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# The Effect of Glutaraldehyde on Hydroxyapatite-Gelatin Composite with Addition of Alendronate for Bone Filler Application

ALFIAN Pramudita Putra[1,a,*], ANNISA Aulia Rahmah[1,b], NIA Fitriana[1,c], SAYYIDATI Arristifanniy Rohim[1,d], MIFTAKHUL Jannah[1,e], DYAH Hikmawati[2,f]

[1]Biomedical Engineering, Department of Physics, Faculty of Science and Technology, Universitas Airlangga, Surabaya, Indonesia

[2]Department of Physics, Faculty of Science and Technology, Universitas Airlangga, Surabaya, Indonesia

[a]pramuditaalfian@gmail.com, [b]auliarahmah.annisa@yahoo.co.id, [c]nea_tbe@yahoo.co.id, [d]s.aristifanniy@gmail.com, [e]miftakhul.jannah66@gmail.com, [f]dyah.hikmawati@yahoo.co.id

*Corresponding author: pramuditaalfian@gmail.com

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**Abstract.** Based on data from Indonesian Health Ministry in 2009, osteoporosis case reached 19.7% of the populations in Indonesia, especially women in menopause period. The treatment was performed by consuming bisphosphonate drugs per oral which was not effective since the absorption intake of the drug was only less than 55% of the intake dosage. Because of that, the bone filler which also has a function as drug delivery system was developed. The hydroxyapatite-gelatin bone filler with the addition of alendronate was studied. To increase the characteristics of this bone filler, glutaraldehyde was introduced in the composite as a crosslinking agent. The concentration of 0.25%, 0.5%, and 0.75% were used. The bone filler was then characterized based on FTIR test, morphology test, compressive strength test, cytotoxicity test, and degradation test. The FTIR result showed that there was no significant difference between the sample with and without glutaraldehyde since the crosslinking bond of glutaraldehyde and gelatin was C=N bond which also presented in the gelatin. The morphology of the samples depicted a bigger pore size for higher glutaraldehyde concentration which also supported by lower compressive strength. All the samples were non-toxic based on the cytotoxicity test which had cell viability more than 100%. The degradation tests showed that with the presence of glutaraldehyde in the bone filler could maintain its form longer than the bone filler without glutaraldehyde. In conclusion, the presence of glutaraldehyde could increase the characteristics of the hydroxyapatite-gelatin composite with the addition of alendronate as a bone filler candidate for osteoporotic bone.

## Introduction

Based on data from Indonesian Health Ministry in 2009, osteoporosis case reached 19.7% of the populations in Indonesia, especially women in menopause period. Osteoporosis is a disease in which the bone density is decreasing. It is mostly discovered on 60 years old men or women who are in menopause period. 40% of 120 million women in Indonesia are above 40 years old and half of them are in the menopause period which experienced osteoporosis. 35 % of them suffered from bone fracture which required bone surgery with a high cost. From 50 % of bone defect due to osteoporosis, 9-10 patients need a bone implant which cost approximately IDR 50 million [1]. The high cost is becoming the main problem for people with osteoporosis in the developing country like Indonesia. Thus, there is a need to avoid the bone fracture due to osteoporosis which is effective and affordable. The treatment for osteoporosis sufferer usually was done by consuming the bisphosphonate drugs, one of them is alendronate. Alendronate could inhibit the bone resorption rate by the osteoclast so that the bone mass will increase because the osteoblast could form bone

more than usual. The bisphosphonate drugs could reduce the bone resorption performed by osteoclast which binds to the bone surface and inhibits osteoclast function by reducing the proton production and lysosomal enzyme under the osteoclast [2]. The intake of alendronate per oral would be absorbed in the small intestine and the absorption was bad, which is less than 55 % of the intake dosage. In the case of osteoporosis, it is needed a direct treatment of the bone which has low density with drug delivery system by implantation [3]. The advancement of biomaterial field could help several health problems, such as bone filler. The hydroxyapatite-gelatin-based bone filler is bone filling material which has function and structure of the main component of the bone.

