

Injectable Bone Substitute of Hydroxyapatite-Gelatin Composite with Alendronate for Bone Defect Due to Osteoporosis

Alfian Pramudita Putra^{1*}, Dyah Hikmawati¹, Aniek Setiya Budiati²

1. Biomedical Engineering, Department of Physics, Faculty of Science and Technology, Universitas Airlangga Surabaya 60115, Indonesia.
2. Department of Clinical Pharmacy, Faculty of Pharmacy, Universitas Airlangga, Surabaya 60286, Indonesia.

Abstract

World Health Organization mentioned that there were 200 million people in the world suffered from osteoporosis in 2013 and half of them has bone fractures. Hydroxyapatite as a bioactive material was explored as a substitute for bone defect or osteoporosis. The conventional bone filler was lack of the ability to let the filler to fill the irregular defect due to osteoporosis. Thus, a new method was introduced called an injectable bone substitute. While the bone filler performs its function to stabilize the mechanical property of the defected bone, the addition of a drug, such as an alendronate, would be beneficial. The injectable bone substitute (IBS) based on hydroxyapatite-gelatin was synthesized with the addition of alendronate. The Fourier Transform Infrared (FTIR) result showed a bond formation of hydroxyapatite and gelatin by shifting of carboxyl group wavenumber from $1332,72\text{ cm}^{-1}$ to $1559-1543\text{ cm}^{-1}$ from gelatin with Ca^{2+} from hydroxyapatite. The IBS viscosity was $(38.7\pm 0.53)\text{ dPa}\cdot\text{s}$ and was able to be extruded from the syringe. The IBS could become suspension again after sedimentation and did not change the pH of SBF solution. The IBS was precipitated with a suitable substrate. The cytotoxicity test showed that the samples were non-toxic. The results of IBS characterizations demonstrated that it has potential to be used as a bone filler as well as drug delivery system to the bone defect due to osteoporosis.

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Introduction

Bone defects caused by traumatic accident, tumor and total joint reconstruction often occurred and needed bone substitute materials. There were some implanted synthetic materials in the bone defect and still encapsulated by fibrous tissue and did not attach to bone.¹ Osteoporosis was bone deformation marked by bone strength reduction and influenced by increasing of bone fracture risk.² World Health Organization (WHO) in 2013 reported that around 200 million people in the world suffer from osteoporosis, and 50% of them had a bone fracture, especially in the upper leg³. Bone defect was caused by external factor and

osteoporosis was caused by an internal factor, such as reduction of bone ability to do bone remodeling process because of an unbalanced process of osteoblast and osteoclast. This case could be handled by increasing the bone density or filling the bone defect with a suitable material. Hydroxyapatite as a bioactive material was explored as a substitute for bone defect or osteoporosis.⁴ Hydroxyapatite is brittle. So, in its application, it needs another material from polymer group, like gelatin to support it. The addition of gelatin was aimed to increase osteoblast adhesion, migration, and mineralization. The hydroxyapatite-gelatin composite was already studied and proved that this composite was suitable to use as a bone substitute material with high biocompatibility and non-toxicity.⁵

The application of bone filler nowadays was not effective for the defect caused by the osteoporosis since the defect was in an irregular shape. The bone filler should fill the irregular defect while maintaining its mechanical function. Because of that, a new method called injectable bone substitute was introduced. Injectable bone

*Corresponding author:

Alfian Pramudita Putra,
Department of Physics
Faculty of Science and Technology
Universitas Airlangga
Surabaya 60115, Indonesia.
E-mail: pramuditaalfian@gmail.com

