Characteristics of Composite Scaffold Chitosan-Chondroitin Sulphate / Hydroxyapatite as the Candidate of Bone Graft

Aminatun[1,2a*]; Tia Rahayu Wijayanti[1b]; Dolfi Varton[2] and Prihartini Widiyanti[1,2c]
[1] *Department of Physics, Faculty of Science and Technology, Universitas Airlangga Kampus C UNAIR-Jl. Mulyorejo-Surabaya-60115- Indonesia*
[2] *Biomedical Engineering, Post Graduate School, Universitas Airlangga- Kampus B UNAIR-Jl. Airlangga 4-6, Surabaya-60286-Indonesia*

Keywords: *Scaffold, Chitosan, Chondroitin Sulphate, Hydroxyapatite, Bone Graft.*

Abstract: Bone defect can be addressed by using bone graft, one of them is in the form of scaffold. Tri-component scaffold consisting of chitosan, hydroxyapatite and chondroitin sulphate is more effective for tissue engineering since it is biocompatible, non-toxic, and biodegradable and additionally, it can be absorbed by the body. The purpose of this study is to know the effect of adding chondroitin sulphate and to determine the best composition of chitosan-chondroitin sulphate/hydroxyapatite as the candidate of bone graft. The process of composite scaffold synthesis used a freeze dry method. The result of composite scaffold chitosan-chondroitin sulphate/hydroxyapatite was obtained through characterization which covers tests on functional group, morphology, porosity, biodegradability and cytotoxicity. The result of a functional group test revealed that there is a stretching vibration of NH_2 on 1638.35 cm^{-1} as a typical group of chitosan, stretching vibration S=O chondroitin sulphate on 1384.41 cm^{-1}, stretching vibration of P-O-C on 1089.34 cm^{-1}, 1050.38 cm^{-1} and 602.36 cm^{-1} are the functional groups of phosphate (PO_4^{3-}) hydroxyapatite. The result of a morphology test obtained pore size range from 26-239 µm which is appropriate as the ideal scaffold pore size. The result of a scaffold porosity test was 90.06 – 93.48% which suited the porosity of cancellous bone. The result of a biodegradability test showed the decrease of mass on each sample which ranged from 27.149 – 60.658% during 4 weeks. The result of a cytotoxicity test revealed that the five samples of the composite were non-toxic toward cells. Based on these characteristics, composite scaffold chitosan-chondroitin sulphate/hydroxyapatite has a potential to be the bone graft with the best variation on samples with hydroxyapatite:chitosan:chondroitin sulphate composition of 50:35:15 wt%.

1 INTRODUCTION

The result of basic research by the Ministry of Health Indonesia showed the comparison of injury escalated in prevalence from 7.5% becoming 8.5% from 2007 until 2013 (RISKESDAS, 2013). Fractures happened due to traffic accidents which reached 24 million cases per year and those caused by osteoporosis reached 350,000 cases per year (KEMENRISTEK, 2014). There are more than 2.2 million cases of bone-grafting in one year in all parts of the world. In Indonesia, the growing need for biomaterials was four times larger and the need for bone grafts will always increase along with the increase of bone defects caused by trauma, tumors, congenital abnormalities, infection and so forth (Ferdiansyah *et al.*, 2011). Nowadays, orthopedics mostly uses bone graft from natural bone such as *autograft*, *allograft* dan *xenograft* (Darwis *et al.*, 2008).

Scaffold is one bone graft which is able to provide a condition needed by cells to proliferate and maintain each function (Humatcher and Dietmar, 2000). Bone scaffolding is a temporal matrix for skeletal growth and provides a specific sphere and a construction form in regards to developing system (Schieker *et al.*, 2006). Osteoblasts and chondrocyte could grow on scaffold which will be absorbed by the body carefully and grow as new skeletal tissue (Humatcher and Dietmar, 2000). The ideal scaffold has three dimensional characteristics and is porous with pore tissue which is interconnected as a place to grow cells and transport the flow of nutrients and