The bone filler application in the bone with osteoporosis was expected to fill the fragile bone directly and stimulate the surrounding bone tissue to form the new bone cells by osteoblast in the bone formation process [4]. The bone filler would be implanted on the bone was based on hydroxyapatite and gelatin with the addition of alendronate. The bone filler would help in the bone reformation and also has a function as drug delivery [3].

Hydroxyapatite is one of the bioceramics which was applied to heal the bone. The biocompatibility level of hydroxyapatite was high in the body[5]. The addition of gelatin could increase the physical features of the bone filler. Gelatin is one of the natural polymers which would control the pore size so that it could suit the bone pore size. The study of drug delivery in bone filler was ever performed by using gentamycin as an antibacterial agent, in which the result showed the suitable characteristics in hardness, porosity, nontoxicity, and the ability to release the gentamycin regularly active [6].

The bone filler synthesis steps started with the mixing of three main materials, which are hydroxyapatite, gelatin, and alendronate. The mixture was then compacted into a pellet. The crosslinking process was performed with glutaraldehyde (GA) so that the bond in the composite would be stronger and then the samples were characterized by Fourier Transform Infrared (FTIR) test, morphology test, cytotoxicity test, compressive strength test, and degradation test.

## Material and Methods

Materials used in this study were hydroxyapatite (HA) powder from the bovine bone in Tissue Bank dr. Soetomo General Hospital, Gelatin (GEL) 150 bloom Rousselot (Guangdong, China) from bovine skin, Alendronate (ALE) Arshine Technology Co., Limited (Wanchai, China), Hydroxypropyl methylcellulose (HPMC) Sigma Aldrich H7509, distilled water, Phosphate buffer saline (PBS), and glutaraldehyde (GA) Sigma Aldrich G6257.

**The Hydroxyapatite-gelatin based bone filler synthesis.** The HA was mixed with ALE with 10 % of its mass. The gelatin solution of 0.2 gr/ml was dissolved in 40°C distilled water. The mixture of 10 gr HA and 1 gr ALE was mixed with 5 ml GEL solution homogenously in a warm mortar. The sample was dried in the oven at 40°C for 24 hours. The dry granule sample was crosslinked with GA with several concentrations, such as 0.25, 0.5, and 0.75 v/v% with immersion method for 24 hours. The sample then was washed with distilled water and PBS and was dried again in the oven at 40°C for 24 hours. The dry-crosslinked granule was compacted into pellet with the diameter of 4 mm and the width of ± 3 mm.

**Fourier Transform Infrared (FTIR) Analysis.** The samples were characterized by Fourier Transform Infrared (FTIR) (Bruker Tensor 27) in Membrane and Polymer Laboratory Surabaya University. The sample with 5 % weight of KBr total weight was used and formed into a pellet. The FTIR test was performed in the range of 4000 - 400 $cm^{-1}$.

**Morphology Test.** The morphology test using Scanning Electron Microscope (SEM) Inspect-S50 Fei Corp. Japan was performed at Central Laboratory in State University of Malang. The dry sample was put in the holder and sputtered with gold-palladium (Au:Pd = 4:1) and then shot with an electron beam. The result was with a magnification of 5000x to 40000x.

**The compressive strength test.** The compressive strength test was performed at Basic Laboratory in Universitas Airlangga with Autograph based on ASTM C39 (Standard Test Method

for Compressive Strength of Cylindrical Concrete Specimens) [7]. Before the test, the thickness and the diameter of the sample were measured. The dry cylindrical bone filler sample was tested in the compression mode. The test was replicated three times. The result of this test was the force needed to break the sample and this force was inserted to Eq. 1 to get the compressive strength.

$$\sigma = 4F/(\pi d^2) \quad (1)$$

**The degradation test.** The degradation test was performed at Material Physics Laboratory in Universitas Airlangga by immersion method. Each sample was immersed in 10 ml PBS at pH of 7.4 over time and was observed the condition of the sample whether there was a cracking or the sample was degraded before 40 days. This test was performed in three replications.