substitute (IBS) was bone substitute material in an injectable form. There were two kinds of IBS, IBS in ready-to-use suspension form and IBS with ionic hydraulic cement that could harden *in vivo* after injection.⁶ Weiss et al. (2007) already conducted research about calcium phosphate-based IBS paste that could harden after injection.⁶ The results showed that the paste could form suspension or paste by using hydroxypropyl methylcellulose (HPMC) polymer 2% w/v as a suspending agent. Another study from Warastuti and Abbas (2012) already succeeded to synthesize IBS from hydroxyapatite and chitosan with HPMC 2% w/v as suspending agent. That research showed the effect of irradiation in physics characterization of IBS. The results showed that irradiation 25 kGy did not affect the IBS.⁷ The function of IBS could be added as drug delivery to help bone defect healing by using a bisphosphonate drug, like alendronate. Alendronate has high-affinity electron to Ca^{2+} ion that could improve the interaction with bone calcium and inhibit the osteoclast in the bone remodeling process.⁸ Thus, there is a need to investigate the potential of hydroxyapatite-gelatin-based injectable bone substitute with the addition of alendronate for application in bone defect due to osteoporosis.

Materials and methods

Materials. Materials used in this research were hydroxyapatite powder from cow bone (Tissue Bank dr. Soetomo General Hospital, Surabaya, Indonesia), Gelatin (150 bloom Rousselot, Guangdong, China) from cow skin, Alendronate (Arshine Technology Co., Limited, Wanchai, China), *Hydroxypropylmethylcellulose* (HPMC) (Sigma Aldrich H7509, Singapore), distilled water, and *Simulated Body Fluid* (SBF) solution.

Methods. The samples were synthesized by dissolving 2% w/v HPMC in distilled water at 90-100°C and dissolving 5% w/v gelatin in distilled water at 40°C. The hydroxyapatite (HA) powder was added to the gelatin solution with a variation of 45:55, 50:50 and 55:45. The alendronate was added to that solution with a ratio of 1 to 10 compared to the mass of hydroxyapatite.⁹ The final step was added HPMC solution to hydroxyapatite-gelatin-alendronate solution gradually until that solution became a white suspension.

Fourier Transform Infrared (FTIR)

Analysis. The samples were characterized by Fourier Transform Infrared (FTIR) in Faculty of Mathematics and Natural Science, Institute of Sepuluh November, Surabaya, Indonesia. The sample was freeze-dried first at -20°C for 4 hours. KBr was added to the sample and compacted into a pellet. The FTIR test was performed in the wavenumber range of 4000 - 400 cm^{-1} .

Viscosity test. This test was performed in the Material Physics Laboratory Faculty of Science and Technology, Universitas Airlangga, Indonesia by using Viscotester VT-04F RION Co. Ltd. The composite suspension of hydroxyapatite-gelatin poured to the 300 ml Becker glass. The test used Rotor Number 1. The motor would stir the solution and the reader would read the viscosity of the solution corresponding to the second scale due to the use of rotor no. 1. The test was replicated 5 times.

Resuspension test. This test aimed to observe the ability of the solution to form suspension again after sedimentation in term of time to reform a suspension again and the pH of the solution. The test was performed by adding PBS to the suspension and shaken them. The time and pH after shaking until forming suspension again were recorded. The pH was measured by using pH-indicator strips (Merck®).

Cytotoxicity Test. This test used MTT assay method to evaluate the cytotoxicity properties of materials. The test was performed at PUSVETMA, Surabaya by using fibroblast cell from Baby Hamster Kidney (BHK)-21. The living cell would react by changing the tetrazolium salt to formazan. The fibroblast cell of BHK-21 in Eagle's media was incubated for 48 hours and cleaned by using Phosphate Buffer Saline (PBS). 86% Eagle's media, 1% Penicillin streptomycin and 100 units of Fungizone/ml was prepared for 100 μl and the cultured cell was inserted to it. The cell culture was moved to 96-microwell plate. The suspension sample was diluted four times to be neutral. 1 ml of that suspension was dissolved in 10 ml aquabidest. 1 μl of that sample was placed in each well and was incubated for 24 hours at 37°C. The micro well plate was rinsed with PBS and was added 10 μl of 5mg/ml MTT solution in each well. Every well was added with 50 μl of DMSO and then centrifuged for 5

minutes with 30 rpm. The measurement process was performed by Elisa reader showing the violet level as Optical Density (OD) which represents the cell viability of the material based on Eq. 2.¹⁰ The test was replicated four times. % cell viability = (OD treatment + OD media control)/(OD cell control + OD media control) (2)

Setting time and morphology test. The setting time and morphology test was performed aimed to obtain the time which the suspension needed to set in the application. Two types of substrate were used, glass and hydroxyapatite-collagen scaffold. After that, the substrate that was treated with the suspension was observed in its physical appearance and furthermore, characterized by Scanning Electron Microscope (SEM) Inspect S20 with magnification of 1000.