Aminatun, ., Wijayanti, T., Varton, D. and Widiyanti, P.
Characteristics of Composite Scaffold Chitosan-Chondroitin Sulphate / Hydroxyapatite as the Candidate of Bone Graft.
DOI: 10.5220/0007540602360243
In *Proceedings of the 2nd International Conference Postgraduate School (ICPS 2018)*, pages 236-243
ISBN: 978-989-758-348-3

metabolical waste. A scaffold must be biocompatible and bioresorbable with controllable degradation levels and absorption levels which are suitable for the growth of cells or tissue. Additionally, scaffold must have mechanical characteristics which are appropriate for tissue located in implantation areas (Humatcher and Dietmar, 2000). Thus, the biomaterial of bone scaffold is a potential alternative as the improvement technique of bone defects caused by trauma, tumor resection, and abnormal development (Mitsak *et al.*, 2011).

The process of making a bone graft which is based on a composite scaffold has been conducted by several researchers, i.e. Venkatesan *et al.* (2008), by synthesizing the composite scaffold chitosan, chondroitin sulphate, and hydroxyapatite. Chitosan was chosen since it has some characteristics of biocompatibility, biodegradability, and also it was expected to be able in shaping pores and be an appropriate media for the cells' growth. Since the ideal scaffold provides a suitable atmosphere for cell proliferation, it is necessary to add materials which could support the process of cell proliferation, such as hydroxyapatite (HA). The biggest potential of bone substitution indicated by HA is the ability to build strong connection with skeletal groups, which is osteoconductive and stable toward biological absorption and preventing bad impacts for humans (Orlovskii *et al.*, 2002). The study showed that the additional chondroitin sulphate with collagen/hydroxyapatite caused the increase of skeletal remodeling, new bone construction, and osteoblast differentiation (Venkatesan *et al.*, 2012). Therefore, by adding chondroitin sulphate, it was expected that the composite scaffold chitosan-chondroitin sulphate/hydroxyapatite could be a bone graft which could stimulate the cell growth and accelerate the process of the skeletal remodeling process. The research will be conducted by using composition variations of hydroxyapatite, chitosan, and sulphate chondroitin with comparison (A) 50%:50%:0%, (B) 50%:40%:10%, (C) 50%:35%:15%, (D) 50%:30%:20% and (E) 50%:25%:25% from the total mass. The objective of this research is to know the effect of adding chondroitin sulphate and determine the best composition of composition variation of chitosan-chondroitin sulphate/hydroxyapatite as the candidate of bone graft.

2 RESEARCH METHOD

(w_1), the weight of scaffold and ethanol which are being marinated (w_2), and the final weight of

2.1 Materials

The materials used are commercial hydroxyapatite produced by Tissue Bank of Dr. Soetomo Hospital Surabaya, chitosan with 70% DA, the synthetic result of Bogor Agricultural Institute, chondroitin sulphate by Interlab CV, 2% acetic acid, 10% NaOH solution, distilled water, ethanol and dehydrated alcohol and the making of *Simulation Body Fluid* (SBF) solution by using $K_2HPO_4.3H_2O$, $CaCl_2.2H_2O$, NaCl, $NaHCO_3$, Na_2SO_4, KCl, HCl, $MgCl_2.6H_2O$ and $(HOCH_2)_3CNH_2$.

2.2 The Synthesis of Scaffold Composite of Chitosan-Chondroitin Sulphate/ Hydroxyapatite

The solution of chitosan-chondroitin sulphate/ hydroxyapatite which has been prepared was moved into a pot bottle. To create a scaffold, the solution was frozen at -80° C temperature for 5 hours. After that a process named *freeze-drying* was done to the frozen solution for 30 hours.

After the *freeze-drying* process, the sample of composite scaffold chitosan-chondroitin sulphate/hydroxyapatite was marinated in 10% NaOH solution for 24 hours to neutralize the acetic acid residual present in the sample. After that, it was cleansed by using equades until it reached the neutral pH. Next, freeze-dying was done once again to relieve the water wastes in the composite sample of chitosan-chondroitin sulphate/hydroxyapatite scaffold.

The next process is the characterization covering functional group testing with a *Fourier Transform Infra-Red* (FTIR) spectrophotometer American Perkin Elmer Co, morphological surface testing by using a *Scanning Electron Microscope* (SEM) inspect S50, FEI Corp., porosity test, biodegradability and cytotoxicity test by using MTT assay.