**The cytotoxicity test (MTT Assay).** The cytotoxicity test using MTT Assay method was performed at PUSVETMA, Surabaya by using Baby Hamster Kidney (BHK)-21 fibroblast cell. This method was based on the change of tetrazolium salt to formazan in the mitochondria of the living cells. The monolayer BHK-21 fibroblast cell culture in Eagle's media was incubated for 2 days and rinsed with PBS afterward. 100 µl of 86% Eagle's media, 1% Penicillin streptomycin and 100 units of Fungizone/ml was prepared and the cell cultured was inserted to it. Then, the cell culture was transferred to the 96 microwell plate. Each bone filler sample was dissolved with 2 ml of Eagle's media and 50 µl of it was inserted to 96 microwell plate and then incubated for 24 hours at 37°C. The sample was then rinsed with PBS and 10 µl 5mg/ml MTT solution was added to the media for each well. After that, each well was added with 50 µl of DMSO and centrifuged for 5 minutes with speed of 30 rpm. Finally, the cell optical density was measured with Elisa reader. The violet level of the formazan was measured by the Elisa reader as Optical Density (OD) to determine the cell viability based on Eq. 2. The sample was replicated for four times.

$$\% \text{ cell viability} = (\text{OD treatment} + \text{OD media control})/(\text{OD cell control} + \text{OD media control}) \quad (2)$$

**The Statistical Test.** The measured data in this study was presented in average and its standard deviation. The result was analyzed statistically by one way ANOVA test with a significance value $p = 0.05$.

## Result and Discussion

In this study, the bone filler was synthesized based on hydroxyapatite and gelatin with the addition of alendronate as osteoporosis drugs and glutaraldehyde as a crosslinking agent with several concentration variations. The results were shown in Fig. 1. The increase in glutaraldehyde concentration resulted in more yellowish samples.

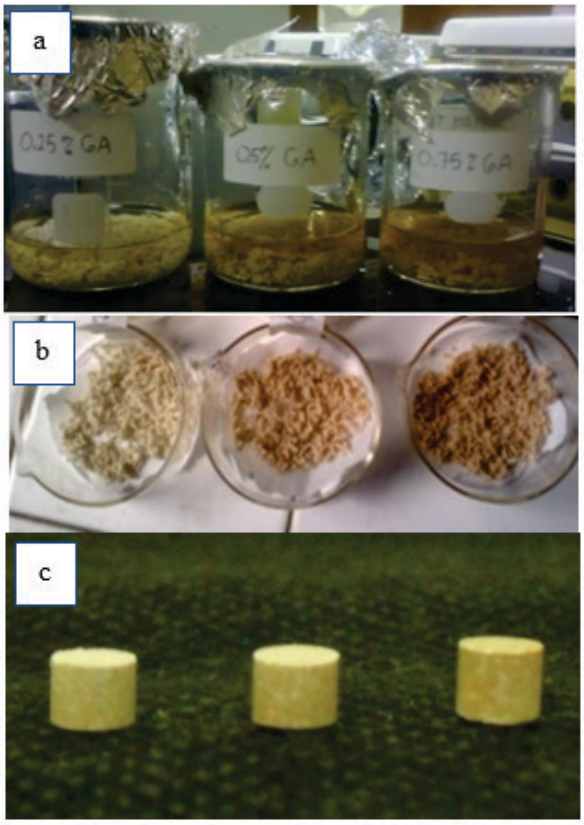


Figure 1. The result of bone filler synthesis based on hydroxyapatite and gelatin with the addition of alendronate and glutaraldehyde as a crosslinking agent. (a) The sample in immersion with glutaraldehyde for crosslinking process. (b) The bone filler samples after drying process in the oven at 40°C for 24 hours. (c) The samples after compaction (Personal document)

**FTIR Test.** The FTIR test was performed to observe the functional group and new bonds formed in the sample, especially the crosslinking bond due to the presence of glutaraldehyde. The result was shown in Fig. 2.

