Composition Variation of Chitosan-Gelatine Scaffolds with Glutaraldehyde Cross linker for Skin Tissue Engineering in Burn Wound Cases

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Abstract

Burn wound is one type of trauma that has high morbidity and mortality. Skin tissue engineering for acceleraterepair of tissue damage in burn woundis needed The purpose of this research was to determine the best composition of the chitosan-gelatine from variation of chitosan-gelatine scaffolds with cross linker glutaraldehyde by the result of characterization of scaffolds. The making of scaffold was conducted by freeze drying techniques. The results of FTIR showed crosslinking between $-NH_2$of chitosan and gelatine with C=O inglutaraldehyde. Tensile test was obtained at range 0,041-0,068 MPa value, all samples have tensile strength over tensile strength of the scaffold for skin application, which is 0,04 MPa. The morphology test resulted the diameter size of sample C and F, from 16,48-84,64 µm that met to pore size of scaffold for skin tissue engineering application. The cytotoxicity test demonstrated non-toxic scaffold with average of living cells above 90%. The degradation test obtained a value of 18,65%- 86,17% of the degraded weight during 21 days of observation. Based on the results of FTIR, tensile strength test, morphology test, cytotoxicity test and degradation test, chitosan-gelatine and glutaraldehyde scaffold is a potential candidate for skin tissue engineering in skin burn cases.

Experimental article (J Int Dent Med Res 2018; 11(3): 778-785)

Keywords: Scaffold, Chitosan, Gelatine, Glutaraldehyde, Skin tissue engineering.

Received date: 18 November 2017 **Accept date:** 24 January 2018

Introduction

Soft tissue, such as skin, has an important functional role in the body. In clinical practice, there are some common skin defects due to burns, chronic wounds and trauma. Burn wound is one type of trauma that has a high morbidity and mortality. Based on data obtained from a medical record at Burn Center Dr. Soetomo Hospital from the period of January 2011 to December 2013, it was found that there were 435 patients consisting of 272 male patients (62.5%) and 163 female patients (37.5%), with the number of death 28.[1] In Cipto Mangunkusumo Hospital, Jakarta, the number of burn patients treated from January 2011 to December 2012 was 275 patients, 203 of them were adults. It was recorded in the hospital that the number of deaths among the patients of all ages was 93 patients (27.6%). Among the patients who died, 78% were caused by fire, electrical burns (14%), hot water (4%), chemicals (3%) and metal (1%) withalmost all the area of the burn woundswere deep dermal burns (Grade 2) and full thickness (Grade 3).[2]

Burn wound triggers uncontrolled inflammation and suppresses the immune system that is likely to cause infection, sepsis and multi-organ failure with a high mortality rate.[3] The handling and treatment of burns to this day still require complex treatments such as taking a long time and multiple surgeriess. Therefore,we need a treatment to speed up the repair process of tissue damage particularly those occured on burns grade 3.

The clinical treatment of skin defects treatment typically focuses only on skin tissue transplantation.[4] In the past, various types of skin substitutes have been applied to the treatment of skin defects such as xenograft, allograft and autograft.[5] Nevertheless, allograft and skin transplants have caused such serious problems

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as the refusal of the patient's body by the immune system and the allowance of the presence of infection or disease from donor patients.[4] As a result skin tissue engineering techniques began to be used for the alternative solution to treat skin damage cases.[6]

In general, the material that is used for skin tissue engineering should have a high absorption power, biocompatibility and anti-bacterial properties to protect the skin from infection, dehydration and tissue damage.[7] One of the important factors in skin tissue engineering is the scaffold structures.[5] As one of the important factors in skin substitutes, scaffold gives extracellular matrix. Recently, the use of natural material in tissue engineering has expanded, one of which is chitosan.