Porosity test was done by using a fluid displacement method. During the test, the sample of composite scaffold which would be used was initially weighed to find the initial weight of the sample. After that, the sample was marinated in 98% ethanol for 48 hours. After marinating, the scaffold sample was re-scaled along with the ethanol to find the weight of marinated scaffold in ethanol. The last step was measuring the ethanol whose sample has been taken over. The final result of porosity testing was acquired from the initial weight of the scaffold ethanol after the scaffold was taken over (w_3). Then, the percentage of porosity of each composite

scaffold sample was then calculated by using Equation 1.

$$Porosity = \frac{W_1 - W_3}{W_2 - W_3} \times 100\% \quad (1)$$

Biodegradability in-vitro test was done by marinating the composite scaffold sample in a *Simulated Body Fluid* (SBF) solution. In this test, the composite scaffold sample was immediately measured to see the basic weight of the scaffold. Further, the sample of composite scaffold was marinated in a *Simulated Body Fluid* (SBF) solution for 4 weeks. The data of the biodegradability in-vitro testing result was collected on 1st, 2nd, 3rd, and 4th week. In each data collection, the scaffold sample was dried initially, a while later it was weighed to reveal its final weight after being marinated. The data acquired from the test was in the form of the initial weight (w_0) of each composite scaffold and the final weight (w_1) after marinating process. The percentage of degradation or lost mass acquired from the data was calculated by using Equation 2.

$$W_L = \frac{(W_0 - W_1)}{W_0} \times 100\% \quad (2)$$

The cytotoxicity testing was conducted by using MTT Assay consisting of tetrazolium salt [3-(4,5-dimetiltiazol-2-yl)-2,5-difeniltetrazolium bromide]. The systematic principal of the MTT *Assay* method is following the ability to live of the living cell based on mothocondrial activities of cell culture. The changes happened on tetrazolium salt [3-(4,5-dimetiltiazol-2-yl)-2,5-difeniltetrazolium bromide] which became formazan in an active mothocondria as the base of the MTT Assay method. The living cell will change MTT which was then cracked through the reduction of reductase enzym in a chain of mothocondrial respiratory system to formazan which was dissolved in purple. The bigger the absorbance, the more the living cells calculated by using Equation 3.

$$\%\ Cell\ Viability = \frac{ODT - ODM}{ODC - ODM} \times 100\ \% \quad (3)$$

where ODT = OD treated cells, ODM = OD media control and ODC = OD cells control.

3 RESULTS AND DISCUSSION

3.1 The Analysis of Functional Group by Using *Fourier Transform Infra-Red* (FTIR)

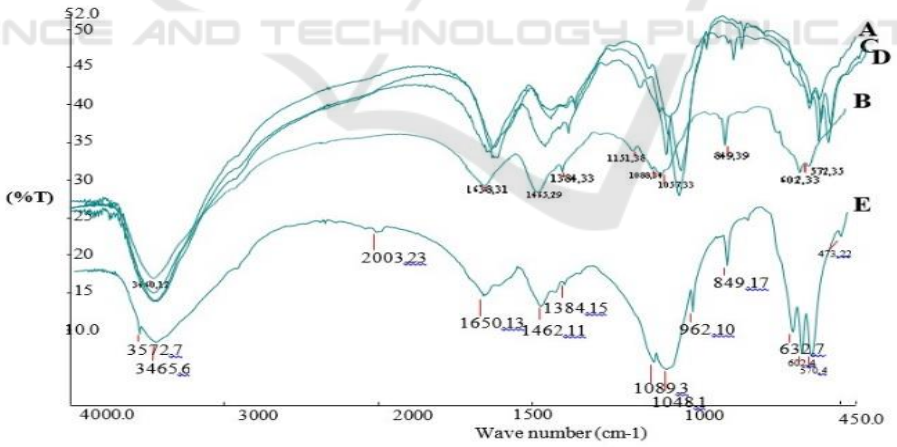
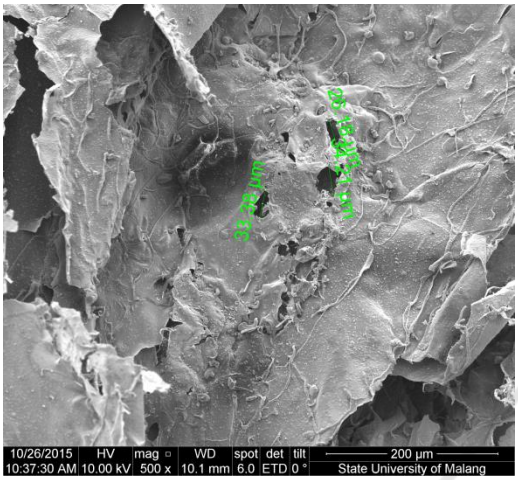