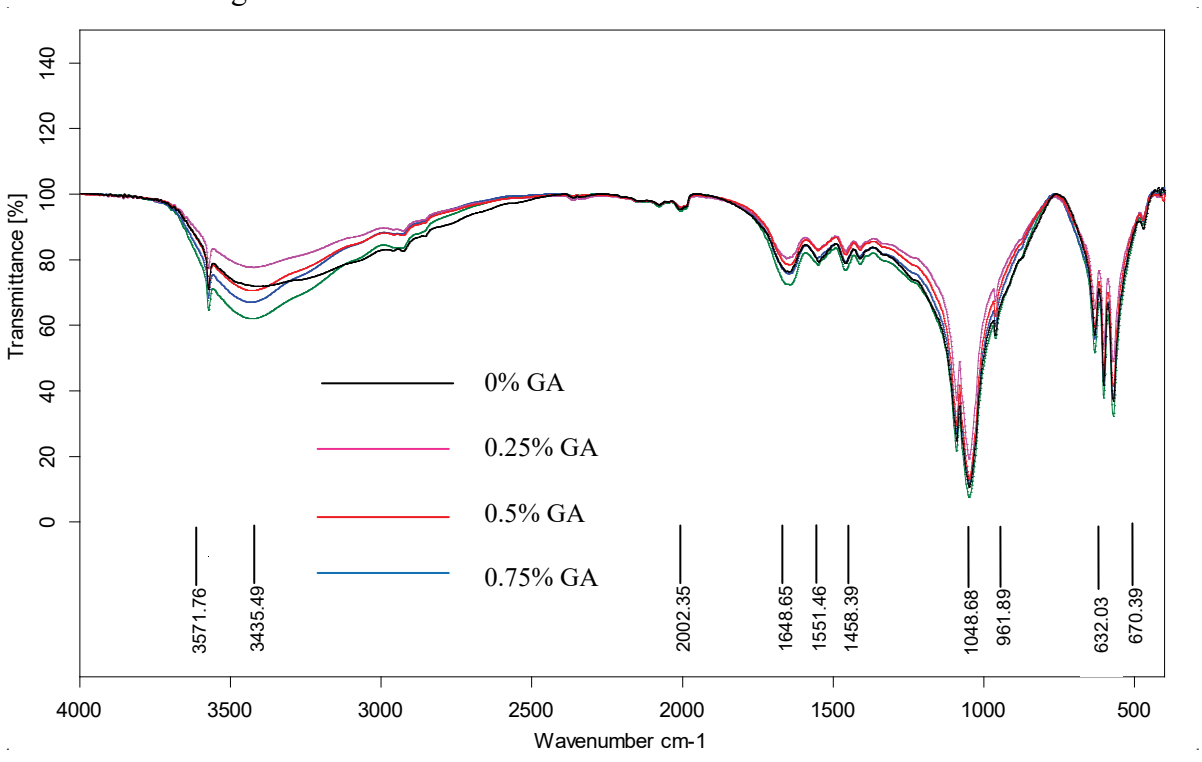


Figure 2. FTIR Spectra of the Sample of 0%, 0.25%, 0.5%, and 0.75% GA

Fig. 2 showed that there was no difference between the sample with and without GA because the formed crosslink bond was C=N group which also presents in the gelatin itself at 1648.65 $cm^{-1}$ and 1667.77 $cm^{-1}$. The same group gave the same peak in the FTIR test. Besides that, the phosphate group from hydroxyapatite and alendronate were present in the sample at 1048.68 $cm^{-1}$ and 1048.86 $cm^{-1}$. The N-H stretching and bending were also present in the sample which represented the characteristics group of gelatins at 3571.70 $cm^{-1}$ and 3571.76 $cm^{-1}$ for N-H stretching and 1551.46 $cm^{-1}$ and 1547.78 $cm^{-1}$ for N-H bending. There was a slight difference in the N-H stretching peak in which the sample with GA had sharper peak compared to that of the sample without GA. It showed that some N-H group reacted to the glutaraldehyde to form the crosslinking bond as C=N group [8]. The presence of amide II that was indicated by an absorbance band at 1540 $cm^{-1}$ showed that the composite formed an α-helix shape [9].

The glutaraldehyde has two aldehyde group on its two sides which could bind to the amine groups from gelatin. The amine groups should be the free ones because some of them are binding to each other forming a cross-linkage. The crosslinking bond between gelatin and glutaraldehyde directly occurred to the free N-H stretching groups of gelatins because glutaraldehyde could easily react to the free amine group of the protein [8].