The statistical test. The data was analyzed by using statistical test which was one-way ANOVA test with p-value of 0.05 and presented as its average and standard deviation.

Results

FTIR test. The sample was freeze-dried and characterized by Fourier Transform Infrared (FTIR) first. The result showed the spectra of the functional bond in the sample as shown in Figure 1.

Based on spectra in Figure 1, there were some absorbance peaks that represented specific functional groups of the material, such as 3262.65 cm^{-1} which represented the hydroxyl O-H group (intermolecular hydrogen bond) that came from HPMC, gelatin, hydroxyapatite, and alendronate.¹¹ The absorbance at a wave number of 1332.72 cm^{-1} represented the carboxyl (COO^-) bond from proline of the gelatin which was the specific characteristic of type I collagen.¹¹ Besides that, the absorbance at a wave number of 1627.82 cm^{-1} represented the amine (NH_2) group from gelatin and alendronate. The most important part was the presence of a new bond shifting from 1332.72 cm^{-1} to 1558.65 cm^{-1} which was an absorbance peak of the interaction of Ca^{2+} from hydroxyapatite and carboxyl group from gelatin.^{11,12} The P-O-C and phosphate group from hydroxyapatite and alendronate was also present at 1042.24 cm^{-1} and 547.13 cm^{-1} , respectively.¹⁰

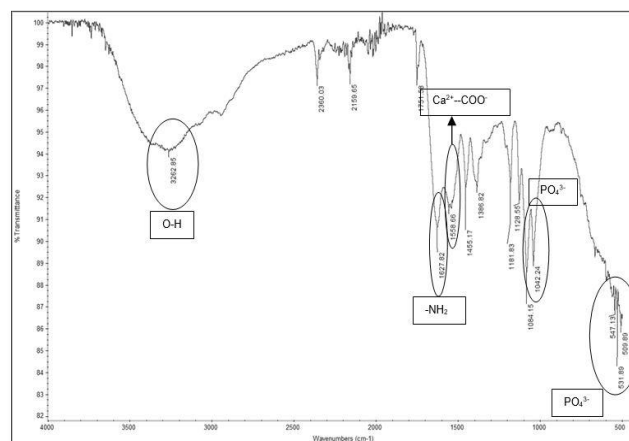


Figure 1. The Hydroxyapatite-gelatin based IBS suspension with addition of alendronate FTIR spectra.

Viscosity test. The viscosity of IBS is essential as the injectability property is defined by the viscosity. The viscosity IBS suspension was characterized by Viscotester VT-04F Rion Co. Ltd. The results of viscosity test showed in Figure 2.

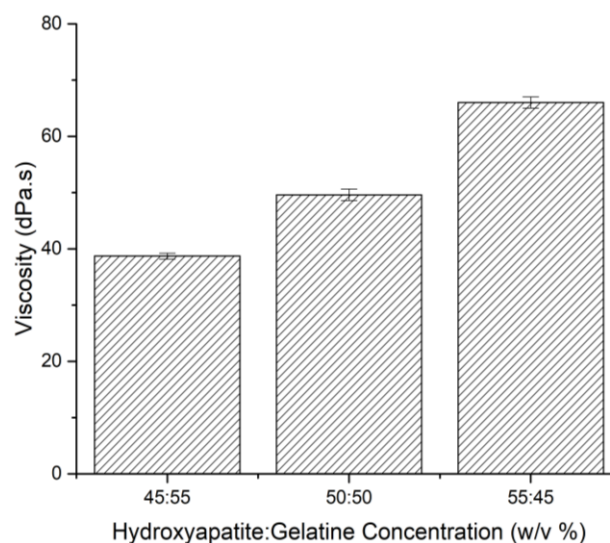


Figure 2. The Average Viscosity of Hydroxyapatite-Gelatin Based IBS Suspension with Addition of Alendronate.