Figure 1: FTIR Spectrum of Composite Scaffold Chitosan-Chondroitin Sulphate / Hydroxyapatite.

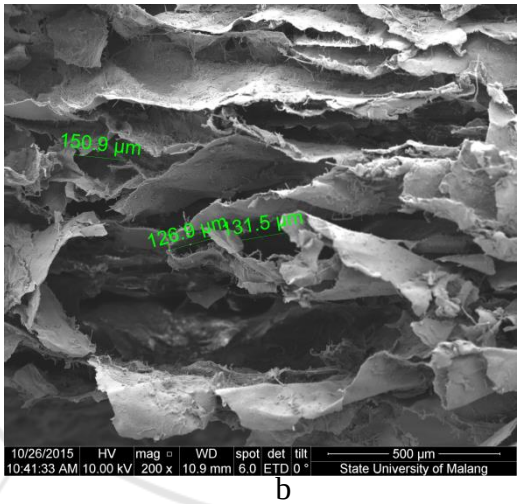
Based on Figure 1, the result of FTIR test, it was acquired wavenumber as the stretching vibration of O-H, the O-H of all materials consisting of OH functional group. The absorption area on the wavenumber 1638.35 cm^{-1} was the stretching vibration of Amina (NH_2) and carbonyl (C=O) functional group. The wavenumber of 1459.39 cm^{-1} is the bending vibration of C-H from CH_2 group. On each absorption, the wavenumber 1384.41 cm^{-1} is the stretching vibration of S-O, the characteristic of SO_3^- functional group as the group of chondroitin sulphate. The stretching vibration of P-O-C was shown by the absorption area on the wavenumber 1089.34 cm^{-1}, 1050.38 cm^{-1} and 602.36 cm^{-1}

included in the phosphate functional group (PO_4^{3-}), the specification of hydroxyapatite. The carbonate functional group (CO_3^{2-}) found on the absorption band on wavenumber 849.49 cm^{-1}. Besides, it was the distinctive characteristic of the chondroitin sulphate absorption band. The absorption band on the waveband 571.35 cm^{-1} belongs to hydroxyapatite. The carbonate functional group (CO_3^{2-}) appearing in the result of FTIR test was originally from the absorption of carbon dioxide from the atmosphere.