**Morphology Test.** The morphology test was performed with scanning electron microscope (SEM) to observe the surface of the sample to obtain the pore size of the bone filler. The result was shown in Fig. 3. The pore size was measured based on the average pore size available in Fig. 3 in several points (at least three points).

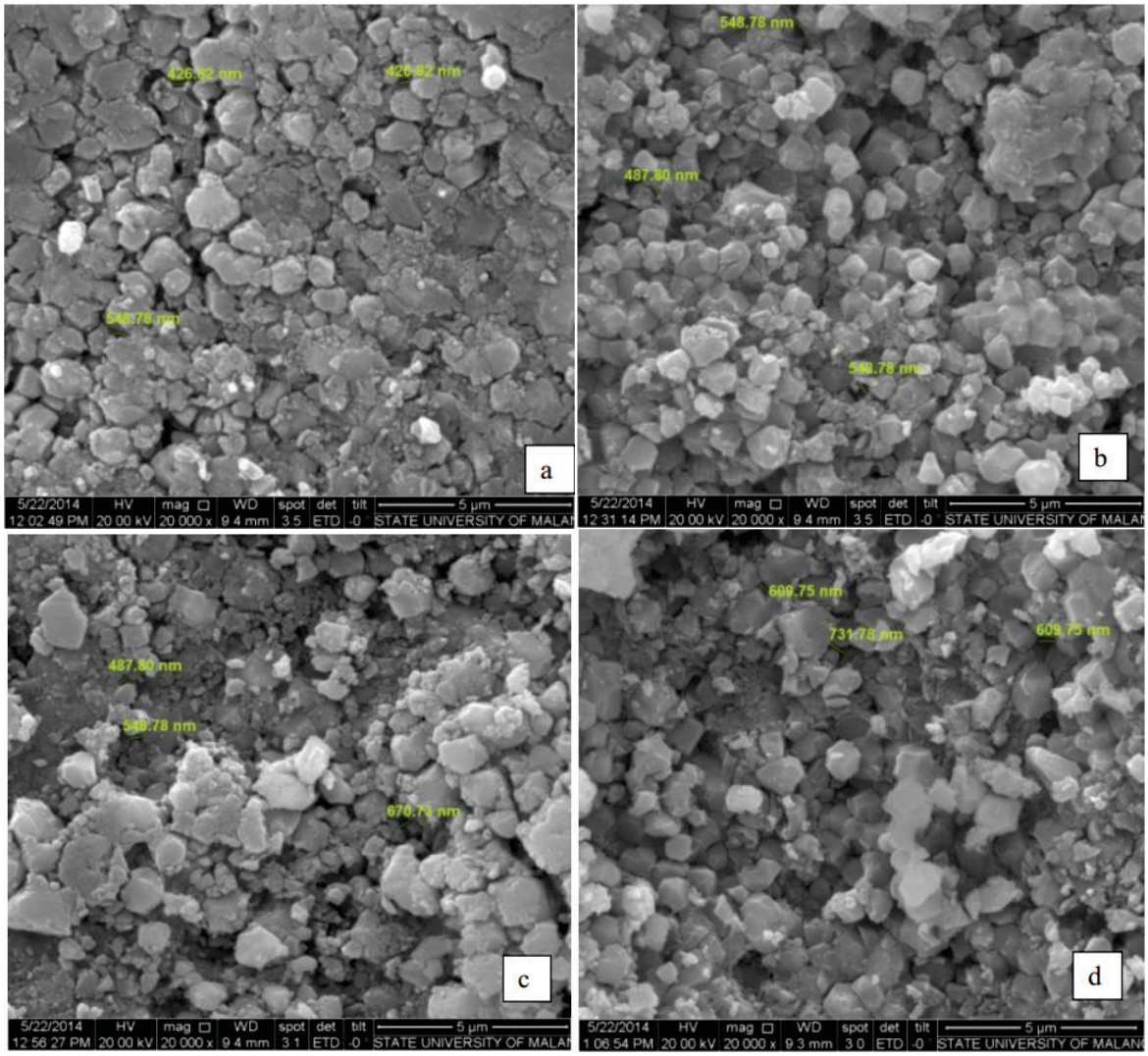


Figure 3. The SEM images of the bone filler with (a) 0 v/v% GA, (b) 0.25 v/v% GA, (c) 0.5 v/v% GA, and (d) 0.75 v/v% GA

