Materials Science Forum
ISSN: 1662-9752, Vol. 966, pp 133-138
doi:10.4028/www.scientific.net/MSF.966.133

Submitted: 2018-11-27
Revised: 2019-03-28
Accepted: 2019-05-09
Online: 2019-08-06

Physicochemical and Cytotoxicity Characterization of Injectable Bone Substitute Based on Hydroxyapatite - Chitosan - Streptomycin for Spinal Tuberculosis Cases

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Keywords: chitosan, cytotoxicity, hydroxyapatite, injectable bone substitute, physicochemical, spinal tuberculosis, streptomycin

Abstract. Injectable bone substitute (IBS) based on hydroxyapatite, chitosan and streptomycin has been developed successfully. The IBS was made by mixturing 20% w/v hydroxyapatite and varying the chitosan ratio of 60:40, 65:35, 70:30, 75:25 and adding streptomycin as antibiotic substance. The mixture was added with hydroxyl propyl methylcellulose. The synthesis process was steady and no chemical reaction occurred as proven by Fourier Transform Infrared Spectroscopy (FTIR). The in vitro characterization were acidity (pH) and cytotoxicity test (MTT assay), while the physical characterization performed included injectability test, setting time, and morphology. The acidity test showed that the pH samples reached the human normal pH (6.8-7.4) in seven days. The cytotoxicity test proved that the samples were non-toxic. The repasta test showed that the acidity reached the human pH and could release the IBS pasta around 111-150 seconds. The injectability test indicated that IBS had ability to be injected for 95-96%. The setting time in all samples needed 72-166 minutes when it was injected into human bone scaffold model that was able to coat the pore of its scaffold model which proven by Scanning Electron Microscope (SEM) imaging. The pore size of human bone scaffold model was decreased from ±800 μm into ±120 μm. So, IBS pasta based on hydroxyapatite-chitosan-streptomycin in physicochemical and cytotoxicity behaviour is preferable to be applied for spinal tuberculosis cases.

Introduction

Based on World Health Organization (WHO) data in 2015, there were top 10 disease that causes death in the world was mostly dominated by pulmonary disease. Indonesia was placed on the second position on tuberculosis disease after India in 2017 [1]. Tuberculosis was caused by *Myobacterium tuberculosis*. This bacteria not only attack human's lung but by the time it would attack human's spinal too. Human's spinal would be infected, got hyperemia, and also got edema [2]. Treating tuberculosis disease nowadays is by using first line of anti-TB drugs such as streptomycin (S), rifampicin (RIF), isoniazid (INH), etc. that should be consumed orally by patient 4 – 5 tablets per day until 6 – 9 months [3]. The second way to treat tuberculosis disease by doing medical operation that would take some part of the patient's infected bone. Not only oral treatment but also medical operation have some limitation. Consuming anti-TB drug is not effective because it is difficult to reach human's lung.

Improving the limitation of tuberculosis treatment, injection treatment is preferred to increase the effectivity of the drug to reach the bone directly and decrease patient's pain that caused by medical operation. The injection treatment or so-called injectable bone substitute (IBS) was made by nano hydroxyapatite (nano-HA) – chitosan as a bone filler for spinal and mixed with streptomycin as anti-TB drugs to inhibit the DNA formation of *Mycobacterium tuberculosis*. Adding hyrdoxyprophyl methylcellulose (HPMC) as a suspending agent is very important to develop IBS pasta formation [4-5]. HPMC function not only as a crosslinker but also restrain the flow of IBS pasta that will be injected [6]. Nano-HA is a bio ceramic material contain calcium and phosphate that easily fabricated become IBS pasta. Nano-HA has a good stability, biocompatible, osteoconductive, and good bioactivity to be applied on human's bone. Nano-HA that contains inorganic material become a matrix for bone cell like osteoblast to proliferate [7-8]. Chitosan is the most abundant polysaccharides that has good biocompatibility, non-toxic, biocompatible, biodegradable, and good as drug carrier would protect nano-HA that contain streptomycin [9].