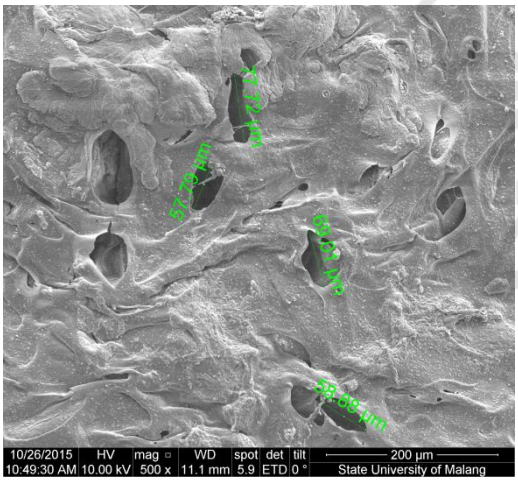
3.2 Morphology Test



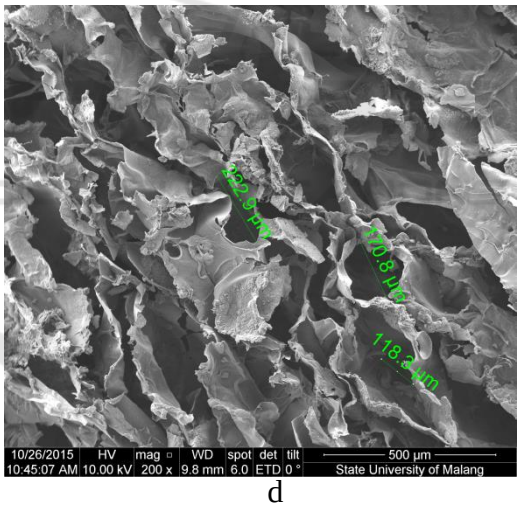
a



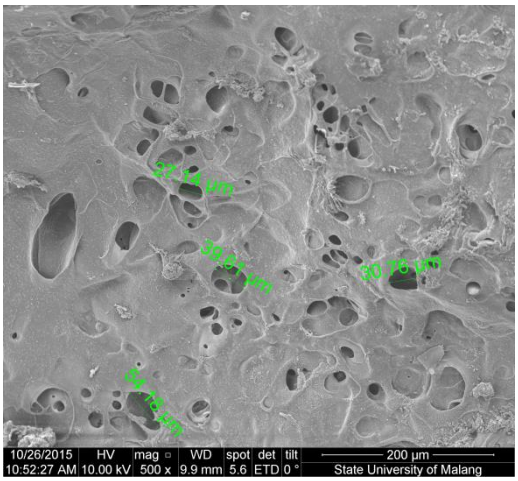
b



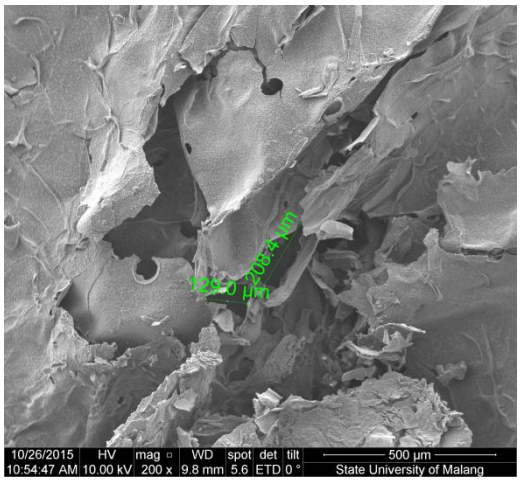
c



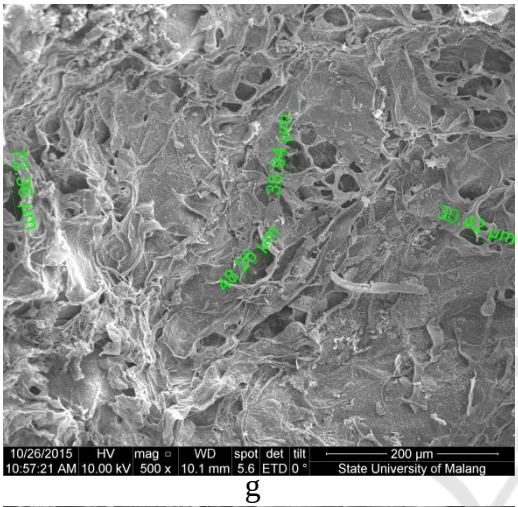
d



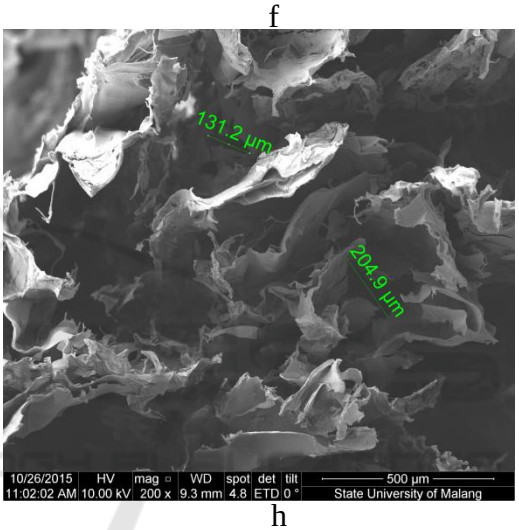
e



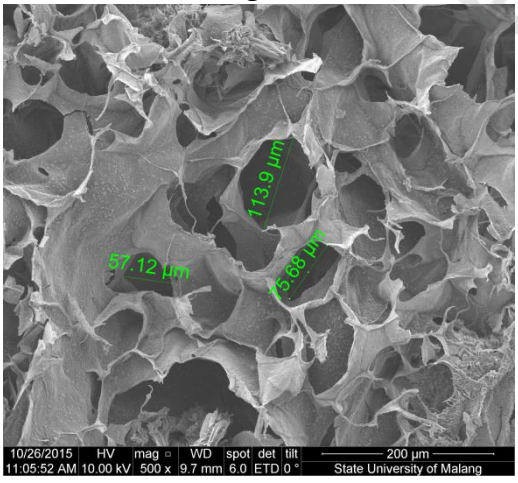
f



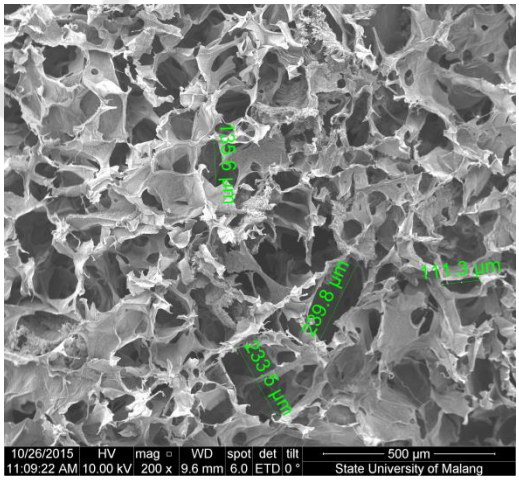
g



h



i



j

Figure 2: Results of Morphology Test of Composite Scaffold Chitosan-Chondroitin Sulphate/Hydroxyapatite with the variation composition of (a)50:50:0 wt% transversal, (b) 50:50:0 wt% vertical, (c) 50:40:10 wt% transversal, (d) 50:40:10 wt% vertical, (e) 50:35:15 wt% transversal (f) 50:35:15 wt% vertical, (g) 50:30:20 wt% transversal (h) 50:30:20 wt% vertical, (i) 50:25:25 wt% transversal dan (j) 50:25:25 wt% vertical

The observation on morphology test was done on the sample by seeing the transversal and vertical longitudinal plane with 200x and 500x enlargement. The measurement of pore on the scaffold sample was done by using a software available in SEM. A ruler was used to meter the diameter of pore seen from the smallest and biggest diameter on the picture as the result of SEM.

A very porous scaffold facilitated the seeding and immigration of cells while the smaller pores enable the growth of tissue. Based on data in Table 1, it was acquired the A, B, C, D, and E samples have fulfilled the criteria of diameter ideal size of a scaffold's pore to help the process of osteoconduction on range 200-350 µm. Meanwhile, the most suitable criteria was on sample C since it has the closest range of diameter size to the pore size of the scaffold which could assist the growth of fibroblast and osteoconduction.