Fig. 3 showed that when the concentration of the glutaraldehyde increased, the pore size increased too. The pore size of the samples was 467.47 nm, 528.45 nm, 569.10 nm, and 650.42 nm for the sample with 0%, 0.25%, 0.5%, and 0.75% GA, respectively. The presence of GA increased the grain size which left a hollow area in the sample so-called pores. The GA bound the gelatin chain which resulted in a compact condition. Moreover, the presence of alendronate also attracted the HA molecule to produce particle grains resulting in a bigger pore size [10]. That pores or interconnectivity is important for osteoconduction.

This result also implied that this scaffold was also compatible with osteoblast cells since one osteoblast cell could cover an area around 700 $\mu m^2$ which equals to a pore size of around 500 μm in diameter. However, to obtain osteoconduction, the pore size of 150 μm was needed. Thus, the pore size of the scaffold is adequate for cells to attach and could have a good osteoconduction [9]. The pore size also possesses a vital aspect of the cell attachment, proliferation, and migration [11].

**Compressive Strength Test.** The compressive test was aimed to observe the ability of the bone filler to hold the compressive force after implantation. The result was shown in Fig. 4.

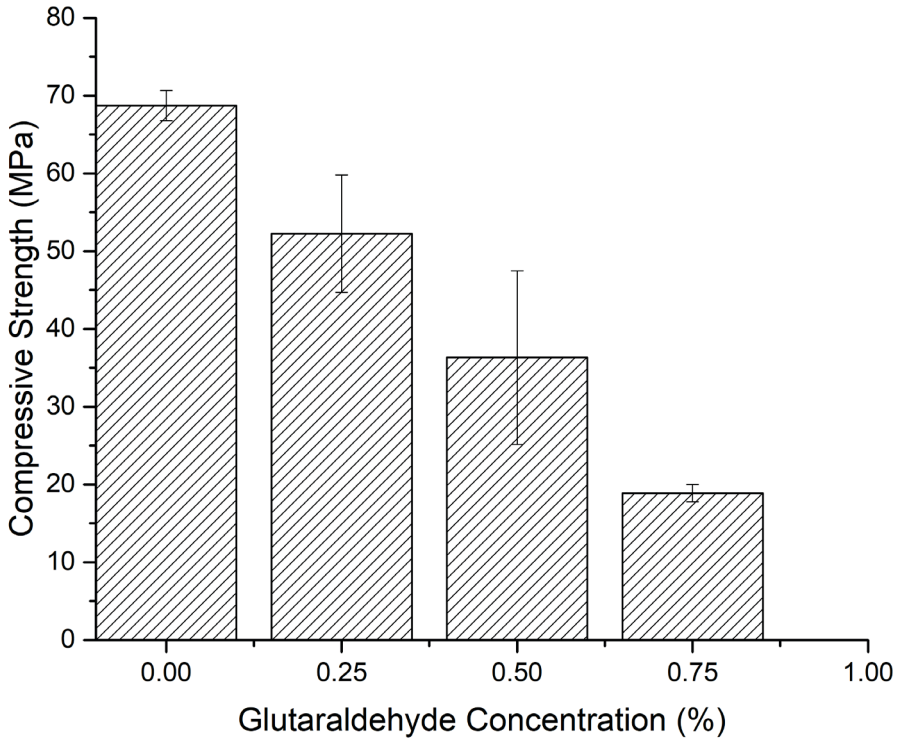


Figure 4. The Compressive Strength of the Hydroxyapatite-Gelatin Composite with Several Glutaraldehyde Concentrations

Fig. 4 showed that the composites with 0, 0.25, 0.5, and 0.75 v/v % GA reached the compressive strength of 68.73, 52.24, 28.35, and 10.9 MPa, respectively. Based on one-way ANOVA test, the result showed that the p-value was 0.00013 which was less than 0.05. That implied that result was statistically different. It was also shown that the compressive strength decreased when the concentration of glutaraldehyde increased. This result was related to the result of morphology test in which the pore size increased when the glutaraldehyde concentration increased. With a bigger pore size, the bone filler would have a lower compressive strength because the force distribution would spread on the grains. The presence of the pores would create an area that could not hold the force from compression [10]. Besides that, the cortical bone in the tibia has a compressive strength of 166 MPa. The result of this scaffold was still below the cortical tibia bone compressive strength [9].