Chitosan is a product with high molar mass results from the deacetylation of chitin, a polysaccharide which is the second largest material abundant in nature.[8] Chitosan has many functions for healing wounds such as homeostasis, accelerate tissue regeneration, and itcan also be applied in skin tissue engineering.[5] This is because chitosan is a biocompatible, biodegradable, non-toxic and anti-bacterial content.[9] However, one limitation of chitosan is that it is a fragile material, so it needs to be combined with other materials to be developed into the corresponding tissue.[7]

Gelatine is a polypeptide results from the hydrolysis of collagen triple helix structures. Gelatine and its derivatives are non-toxic, biocompatible and biodegradable. Therefore, gelatine has the potential to be used in biocomposites synthesis with various substances included, such as organic molecules, drugs and nanoparticles.[7]

The mechanical characteristics one of the important factors in the scaffold. The mechanical strength of Scaffold chitosan-gelatine can be enhanced by adding cross linker such as glutaraldehyde.[10] Glutaraldehyde has been widely used to crosslink biopolymers for medical applications.[11] Crosslinking method is one of the important parameters to create a scaffold.[12]

In the previous research that has been done by Han et al[7], it is shown that chitosan-gelatine scaffolds with composition (3:7, 5:5, 7:3) have similarporous structures with pore size between 120-140 µm and high porosity. It also has high water absorption capacity and is biocompatible against cell adhesion and proliferation of cells that can be developed as skin tissue engineering material. In addition to these characteristics, there are other important factors through the addition of glutaraldehyde cross linker mechanical properties. The mechanical property is one important factor in skin tissue engineering, in which the skin should have the ability to resist the pull and pressure during the recovery process of the wound.[12] Thus, this research was conducted to evaluate the mechanical characteristic of the scaffold by analysing the mechanical characteristic through tensile strength test.

Based on this background, it is necessary to study the production of chitosan scaffolds with the variation of composition chitosan-gelatine (3:7, 5:5, 7:3) with glutaraldehyde as cross linker as skin tissue engineering for burn wound cases. the characterization of chitosan-gelatine scaffold composition variation with glutaraldehyde cross linker as skin tissue engineering for burn wound cases through an analysis of functional group using instrument of Fourier Transform Infrared Spectroscopy (FTIR), a surface microstructure test using Scanning Electron Microscopy (SEM) instrument, cytotoxicity test with MTT assay, analysis of physical characteristic through degradation test, and analysis of mechanical strength through the tensile strength test. By adding the tensile strength test, it is expected to find that the resulting scaffold has suitable mechanical characteristics for skin tissue engineering.

Materials and methods

The equipments used in this study includes a digital scale, glass beaker, magnetic bar, magnetic stirrer, measuring cups, spatulas, freeze dryer, a 24-well cell culture plate, aluminum foil, plastic wrap, horizontal shaker, FTIR (4000 Shimadzu), SEM (Inspect S50, FEI Corp., Japan), Tensile Strength with Autograph Imada HV-500NII), MTT Assay (Elisa reader). The materials used are chitosan, gelatine, acetic acid 1% v/v, glutaraldehyde 0.25% w/v, distilled water, a solution of Phosphate Buffered Saline/PBS including NaCl, KCl, Na_2HPO_4, KH_2PO_4.

Glutaraldehyde fluid was made by dissolving 1 ml of glutaraldehyde (0.25% w/v) in 100 ml of distilled water and mixed distirrer) to achievehomogenity.[7] Synthesis Scaffolds from

Chitosan- Gelatine- Glutaraldehyde were produced by making liquid of chitosan- with a variety of different compositions 3:7, 5:5, 7:3, and the total weight for both materials was 0.6 grams. For example, to make liquid of chitosan-gelatine at a ratio of 5:5, it was preceded by dissolving 0.3 grams of chitosan and 0.3 grams of gelatine in 27 ml of acetic acid liquid (1%, v/v) and were mixed using a magnetic stirrer at a temperature of 40 °C, and then added 3 mL of glutaraldehyde (0.25% w/v). Then, it was stirred with a magnetic stirrer to achieve homogenity for 2 hours. Homogeneous liquid was placed on a petri dish and then in freeze dryer at temperatures below -80 °C. The same process were done for the variety of other compositions.[7] The variation composition were labelled with the condition below : Sample A with Chitosan:Gelatine 3:7 with glutaraldehide, sample B = chitosan : Gelatine 5:5 with glutaraldehide, sample C = Chitosan : Gelatine 7:3 with glutaraldehide, sample D = Chitosan:Gelatine 3:7 without glutaraldehide, sample E = Chitosan:Gelatine 5:5 without glutaraldehide, sample F = Chitosan:Gelatine 7:3 without glutaraldehide

Fourier Transform Infra Red (FTIR) test

The tests using Fourier Transform Infrared spectroscopy (FTIR) aimedatdetermining the clusters formed from the resulting sample and also predicting the reaction of polymerization occurs.[15] IR spectroscopy test was done in a way to grind together scaffolds with KBr. The mixture was then pressed into pellets into solid. The process resulted in the form of the IR spectrums illustrate the value of % transmittance and wave number, and therefore any functional groups contained in the scaffolds can be known.