Table 1: Pore Size of Composite Scaffold Chitosan-Chondroitin Sulphate/Hydroxyapatite.

Samples: Hydroxyapatite: Chitosan:Chondroitin Sulphate (wt%)	Pore Size (µm)
A (50:50:0)	26 - 150
B (50:40:10)	57 - 223
C (50:35:15)	27 - 208
D (50:30:20)	30 - 205
E (50:25:25)	57 - 239

3.3 Porosity Test

The porosity of a scaffold has an essential role in regenerating tissue. It provides the temporal mechanical function and facilitates the migration of cells. High porosity (90%) was chosen for various scaffold designs since it is possible to diffuse adequate nutrition during the tissue growing and provide sufficient area for cells and biomaterial to interact with each other. The biggest percentage of porosity (Figure 3) was on sample A, the composite scaffold of chotisan/hydroxyapatite with 50:50% composition compared to samples B, C, D, and E by adding variation of chondroitin sulphate composition 40%wt, 35%wt, 30%wt, and 25%wt each. The declined percentage of porosity of sample B, C, D, and E, if compared to sample A, happened due to the additional chondroitin sulphate on the composite scaffold sample causing an intermolecular knot of hydrogen between chitosan and hydroxyapatite. From the results of the porosity test using a fluid displacement method, it can be concluded that sample C was the best result with porosity percentage of 90.06%.

