Chemical Characterization of Demineralized Freeze Dried Bone Bovine Xenograft Nanoparticle Scaffold for Enhance Bone Repair

Ariyati Retno Pratiwi[1*], Mohd Zobir Hussein[2], Feni Istikharoh[3], Irma Istiqamah[4], Fadhillah Marzely[4], Salwa Jasmine[4], Laura Naibaho[4]

[1]*Department of Oral Biology, Faculty of Dentistry, Universitas Brawijaya, Indoensia*
[2]*Nanomaterials Synthesis and Characteization Laboratory, Institute of Nanoscience and Nanotechnology, UPM, Malaysia*
[3]*Department of Dental Material, Faculty of Dentistry, Universitas Brawijaya, Indoensia*
**Corresponding Autor's Email: ariyatiretnop@ub.ac.id*

Abstract: This study aimed to determined chemical characterization of demineralized freze dried bone bovine nanoparticle scaffold for enhance bone repair. Demineralized Freeze-dried Bone Bovine Xenograft Nanoparticle (DFDBBX-NPs) was extracted from Bovine. Characterizations of demineralized freeze dried materials were performed by FTIR, WCP (Water Content Percentage), and Enzimatic Degradation. Based on FTIR, there are 29 characterization absorpsion bands, including hydroxyapatite absorption, collagen, and carbonate apatite. Water content percentage result showed about 201,98% on day 3, 201, 19% on day 7, 214,5% on day 14. Meanwhile enzymatic degradation percentage showed 0,0979% on day 4, 0,207% on day 7, and 0,24 on day 14.

***Keywords*:** DFDBBX-NPs, FTIR, WCP, Enzimatic Degradation, Bone Repair.

INTRODUCTION

There are several conditions in which injured bone may not be capable of healing itself. The current gold standard for the treatment of these critical-sized defects is autogenous bone grafting. The use of bonegrafts as regenerative materials is expected to fulfil three main attributes: osteogenic, osteoinductive, osteoconductive. Osteogenic means that they contain living cells which can differentiate into osteoblasts. Osteoinductive describes the ability to stimulate local or added cells to differentiate into osteoblasts and thereby increase bone healing. Bone grafts also act as scaffolds: on their surface new bone material can be generated [1].

Demineralized Freeze-Dried Bovine Bone Xenograft Nanoparticles are bonegrafts produced from the demineralization process of cow bones, leaving organic components containing growth factors such as BMP. The ideal scaffold should have good compatibility, biodegradable, and not toxic to cells. For this reason, it is necessary to observe the chemical characterization of DFDBBX-NPs.

MATERIALS AND METHODS

Synthesize of DFDBBX-NPs

Demineralized freeze-dried bone bone comes from the part of the bovine bone, then separates it from the soft tissue. The bone cut 3 mm x 3 mm, then washed using H_2O_2/peroxide solution until clean from blood and free of fatty tissue. After that it was rinsing using NaCl/aquadest solution to remove residual peroxide, the soaked in 1% HCL solution until the minerals disappear. The sample put in deep freze and dried through a lyophilizer system.

Characterization of DFDBBX-NPs

FTIR (Fourier Transform Infrared)

The FT-IR spectra of the samples were recorded by Thermo Scientific, Nicolet iS10. The scanning range was 400-4000 cm^{-1} at a resolution of 4 cm^{-1}. For the preparation of the pellets. KBr was used as a spectroscopic quality diluent, melted and then stored in an oven. The spectrum of the AEA was collected from the commercial ethanolic solution of the endocannabinoid using ZnSe windows. FTIR test results will be in the form of a graph.

Water Content Percentage

The scaffold used as a sample is weighed to determine initial weight / dry weight (Wd). The scaffold was put into an Eppendorf tube and immersed in 1 ml phosphate buffer saline solution (PBS, pH = 7.4) for 24 hours, 3rd day and 7th at 37^{o}C. The scaffold is taken slowly using small tweezers so that it is scaffold undamaged, then dry using filter paper then weighed to determine the final weight / Wet weight (Ww). Then it is calculated using the formula [2].

Enzimatic Degradation

The scaffold used as a sample is weighed to determine the initial weight (Wo). The scaffold was immersed in 1 ml of solution (phosphate buffer saline and 50 μg/ml collagenase enzyme) for 3 days, and 7 days. Then the scaffold was taken from the medium and washed with distilled water. Then frozen at -80oC and freeze-dried for 2x24 hours. The dry scaffold is weighed to determine the final weight (Wt). This procedure was repeated for 3 days and 7 days. The degradation rate is calculated based on the formula [3].