Morphology Test using Scanning Electron Microscope (SEM)

To find out the micro-structures of the scaffolds, the surface morphology and cross-sectional view of the scaffolds were taken through SEM test.[14] By doing this test, SEM pore size and thickness formed from chitosan-gelatine scaffold with glutaraldehyde cross linker would be known. In the test sample, it was coated with ultrathin layer of gold. Samples coated were observed using SEM (Inspect S50.FEI Corp., Japan) at a voltage of 5 kV in the scaffold surface.

Cytotoxicity Test

In the cytotoxicity test, there were several stages as follows. First, fibroblast cell tissue preparation was done in laminar flow. BHK-21 cell tissue in monolayer with Eagle's medium and FBS 5% was planted in roux tissue bottle and then incubated at 37 °C for 48 hours. Next, the cell tissue was washed with PBS for 5 times to remove any residual serum left. Trypsin versene was then added to detach the cell from the wall of the bottle and separate the bond among cells that do not gather.[15]

Cells with a density of $2x10^5$ were included in the 100 µL medium (media eagle's 86%, penicillin-streptomycin 1%, fungizone 100 units/mL), and then transferred into 96-microwell plate in accordance to the sample amount and control. Each sample was sterilized with UV light for longer than overnight, then 0.05 gram sample was dissolved in 1 ml of ethanol. The blended liquid sample was then put in 96-microwell plate in a 50µL. Then it was incubated for 24 hours at 37 °C.

MTT 5 mg/ml reactor which has been diluted in PBS was added to the medium with 10 µL to each well and then incubated for 4 hours at 37 °C. The DMSO solvent was added to each well with 50 µL and then centrifuged 30 rpm for 5 minutes. The optical density (OD) of formazan was measured by Elisa reader at a wavelength of 630 nm.[15]

Degradation test

Degradation test was measured through weight change of the sample at a certain time and the sample was placed on a fluid-like condition of human body fluid. The Scaffold samples wereall at the same size and weight. The initial weight of the sample was (W_0). The samples were immersed in 5 ml of Phosphate Buffered Saline (PBS), pH 7.4 at 32 °C. In every seven days, the samples were taken from the medium and then dried and recorded as dry weight (wt). The degradation test was conducted for 28 days and observed on days 0, 7, 14, and 21.[7]

Tensile stength test

An important parameter of the mechanical characteristic that was measured and observed

from a chitosan-gelatine-scaffold with glutaraldehyde cross linker is the pull test by using a tensile strength machine with Autograph Imada HV-500NII. The determination of mechanical characteristics was done by cutting scaffold with a dogbone-shaped. Rectangular-shaped test specimens measured about 4 cm x 1 cm x T, where T is the thickness of the scaffold, cut from each sample and pulled at a constant speed of 2 mm/min until breaking.[14] The tensile strength of the scaffold can be seen from the value of its load and stroke. The load value (kgf) refers to the tensile strength at break, whereas stroke (mm/ min) refers the tensile strain at break. The load values and stroke values are usually inversed.[16]

Results

Chitosan - gelatine- - -glutaraldehyde scaffolds were synthesized by mixing chitosan-gelatine with a variety of different compositions 3:7, 5:5, 7:3, with the total weight for both materials was 0.6 grams. Then, the synthesis was added with 27 ml of acetic acid fluid (1%, v/v), and 3 ml of glutaraldehyde (0.25% w/v) was mixed with a magnetic stirrer at a temperature of 40 °C for 2 hours. Anon-glutaraldehyde samples were also made as a control. Then the sample was printed and put in freeze drier to -80 °C for subsequent samples were characterized using several tests. Freeze drying process was needed for the formation of pores in the scaffolds: the texture of the surface of the scaffold seems fine so the pores are not so visible.