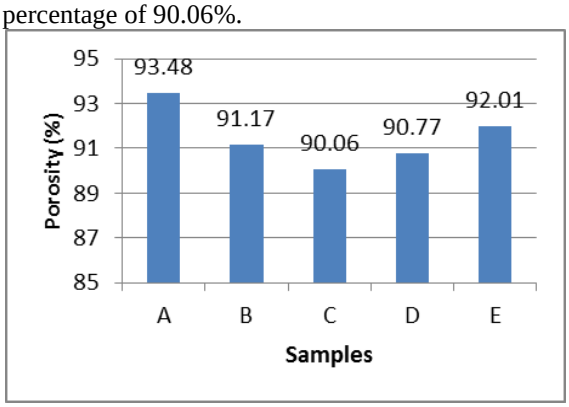


Figure 3: Porosity Percentage Samples of Composite Scaffold Chitosan-Chondroitin Sulphate/Hydroxyapatite

3.4 Biodegradability In Vitro Test

The ideal scaffold has a controllable degradation level which suits the skeletal reparation process. The purpose of a biodegradability in vitro test on a composite scaffold sample is to know the level of composite scaffold biodegradation in the environment of body fluid. The result of a biodegradability in vitro test using SBF fluid increased the degradation level from the first until the fourth week. In the fourth week, it can be seen that composite scaffold still has not been degraded thoroughly, so that it was not good enough for the development of skeletal tissue. While vascular development was in progress, the collagen matrix was secreted by osteoid then mineralized, which directed the formation of soft callus around the reparation area. The callus is broken in 4-6 weeks from the recovery process and needed adequate protection in the form of bracing or internal fixation. Thus, it can be concluded that after 4 weeks, the composite scaffold chitosan - chondroitin sulphate /hydroxyapatite could still provide space for the growth of skeletal tissue so that it could increase in vitro bioactivity on the scaffold, with sample C as the best result with lost mass of 27.1485% during 4 weeks (Figure 4).

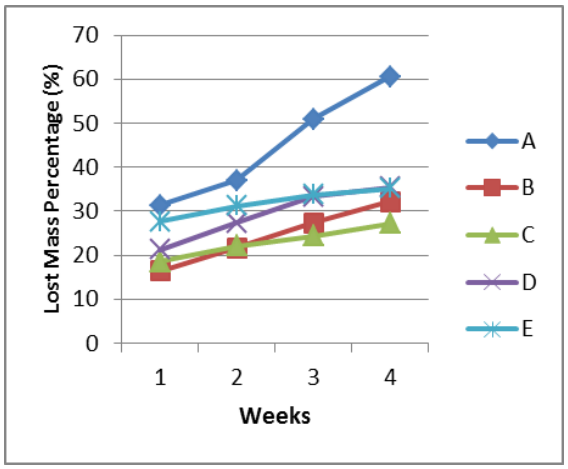


Figure 4: Graphic of Lost Mass Percentage of Samples Composite Scaffold Chitosan-Chondroitin Sulphate/Hydroxyapatite

3.5 Cytotoxicity Test

Cytotoxicity test aims at knowing the nature of cytotoxicity in a sample of composite scaffold chitosan-chondroitin sulphate/hydroxyapatite toward the living cell perceived from the viability cell or living cell percentage. A material is called non-toxic if the percentage of cell viability is more than 50% (Spielmann *et al.*, 2007). On the cytotoxicity test using MTT Assay on the composite scaffold sample A, B, C, D, and E, it obtained the percentage of the living cell as high as 75.15%, 68.56%, 65.52%, 79.89 and 71.56% (Figure 5). This shows that by adding chondroitin sulphate on the composite scaffold sample, the sample became intoxicating for the living cell.