It showed the viscosity of IBS suspension from different hydroxyapatite-gelatin composition variation in five replications. The results of hydroxyapatite-gelatin composition variation viscosity 45:55, 50:50 and 55:45 were (38.7 ± 0.53) dPa.s, (49.6 ± 1.02) dPa.s, and (66 ± 1) dPa.s, respectively. The One-way ANOVA test showed that the result were significantly different with p value < 0.05.

Resuspension test. There were two parameters observed in the resuspension test, which were time and pH to show that the IBS suspension was able to reform and the sediment part could disperse again, did not harden and did not change the pH of Simulated Body Fluid (SBF). The test was performed by applying SBF solution to the suspension samples and measuring the pH and time to form suspension again after mixing or shaking the suspension. The result of resuspension test showed in Figure 3, 4 and 5. Figure 4 showed the results of resuspension test based on pH. The pH was around 7 to 7.9. All samples indicated a stable result and above 7.



(a) (b) (c)

Figure 3. The resuspension test of hydroxyapatite-gelatin based IBS suspension with addition of alendronate (a) Before mixing with SBF (b) After mixing (c) After sedimentation.

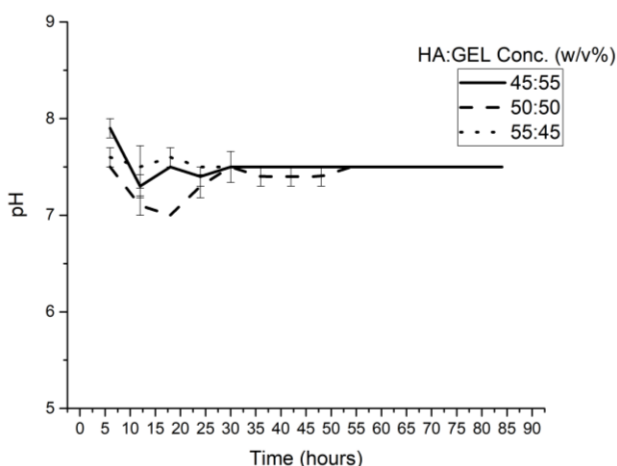


Figure 4. Resuspension test result of hydroxyapatite-gelatin based IBS suspension with addition of alendronate based on pH.

Besides that, it did not show any abnormality and did not change pH of SBF solution drastically which had pH around 7.4.

Figure 5 showed the resuspension test result based on IBS suspension time of forming suspension again. In the first to second day, the time of resuspension was not stable because of the particles in the suspension was still spread in the suspension. After the third to seventh day, the result showed the stable line of resuspension time.

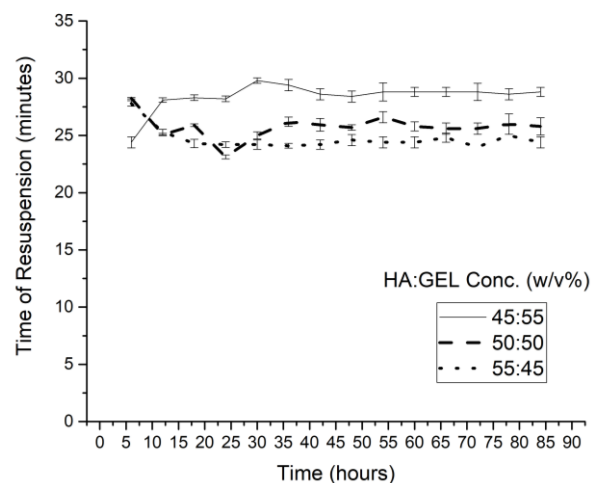


Figure 5. The Resuspension test result of hydroxyapatite-gelatin based IBS suspension with addition of alendronate based on time.