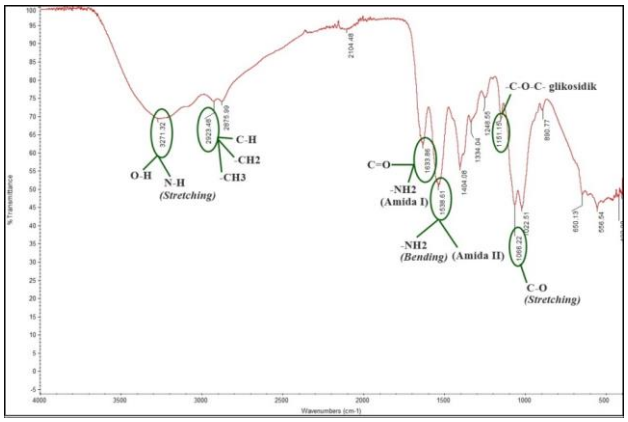

Figure 1. Spectrum Chitosan Infrared of Gelatine with Glutaraldehyde with Ratio 7:3.

Chitosan typical absorption peak at the wave number 3271.32 cm^{-1} is a group of the hydroxyl group (OH). Aliphatic group (-CH_2 and -CH_3) lies in the absorption wave 2923.48 cm^{-1}. At the peak absorption wave number 1633.86 cm^{-1} has a functional group C = O stretching. In the absorption wave number 1538.61cm^{-1} is -NH2 bending. Uptake wave 1151.15 cm^{-1} is -C-O-C- glycosidic which is a connection between chitosan monomer. C-O group stretching from primary alcohol groups are shown in the absorption wave number 1066.22 cm^{-1}. Typical absorption peak of gelatine lies in the absorption wave 3271.32cm^{-1} which is a group of the hydroxyl group (-OH). NH group stretching absorption peak is not seen in the infrared spectra due to the fact that the absorption spectra overlap with the OH group stretching. In the absorption wave 1633.86 cm^{-1} there is Amida I. The presence of amide II is shown in absorption wave number 1538.61 cm^{-1}. Glutaraldehyde absorption spectrum has a characteristic in the aldehyde group (C = O) 1633.86 cm^{-1}.

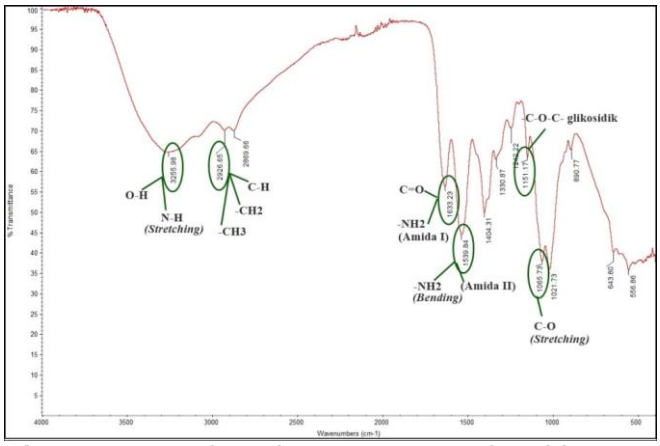

Figure 2. Infrared Spectrum of Chitosan: Gelatine 7: 3 + 0 ml of Glutaraldehyde.

The results showed that the most dominant interactions between chitosan molecules, gelatine molecules and glutaraldehyde molecules are chemical interactions. It is characterized by the emergence of imine bond (-C = N) at wave number 1620-1680 cm^{-1}.

Morphology test result

Scanning Electron Microscopy (SEM) test was conducted to determine the morphology of the scaffold surface. This test is more specifically carried out in this study to compare the scaffold of chitosan-gelatine-glutaraldehyde cross linker and chitosan-gelatine scaffold without

glutaraldehyde cross linker through the observation of the surface and pore size. SEM tested samples were the samples of the scaffoldswith the highest value of the tensile strength, namely sample C and F. The measurement of the scaffold pore diameter is shown in Figure 3 and Figure 4.