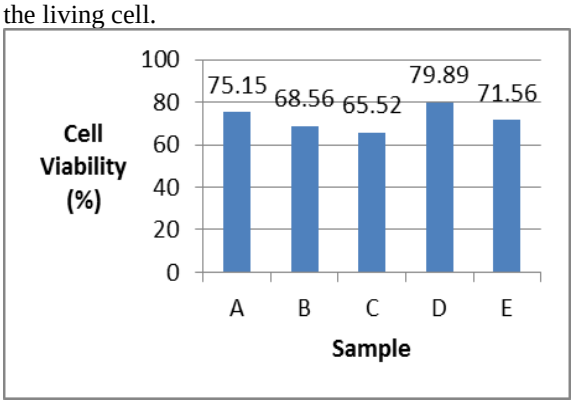


Figure 5: Cell Viability of Composite Scaffold Chitosan-Chondroitin Sulphate /Hydroxyapatite

4 CONCLUSIONS

Composition variation of chondroitin sulphate affects the pore size, porosity, and the percentage of lost mass on a composite scaffold. Composite scaffold chitosan - chondroitin sulphate/hydroxyapatite can be used as the candidate of bone graft. The best result was acquired from sample C (35:15:50 wt%) with pore size range 27-208µm, porosity 90.06%, lost mass percentage was 27.187% for 4 weeks, and cell viability was 65.52%.

REFERENCES

Badan Penelitian dan Pengembangan Kesehatan Kementrian Kesehatan RI. 2013. Riset Kesehatan Dasar (RISKESDAS 2013). Downloaded from: *http://www.litbang.depkes.go.id/sites/download/rkd2013/Laporan_Riskesdas2013.PDF* on 10 December 2014 at 06.09 WIB.

Darwis, Darmawan, dan Yessy Warastuti. 2008. Sintesis dan Karakterisasi Komposit Hidroksiapatit (HA) Sebagai Graft Tulang Sintetik. Jurnal Ilmiah Aplikasi dan Radiasi Vol 4 No.2 Desember 2008.

Ferdiansyah, Djoko Rushadi, Fedik Abdul Rantam, Aulani'am. 2011. Regenerasi pada *Massive Bone Defect* dengan *Bovine Hydroxyapatite* sebagai *Scaffold Mesenchymal Stem Cell*. JBP Vol 13, No.3.

Hutmacher, Dietmar W. 2000. Scaffolds in tissue engineering bone and cartilage. Elsevier B.V: Biomaterials 21 (2000) pp. 2529-2543.

Kementrian Riset dan Teknologi Republik Indonesia. 2014. Accessed on November 2014 at 17.58 WIB in: *http://www.ristek.go.id/index.php/module/News+News/id/12533/pdf*

Mitsak, Anna G, Jesscia M. Kemppainen, Matthew T, Harris, Scott J Hollister. 2011. Effect of Polycaprolactone Scaffold Permeability on Bone Regeneration in Vivo. Michingan: *Tissue Engineering*: Part A, Vol 17, Numbers 13 and 14.

Orlovskii, V.P., V. S. Komlev, dan S. M. Barinov. 2002. Hydroxyapatite and Hydroxyapatite-Based Ceramics. Russia: Inorganic *Materials*, Vol. 38, No. 10, 2002, pp.973-984.

Schieker, Matthias, Herman Seitz, Inga Drosse, Sebastian Seitz, Wolf Mutschier. 2006. Biomaterials as Scaffold dor Bone Tissue Engineering. German: European Journal of Trauma 2006 No.2.

Spielmann, H. Hoffman, S. Botham, P. Roguet, R. Jones, P. 2007. The ECVA International Validation Study on In Vitro Test for Acute Skin Irritation Report on the Validity of the EPISKIN and EpiDerm Assays on the Skin Integrity Function Test. Germany: ATLA.

Venkatesan, Jayachandran, Ramjee Pallela 2012. Chitosan-amylopectin/hydroxyapatite and chitosan-chondroitin sulphate/hydroxyapatite composite

scaffolds for bone tissue engineering. *International Journal of Biological Macromolecules* 51 (2012) halaman 1033-1042.