RESULTS AND DISCUSSION

Bovine bone xenograft is often an alternative because it has structures and physical and chemical characteristics that resemble human bones. In addition, it is also an unlimited source of raw materials when compared to allograft [4-5]. An ideal scaffold has several criteria or characteristics of biomaterials that must be met, including biocompatible, biodegradation, mechanical characteristics, scaffold structure, and manufacturing technology [6]. Physical and chemical characteristics and crystallinization of a material can affect the osteogenic properties of cells in the formation of new bone [7]. Xenograft comes from a different species from humans, so it is necessary to examine the osteological characteristics. In the process of making a material, it will go through chemical processes and temperature changes, this will affect the physical and chemical properties. For this reason, it is necessary to check in every preparation of xenograft material whether it has approached the physical and chemical characteristics suitable for bone

regeneration. Bone grafts that have undergone a demineralization process are expected to further accentuate their osteoinductive properties. For this reason, in this study the researchers investigated the chemical characterization of a new nanoparticle namely Demineralized Freeze Dried Bone Bovine Xenograft.

Observations using Fourier Transform Infrared (FTIR) aim to see the composition of the scaffold. It is known that the main composition of human bone is collagen and hydroxyapatite carbon which are very good at absorbing light from FTIR waves. A bone graft is expected to approach the structure and composition of human bones. In the DFDCBBG spectrum, 29 characteristic absorption bands at frequencies 3539.38, 3485.37, 3375.43, 319.49, 302.13, 3223.05, 3203.76, 3165.19, 3084.18, 3068.75, 2960.73, 2879.72, 2318.44, 2268.29, 2131.34, 2090.84, 1990.54, 415.75, 1336.67, 1234.44, 1205.51, 11.86, 35, 991.41, 960.55, 873.75, 752.24, 601.79, and 569 cm^{-1} (Fig 1.) Hydroxyapatite absorption bands are in the range of 500-700 cm^{-1} and 900-1200 cm^{-1}. Meanwhile, the absorption bands of collagen are in the range of 1200-1700 cm-1 and 2800-3700 cm^{-1}. Apatite carbonate appears at 870-880 cm^{-1} (as a single band) and at 1400-1450 cm^{-1} (as a double band). Based on the results of the FTIR, most of the absorption bands 1336.67, 1234.44, 1205.51, 3539.38, 3485.37, 3375.43, 3223.05, 3203.76, 3165.19, 3084.18, 3068.75, 2960.73, 2879.72 cm^{-1} are collagen. Specifically, type I collagen has waves as an amide group. There is amide A (close to 3300 cm^{-1}), amide B (close to 3100 cm^{-1}), amide I (1600-1700 cm^{-1}), amide II (1500-1600 cm^{-1}), amide III (1200-1300 cm^{-1}), amides IV-VII (500-750 cm^{-1}). The demineralization process of this scaffold remain collagen. On the other hand, freeze dried process still contains protein and minerals.

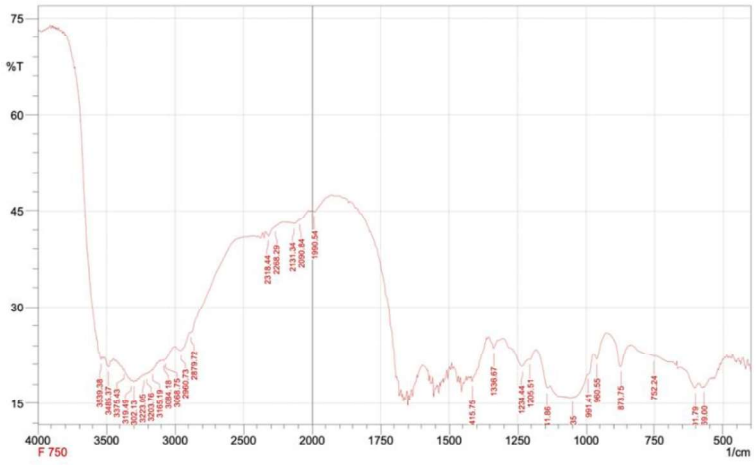

Fig. 1. Graphic of FTIR

Testing of water content or Water Content Percentage (WCP) in the scaffold was carried out to determine the ability of the scaffold to absorb water, by calculating the initial weight of the DFDBBX-NPs scaffold. The research time was determined for 3, 7, and 14 days. At each specified research time, the final weight of the scaffold was measured. After the initial weight and final

weight, the proportion of water content is calculated using the formula. The average value of the research results for the proportion of water content is shown in table 1. A scaffold that exhibits a higher proportion of water content or Water Content Percentage (WCP) has a greater ability to filter cells. The average proportion of water content in the DFDBBX scaffold on the 3rd day it was 201%, on the 7th day it was 201.19%, and on the 14th day it was 214%.