Cytotoxicity test. The cytotoxicity test in this study was using MTT assay method to observe the viability of Baby Hamster Kidney-21 (BHK-21) fibroblast cells towards the IBS suspension. This method was evaluating the change from the tetrazolium rings from MTT due to the activity of mitochondria of the living cells. The amount of the change could be measured by using Elisa reader to obtain the optical density which indicated the viability of the cells. The result showed in Figure 6 and in indicated that all samples were non-toxic since the cell viability exceeded 50%. The sample with a hydroxyapatite-gelatin ratio of 55:45 exceeded 100%.¹³ Based on the One-way ANOVA test, the result was significantly different with p value < 0.05.

Morphology test. The setting time test was performed to obtain the time of hardening process of IBS suspension. This test was conducted in a petri dish. The result showed that the suspension could not harden until seven days. This IBS suspension needed a suitable substrate for hardening process with a similar component to the human bone. Because of that, a freeze-dried hydroxyapatite-collagen scaffold was used

in this research as a substrate media. That scaffold was immersed in IBS suspension for an hour and was dried at room temperature. The scaffold needed 5-6 hours for setting and hardening process.

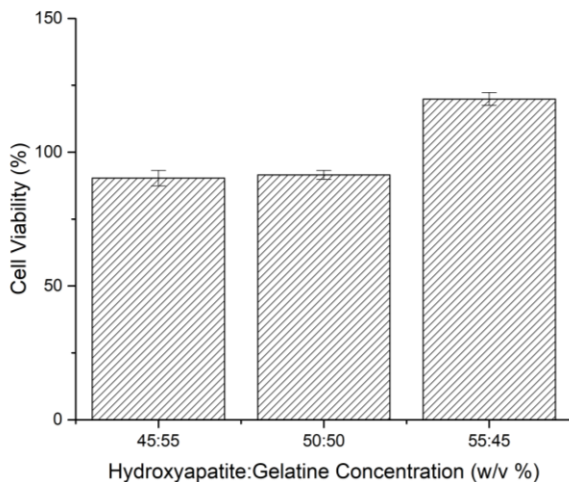


Figure 6. The cytotoxicity test result of hydroxyapatite-gelatin based IBS suspension with addition of alendronate.

The change in scaffold mass was measured before and after the immersion in IBS suspension. The result showed that the mass increased from 0.12 gram to 0.26 gram in wet condition and 0.21 gram in dry condition. The height and diameter of the scaffold also changed from 9.2 mm and 4.2 mm to (9.178 ± 0.053) mm and (3.824 ± 0.156) mm. This result showed that the IBS suspension was able to set in the suitable substrate. Hydroxyapatite-collagen that was synthesized in the freeze-dried process produced some pores that facilitated the IBS suspension to infiltrate the scaffold. The dried scaffold after immersion was characterized by Scanning Electron Microscope (SEM). The SEM image of dried hydroxyapatite-collagen scaffold after immersion was shown in Figure 7. The average of pore size was (19.125 ± 0.201) μm . This size was bigger than scaffold pore size before immersion. This size indicated that alendronate in this IBS suspension could bind the calcium ion of hydroxyapatite and formed some pores with increasing its size. This could occur because alendronate had high electron affinity to Ca^{2+} ion.⁸