The use of crosslinker, such as glutaraldehyde in the bone filler affects the mechanical strength of the bone filler. The increase of crosslinker concentration in the bone filler more than 0.2 % could

decrease its mechanical strength [12, 13]. The presence of gelatin in the composite slowed down the initialization of cracking during compression and increase the endurance under compressive load [14]. It is known that the more porous structure is preferable for bone filler since it could provide faster ingrowth. However, a denser structure also provides better mechanical properties. The presence of crosslinker could form a denser structure in the bone filler. Thus, it could have better mechanical properties [15].

**Cytotoxicity Test.** The cytotoxicity test was performed using BHK-21 fibroblast cells for MTT assay. The MTT assay mechanism is shown by the tetrazolium salt that would be decreased while the cell has metabolic activity. The mitochondria of the living cell could produce dehydrogenase and when it is not functioning because of the cytotoxicity effect, the violet substance, formazan, will not be formed. This formazan substance indicated the activity of a living cell. Thus, it shows the viability of the cell [16]. The result was shown in Fig. 5.

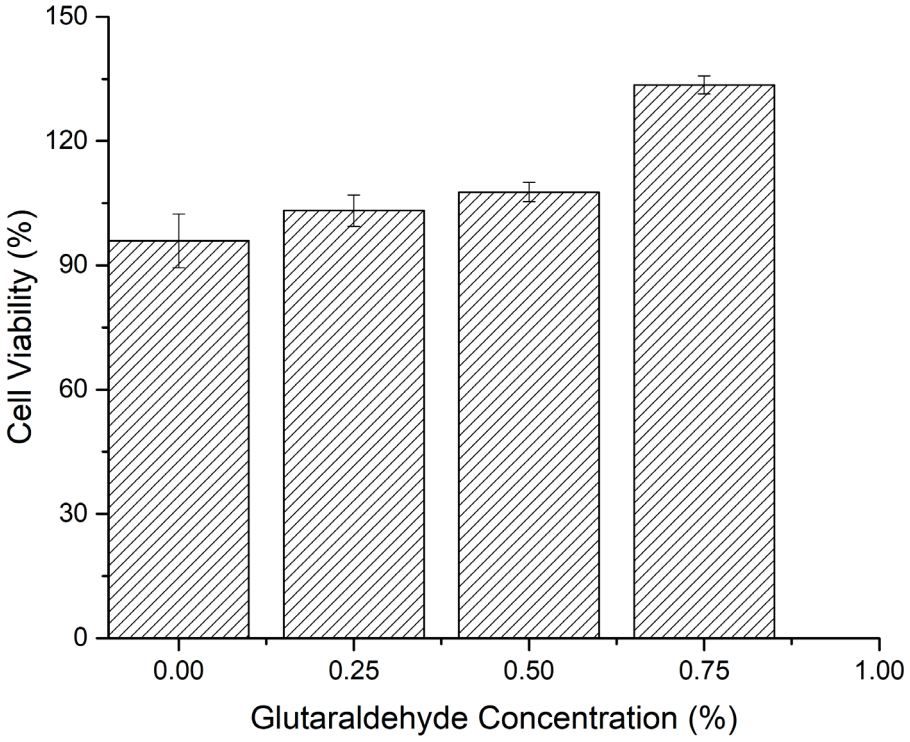


Figure 5. The Cell Viability of the Hydroxyapatite-Gelatin Composite with Several Glutaraldehyde Concentration