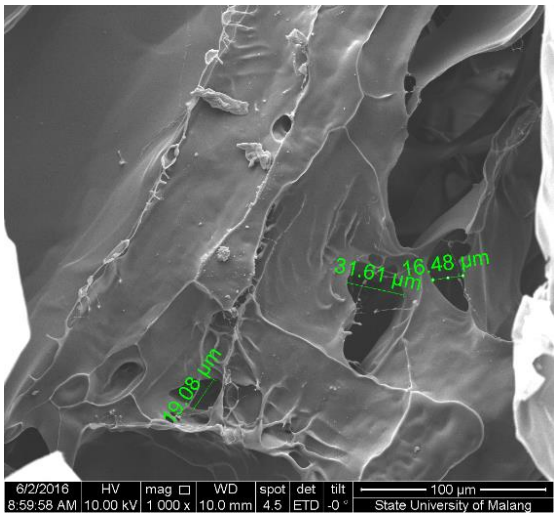

Figure 3. SEM test results of scaffold sample with a variety of chitosan: gelatine 7: 3 with glutaraldehyde (Sample C).

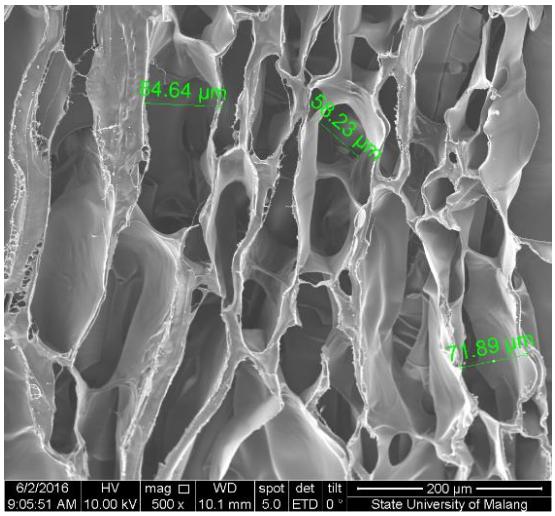

Figure 4. SEM Test Results of Scaffold Sample with A Variety of Chitosan: Gelatine 7: 3 Without Glutaraldehyde (Sample F).

The measurement results of the pore diameter showed that the pore diameter of the scaffold increased at thescaffolds without cross linker. In sample C, the variation in the composition of the chitosan-gelatine 7:3 with glutaraldehyde, it is obtained that the range of pore is 16.48-31.61 µm. In sample F, the results of the scaffold without cross linker and with chitosan-gelatine composition variation 7: 3 without glutaraldehyde showed an increase in the size range of pores from pore diameter of 56.23 to 84.64 µm.

Cytotoxicity Test Result

Cytotoxicity test or MTT assay is a cytotoxicity test with enzymatic test using MTT reagent. MTT-assay (3- [4.5-dimethylthiazol-2-yl] -2,5diphenyltetrazolium bromide) is based on the conversion of MTT into formazan crystals by living cells, which determines the activity of mitochondria.[17] The test is performed using hepatocytes cells, and there are medium control and cell control. Medium control is the mixture of eagle medium, whereas cell control is composed of hepatocyte cells and Eagle. MTT Assay results from the test were then read using Elisa Reader. The results of cytotoxicity test, indicate the percentage of living cells with a scaffold sample composition variation chitosan-gelatine glutaraldehyde, as shown in Figure 5.

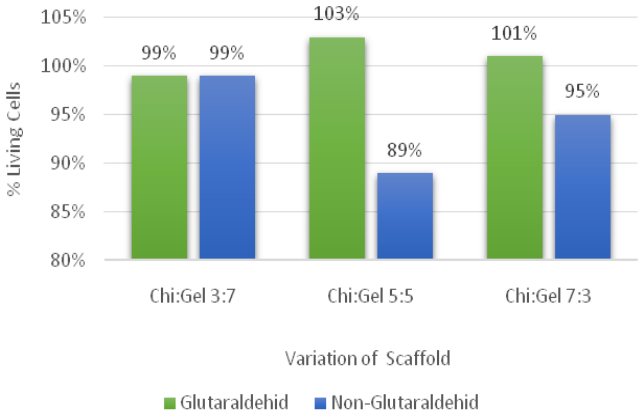

Figure 5. Graph Relationship between% Living Cells Against Variation Composition of Scaffold.