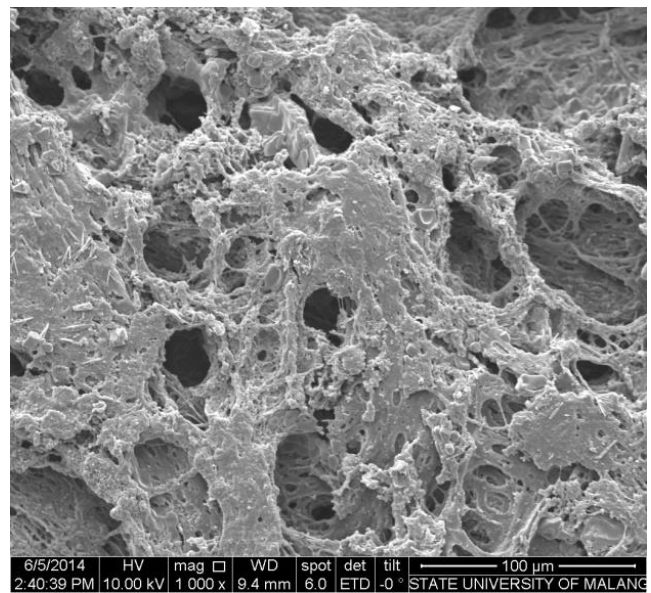


Figure 7. SEM image of dried hydroxyapatite-collagen scaffold after immersion in hydroxyapatite-gelatin based IBS suspension with addition of alendronate.

Discussion

In this study, an Injectable Bone Substitute (IBS) based on hydroxyapatite and gelatin with the addition of alendronate was developed. The presence of HPMC aimed to form the composite as a suspension. The Fourier Transform Infrared (FTIR) study of Injectable Bone Substitute (IBS) showed that there was a new bond between Ca^{2+} ion from hydroxyapatite and carboxyl group (COO^-) from gelatin, as mentioned by Narbat et al. and Wang et al. in their studies.^{11,12} This bond was a covalent bond which presented in the composite. The presence of HPMC would provide more interaction between the Ca^{2+} ion from hydroxyapatite and the carboxyl group from gelatin. The alendronate was also still present in the composite as shown in absorbance peak at 1627.82 cm^{-1} in the FTIR test result.¹⁴

The standard viscosity for IBS application based on Bourges et al. study was 40 dPa.s.¹⁵ The IBS composition that has viscosity value around the standard was the combination of hydroxyapatite and gelatin at 45:55 which was 38.7 dPa.s. The viscosity of IBS suspension had an important role in its application because it determined the injectability. The sample in suspension form was able to sediment and need a mixing or shaking to make it in homogeneous

state again.⁶ The samples that were characterized by viscosity test were also tried in 10 ml syringe with 2 mm inner diameter tip.¹⁶ All the IBS suspensions can be injected from that syringe easily. It showed that the samples had good injectability.

The resuspension test observed the change in pH and time to form suspension again. It indicated that the alendronate addition in the IBS suspension could not change the pH of the body when it applied in the bone fracture. Alendronate had low pH or prominent acidity around 4 – 4.5, and it could affect human body when it applied to the body directly. The pH of IBS solution also affected the material ability in setting time when applied to the bone. The average pH of hydroxyapatite for an application that could set in the bone was 6.¹⁶ The amount of hydroxyapatite used in IBS solution increased the resuspension time because hydroxyapatite powder was affected by gravitation although there were still hydroxyapatite powders that flew around in the IBS suspension.⁶ Its characteristic as suspension caused its particle sediment less than an hour and could form suspension again after shaking and did not harden at the bottom of the glass vial.

The setting time test result indicated that the IBS suspension needs a suitable substrate to set. In the application case, the bone is the most ideal substrate. Thus, the IBS suspension would set in the bone. The use of hydroxyapatite-collagen scaffold with the application of IBS suspension showed that it could set and also infiltrate the scaffold which represented the bone. The dimensional change of the scaffold also emphasized the result of the FTIR test which showed that there was an interaction between the hydroxyapatite and gelatin.

All of the results mentioned above was also strengthened by the result of the cytotoxicity test that all the samples were non-toxic with the cell viability of around 100% and even stimulate the proliferation of the fibroblast cells.¹⁰ The other studies need to be conducted to investigate the behavior of this IBS suspension thoroughly, especially in the injectability, alendronate release, the stability, and also in vivo study.

Conclusions

The hydroxyapatite-gelatin based injectable bone substitute with addition of alendronate can

be synthesized and showed good characteristics in FTIR, viscosity, resuspension and setting time.

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Declaration of Interest

The authors declare there is no conflict of interest.

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