The result was shown that the cell viability of the samples with glutaraldehyde exceeded 100% which means that the samples were non-toxic [9]. The cytotoxicity properties were based on the $CD_{50}$. If the percentage of the cell viability was below 50%, then the material is toxic. As mentioned before that the samples had the cell viability more than 100%, thus they were non-toxic. Based on one-way ANOVA test, the result was statistically different with a p-value of 0.00017 ($p < 0.05$). The fibroblast cells could grow in the sample and did not show any rejection which showed its biocompatibility. The result of more than 100% means that the cell did not only accept the sample but they could also proliferate in the sample[10]. The following in vitro and in vivo test were needed to observe this behavior thoroughly. The glutaraldehyde had a restriction in its use due to its toxicity. But, the glutaraldehyde concentration used in this study was still in the tolerable range of the body which was below 2% v/v [9]. Azami et al. (2010) also studied the effect of glutaraldehyde on the cytotoxicity as a bone filler and the result showed that the scaffold using 2.5 v/v % GA as a cross-linker did not threaten the cell viability. Thus, the higher concentration of GA was preferable as long as it is still in the range of tolerable concentration [11]. The fact of the fibroblast cell could

proliferate indicated that the cell could attach and spread in the composite surface. The cell proliferation is important because the osteoconduction could occur if the cell could attach and proliferate on the implant or in this case, is the bone filler.

**Degradation Test.** The degradation test was performed using immersion method in PBS to observe the condition of bone filler after a specific amount of time to degrade. The result was shown in Table 1 The observation was performed when the samples showed the signs of degradation or crumbling.

Table 1. The Degradation Time of the Hydroxyapatite-Gelatin Composite with Several Glutaraldehyde Concentration

| Glutaraldehyde Concentration (v/v%) | Time Starting Degradation | Notes |
|---|---|---|
| 0 | 6 hours | Degraded |
| 0.25 | 28 days | No degradation, only erosion |
| 0.5 | 35 days | No degradation, only erosion |
| 0.75 | More than 40 days | No erosion after 40 days |

The sample without glutaraldehyde had a degradation time of 6 hours and the samples with glutaraldehyde could withstand in the PBS and do not show any degradation, but erosion. The sample without glutaraldehyde could easily degrade because there was no crosslinking bond in the gelatin chain and it tends to be swollen over time [6]. The sample with 0.25 % and 0.5% GA could withstand until 28 and 35 days after immersion. For the sample with 0.75 % GA, it even did not erode after 40 days. This behavior showed that the presence of the crosslinking bond due to glutaraldehyde could maintain the condition of the bone filler and also inhibit the degradation. The process happening in the immersion in PBS was the soaking of the PBS into the bone filler and the drug, alendronate, would be released. Because of that, the optimum glutaraldehyde concentration needs to be observed thoroughly so that the release of the drugs could be periodically controlled, around 30 days of bone healing period [12]. The presence of glutaraldehyde could give a higher stability of the bone filler. A previous study was also mentioned that GA enhanced the biostability of the scaffold [11].

When the GA concentration is increasing, the degradation time is longer than it was before. The crosslink of GA in the gelatin chains affects the expansion of the gelatin structure because the liquid insert to the bone filler. All the samples with GA had a crack that showed the presence of liquid inserting the bone filler since the immersion process [20]. The erosion of the bone filler with 0.25% GA and 0.5% GA was at $28^{th}$ and $35^{th}$ day, respectively. This behavior showed that the samples released the alendronate slowly with the bone healing time of 4-6 weeks until the callus formation [21].

## Conclusion

The hydroxyapatite-gelatin composite with the addition of alendronate was synthesized with several glutaraldehyde concentrations. The FTIR test was shown no significant difference between samples with and without glutaraldehyde because the C=N crosslinking bond peak presented in the gelatin. The morphology of the samples showed a bigger pore size for higher glutaraldehyde concentration which also supported by the lower the compressive strength. The result of compressive strength was statically different. All the samples were non-toxic based on the cytotoxicity test and the group was statistically different. The degradation tests showed that with the presence of glutaraldehyde the bone filler could maintain its form longer. From all the characterization results, the presence of glutaraldehyde could increase the features of the hydroxyapatite-gelatin composite with the addition of alendronate as a bone filler candidate for osteoporotic bone.

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**Conflict of Interest**

The authors report that there is no conflict of interest.

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