Figure 5 shows the results of the calculation of any samples that have been through the process of testing and reading using Elisa Reader. The percentage of living cells obtained for sample A, B, C, D, E, and F were respectively 99%, 103%, 101%, 99%, 89%, and 95%. The sixscaffold samples showed values above the standard value of the toxicity of a sample.

Degradation Test Result

This degradation test is a form of simulation when the scaffold is applied to the skin. Degradation test is measured through changes in sample weight at a certain time and the sample is placed on a fluid-like condition of human body fluids. [7] The rate of degradation of the material will determine the length of the time the material remains in the body. [18] Being degraded does not mean loosing directly, but the scaffolds shed gradually as time wound healing. Material degradation can be said to occur when there is a decrease of material weight.The testing method in vivo or in vitro was conducted with a medium of Phosphate Buffered Saline (PBS) liquid. [19]

In Figure 6, the scaffolds without glutaraldehyde cross linker were most rapidly degraded. The existence of crosslinker at chitosan chain will protect the scaffold from degradation. [14] Scaffolds without crosslinker have larger diameter pore size so that they will more quickly degrade.

Tensile Stength Result

An important parameter of the mechanical characteristic measured and observed from a scaffold is the tensile strength to determine the tensile strength of the scaffold sample. For this reason,the tensile strength test or tensile test was conducted.

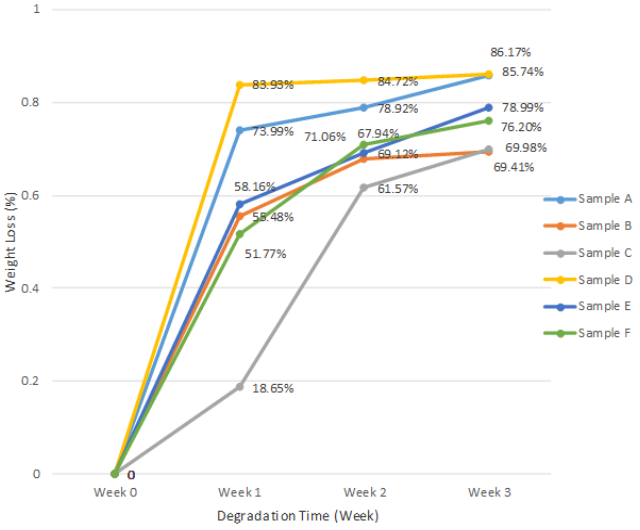

Figure 6. Graph of the relationship betweenn percentage of degradability to the PBS soaking length time on Composition Variation of Scaffolds.

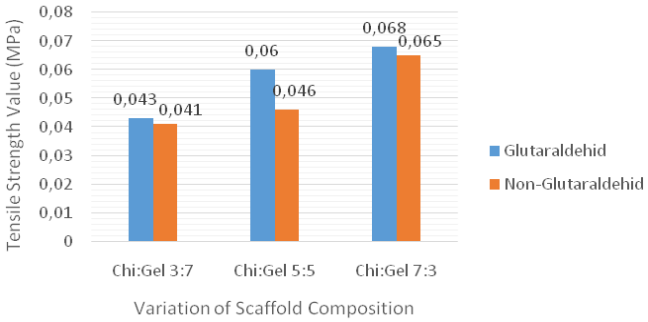

Figure 7. Graph Relation Between Tensile Strength Values to the Various Composition of Scaffolds.

Figure 7 shows the relationship between the value oftensile strength to various composition of the scaffolds. From the test results, the values of tensile strength of each variation of the scaffold composition were obtained. Sample A has a tensile strength of 0.043 MPa. Sample B has a tensile strength of 0.060, and a row for the samples C, D, E, and F amounted to 0.068 MPa; 0,041 MPa; 0.046 MPa and 0.065 MPa. The values of the sixth tensile strength samples were better than those of the tensile strength of scaffolds found on the research conducted by Liu et al,[12] which amounted to 0.04 MPa. The increase of the tensile strength value is comparable with that of chitosan composition. Chitosan has a unique porous formation that can increase the absorption capacity of liquid and cell interaction thereby the mechanical strength has increased.[7] Another factor that affects the tensile strength is cross linker. From the Figure 3, it can be seen that a sample with glutaraldehyde cross linker has a tensile strength value higher than that without glutaraldehyde.