Table 1. Water Content Percentage of DFDBBX-NPs on Day 3, Day 7, and Day 14

Time	W_0 (mg)	W_t (mg)	Water Content Percentage (%)
Day-3	12,65	38,2	201,98
Day-7	15,25	45,95	201,19
Day-14	13,1	41,2	214,5

Water content percentage (WCP) is the ability of the scaffold to bind water. In this study the results of the swelling ratio graph with WCP were similar. The natural hydrophilic ability of the scaffold is important in the interaction of biological fluids to aid cell migration to the interior of the scaffold. The results showed that the largest WCP was in the scaffold of the third day treatment group 61.17%. The hydrophilic ability of the treatment group was significantly greater when different tests were carried out between the two groups on the first, third and seventh days. There was an increase in the control group from the first to the third day, but it was not significant. On the seventh day, the ability to absorb water is no longer increasing. The ability of the scaffold to absorb water is not only a medium for cell transfer, it also facilitates the distribution of nutrients, metabolites and growth factors through the extracellular media.[7] Therefore, hydrophilic properties are necessary for the success of bone regeneration. Hydrophobic nature itself causes higher protein adsorption than hydrophilic. Protein adsorption is useful on the surface of the scaffold as a mediator of cell attachment, signaling to cell through cell adhesion receptors (especially integrins). However, if it is too hydrophobic it will cause a thick deposition of protein on the surface of the scaffold [8].

Observation of scaffold degradation was carried out to determine the degradation rate of the DFDBBX-NPs scaffold. The initial weight calculation was carried out for each sample. The study time was determined at 4, 7 and 14 days. At each specified research time, the final weight of the scaffold was measured. After obtaining the initial weight and final weight, the weight degradation of the scaffold is calculated using a formula. DFDBBX-NPs scaffold degradation on the fourth day of immersion was 0.0979%, on the seventh day it was 0.207% and on the fourteenth day it was 0.24%. The average value of the research results for the analysis of the rate of heavy degradation of the scaffold is shown in table 2.

Table 2. Percentage of Enzimatic Degradation on Day 4, Day 7, and Day 14

Time	W_0 (g)	W_t (g)	Enzimatic degradation (%)
Day-4	19,2	17,55	0,0979
Day-7	19,1	16,4	0,207

Day-14	27,75	23,2	0,24

The rate of biodegrability is also an important parameter to avoid a second surgery to remove the scaffold. The successful regeneration of a defect requires at least 6 months which determines the rate of biodegradation of a scaffold. In order to act as a barrier, a resorbable scaffold must at least last for 3-4 weeks.

Biodegradation is an important parameter in the occurrence of regeneration. The rate of biodegradation must be in line with the rate of formation of new bone, so that when bone regeneration has been completed the scaffold material has all been degraded. The rate of degradation is influenced by hydrophilic properties, chemical composition, degree of crystallinization, and scaffold geometry [9]. The main composition of the scaffold is collagen as shown in the FTIR results. The combination of scaffold with nanostructure is known to slow down the rate of degradation, because it neutralizes the acid degradation products [8]. The rate of degradation can be controlled by modifying the chemical composition and surface structure. By adding some cross-linking materials it can also restrain the rate of degradation [10].

CONCLUSIONS

DFDBBX-NPs contain of collagen, hydroxyapatite, and carbonate apatite. Water content percentage of DFDBBX-NPs on day 14 is 214,5%, meanwhile the rate of degradation showed that DFDBBX-NPs is slow (0,24% on day 14).

REFERENCES

[1] Lichte P, Pape HC, Pufe T, Fischer H. Scaffolds for bone healing: Concepts, materials and evidence. 2011, 42(6), 569-573

[2] Guo, Z., Yang, C., Zhou, Z., Chen, S., Li, F. Characterization of biodegradable poly(lactic acid) porous scaffolds prepared using selective enzymatic degradation for tissue engineering. RSC Adv. 2017, 7, 34063–34070.

[3] Park, H.J., Min, K.D., Lee, M.C., Kim, S.H., Lee, O.J., Ju, H.W., Moon, B.M., Lee, J.M., Park, Y.R., Kim, D.W., Jeong, J.Y., Park, C.H., 2016. Fabrication of 3D porous SFb-TCP hybrid scaffolds for bone tissue reconstruction Fabrication of 3D porous SF / b -TCP hybrid scaffolds for bone tissue reconstruction. https://doi.org/10.1002/jbm.a.35711

[4] Figueiredo, M., Henriques, J., Martins, G., Guerra, F., Judas, F., Figueiredo, H. Physicochemical characterization of biomaterials commonly used in dentistry as bone substitutes - Comparison with human bone. J. Biomed. Mater. Res. - Part B Appl. Biomater. 2010, 92, 409–419

[5] Galia, C.R., Lourenço, A.L., Rosito, R., Souza Macedo, C.A., Camargo, L.M.A.Q. Physicochemical Characterization of Lyophilized Bovine Bone Grafts. Rev. Bras. Ortop. English Ed. 2011, 46, 444–451

[6] O'Brien, F.J. Biomaterials & scaffolds for tissue engineering. Mater. Today, 2011, 14, 88–95

[7] Rodríguez-Vázquez, M., Vega-Ruiz, B., Ramos-Zúñiga, R., Saldaña-Koppel, D.A., Quiñones-Olvera, L.F. Chitosan and Its Potential Use as a Scaffold for Tissue Engineering in Regenerative Medicine. Biomed Res. Int. 2015, 821279

[8] Yassin, M.A.A.Y. Surface Hydrophilicity: a Key Factor in Developing Bone Tissue Engineering Constructs. 2017