-chitosan solution has enabled scaffold mass to survive [18]. In addition, the presence of AV also affected degradation. Therefore, AV is able to reduce the activity of the enzyme in breaking down collagen molecules [8].

On the 12th day after the burn, substances that serve to heal burns and histocytes including fibroblasts would be stimulated. As the substances were stimulated within the body, the presence of scaffold would then provide a place for cell proliferation. On the 14th day, the research sample result still remained on the PBS media; therefore it corresponds with the time required for cell regeneration, which takes 12 days [19].

Five samples having tendency increased degradation mass as long as increased time degradation..Based on this data, whole sample not discharged in two weeks,because chitosan content who reached 50 % from total volume of collagen – chitosan solution [18]. In addition, the existence of AV also affect the degradation. AV capable to reduce the enzyme activity in break up the collagen molecules [8]. On the day 12^{th} after burns, fibroblasts and histocytes are releasedand scaffold is ready for proliferation [19].

Conclusion

Composite of Chitosan-Collagen-*Aloe vera* for Scaffold was successfully synthesized as the Aloe vera formed beige sponge-like scaffold sheets with a rough texture on a smooth surface. Identification result of the scaffold was presented an absorption band of N-H stretch with typical AV at 3474.41 cm^{-1}. Collagen was characterized with C=O stretch by amide group I at 1640.94 cm^{-1}. Chitosan is characterized by the N-H stretch at 1540.28 cm^{-1}. The more AV content, the lower the value of its tensile strength was. Meanwhile, sample performed best in tensile test with AV addition was the 0.1% AV sample, with a score of 0.017 Mpa and a pore size of 17.61μm on SEM test. MTT Assay proved that the samples were not toxic, with the best percentage of living cells was 72% for 0.15% AV sample. Degradation test showed that the sample has not been exhausted at > 12 days, which corresponds to the time required for cells to regenerate. Within two weeks, the 0% AV sample (A) was degraded by 71%; 0.1% AV (B) by 67%; 0.15% AV (C) by 74%; 0.20% AV (D) by 71%, and 0.25% AV (E) by 83%. Scaffold with 0.2% Aloe vera demonstrated the best results of the overall AV variations based on the 0.014 MPa in tensile strength test review, 70% of living cells in cytotoxicity test, and 71% of degraded masses in degradation test for two weeks.

Scaffold is needed for the supporting structure for wound healing. Scaffolds are play important role as a template for tissue formation that would be seeded with cells and occasionally growth factor which could stimulate many biochemistry process underlaying the healing. The composite of chitosan-collagen-Aloe vera could be a very good composite while each 3 base material have proper characteristic for good scaffold. Collagen which biocompatible and support the mechanical strength; chitosan which biocompatible, biodegradable and have antibacterial effect and Aloe vera which act to support the mechanical strength, act as detoxifying agent and has antiinflamatory effect could be promising biomaterial for treating wound and accelerating wound healing.

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