Discussion

Based on the comparison of the spectrums in FTIR, it is known that there is no significant difference between the spectra of chitosan-gelatine with glutaraldehyde cross linker and without cross linker. However, there was a shift wave number at typical functional group. There was a shift in the wave number range of typical OH absorptions due to changes in the location of the hydroxyl group (OH) on chitosan produced from the reaction of free radical formation in group O and binds to the aldehyde group (C = O) in glutaraldehyde. [20]

According the morphology analysis, the smaller the pore size in the sample, the stronger the tensile test of the materials.[21] This is because the glutaraldehyde cross linker on a scaffold produce a smaller pore size. The size of the pores in the scaffold can be influenced by the temperature at the time of freeze drying stage. Temperature regulation in the process of freeze drying at a constant rate from room temperature to a final temperature of freezing will improve the uniformity of the pore diameter of the pores on the scaffold. The pore size of sample C and F meet the pore size of the scaffold for skin tissue engineering applications of 20-120 µm.[22]

All of the percentage of living cells scaffold samples were showed above 50%. The value corresponds to what was expected, because the sample is said to be non-toxic when the percentage of living cells is over 50%. [23] The scaffold samples are not toxic because chitosan is biocompatible and not toxic. [14] In addition, gelatine and its derivatives are also non-toxic and biocompatible so that it has the potential in the synthesis of biocomposites with various substances.[7] Again, the use of glutaraldehyde cross linker concentration below 8% is notably non-toxic. [11]

Scaffold A and D where the composition of the gelatine were higher, were degraded faster. It can be noted that the more gelatine composition on the scaffold the faster the degradation time, while the more chitosan composition the longer the time of degradation. Chitosan has lower degradation time because it has long chain.[24] Thus, the degradation time of the scaffolds is influenced by the composition of the chitosan and gelatine. Based on the degradation test results of the chitosan scaffold, gelatine and glutaraldehyde degraded 86.17% for a maximum of 21 days of observation. Degradation length of time on these samples is in accordance with a process of regeneration of skin tissue from burn wound that commonly requires within a period of 17-21 days. [25]

The increase of tensile strength value generates higher chemical resistance. The increase of tensile strength was caused by the crosslinking between $–NH_2$ on chitosan and gelatine with group C = O in glutaraldehyde. However, the six samples--when compared to standard tensile strength to scaffold of human skin on the abdomen based on the research conducted by Annaidh et al,[26] have not met the range of values that is1-24 MPa UTS. Thiscould possibly be due to the unoptimum use of the concentration of crosslinker. In fact, increasing concentrations of glutaraldehyde will also increase the tensile strength. [17] The crosslinked scaffold is compounding material to obtain suitable scaffolds that could be applied well on tissue regeneration.[27]

Conclusions

The characteristic of functional groups of variation composition chitosan-gelatine scaffold with glutaraldehyde crosslinker by of Fourier Transform Infrared Spectroscopy (FTIR) test showed a crosslinking bond between chitosan with gelatine and chitosan with glutaraldehyde. Tensile strength values increased along with the increase of chitosan composition. In the morphology test by SEM, it is shown that the samples had a pore size that met the standard of the scaffold pore size for skin tissue engineering applications. Cytotoxicity test (MTT Assay) showed that the scaffolds were not toxic to the living cells on average above 90%. In addition,the degradation test showed that all samples can be degraded in the time range of regeneration process. The best chitosan – gelatine - glutaraldehyde variation composition is 7: 3 based on the parameter of the functional group, tensile strength, morphology, cytotoxicity status and degradation level. This variation composition is quite promising as scaffold for burn wound cases, despite it still need the further research to optimalize all the parameters.

Acknowledgements

The author would like to thank to the Material Physic Laboratory, Chemistry Laboratory, Faculty of Science and Technology Universitas Airlangga, Pharmacy Faculty Universitas Airlangga, Gastroenterology Laboratory in Institute of Tropical Disease Universitas Airlangga, Central Laboratory of Universitas Negeri Malang and Material Engineering Laboratory Sepuluh November Technology Institute for support in material synthesis and characterization.

Declaration of Interest

The authors report no conflict of interest and the article is not funded or supported by any research grant.

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