The combination of nano-HA – chitosan – streptomycin was chosen as the raw materials because nano-HA itself would attract osteoblast to proliferate, chitosan would protect the interaction between nano-HA – streptomycin, and the streptomycin itself as an anti-TB drug to destroy TBC's bacteria in the spinal and lung directly. The characterizations in this study were Fourier Transform Infrared (FTIR), injectability test by syringe, and setting time using Scanning Electron Microscope (SEM) as physical characterization. Acidity test and repasta test for chemical characterization. The biological test used was MTT-Assay for observing the degree of cytotoxicity.

Materials and Methods

Materials. Nano hydroxyapatite and chitosan was obtained from Badan Tenaga Nuklir Nasional (BATAN) Jakarta, Indonesia. The streptomycin sulfate (powder for injection) was obtained from PT. Meiji Indonesia. The hydroxypropyl methylcellulose (HPMC) was obtained from Sigma Aldrich H7509. The materials used for characterization were human bone scaffold model from Bank Jaringan Dr. Soetomo Hospital (Surabaya, Indonesia) for setting time test and SBF solution that contain from $NaHCO_3$, NaCl, KCl, $K_2HPO_4.3H_2O$, HCl, Na_2SO_4, $CaCl_2.2H_2O$, $MgCl_2.6H_2O$, and $(HOCH_2)_3CNH_2$.

Methods. The injectable bone substitute (IBS) was synthesized by dissolving 20% w/v chitosan in acetic acid at 40ºC for one hour. The hydroxyapatite (HA) powder was added to that solution with several ratio of HA:chitosan (60:40, 65:35,70:30 and 75:25). 10 wt% of streptomycin was added in the mixture. 4% w/v HPMC was dissolved into distilled water at 90ºC. Then, the suspension of HPMC was added into hydroxyapatite-chitosan-streptomycin suspension at 40°C in six hours to generate white pasta of IBS.

The FTIR test was used to observed whether there was no interaction among the materials used in this study based on the functional groups. All the IBS pasta sample would be freeze-dried to make it solid. The solidified IBS was mixed with KBr and compressed into pellet to be characterized by FTIR tools.

The injectability test was aimed to observe the ability of the IBS to be extruded from a syringe within a range of time. The test used 10 cc syringe with the inner diameter of the needle was 1.2 mm [10]. The percentage of injectability could be calculated by measuring the weight before and after injection. By using Eq. 1, the injectability of the IBS could be obtained. The test was repeated five times.

$$\text{Injectability (\%)} = (\text{Mass extruded from syringe/Total mass before injection}) \times 100\% \quad (1)$$

The setting time test was performed by applying human bone scaffold model that was injected by IBS pasta vertically. In the setting time, the mass of human bone scaffold model was measured before and after injected IBS pasta and the morphology of the covered human bone scaffold model could be characterized by SEM.

The acidity test was used to observe the stability of the IBS pasta itself before injected into the human's body. The acidity of the IBS was measured overtime by using pH meter. The materials were considered on pH 6.8 – 7.4 as human's pH inside the body.

The repasta test was aimed to observe the needed time for solidify IBS becoming pasta again when the solidify IBS dissolved into Simulated Body Fluid (SBF). SBF is a solution that has some composition like human's body. The other purpose of this test also to observe the stability IBS pasta in pH compound between SBF and IBS pasta.

The four best samples based on all characterization mentioned above were continued to the biological characterization. The cytotoxicity test was performed by using MTT assay method which used 3-(4,5-dimethyl-2-thiazolil)-2.5-diphenil-2H-tetrazolium bromide (MTT reagent). This substance would give the information of viability cell while it changed to formazan salt due to the activity of mitochondria of living cell. The cell used in this test was fibroblast cell from Baby Hamster Kidney (BHK-21). The optical density of formazan salt would be measured by using Elisa Reader. The cell viability would be calculated by using Eq. 2. The materials were considered as not-toxic if the cell viability is more than 50% [11].

$$\text{Cell viability (\%)} = ((OD_{\text{treatment}} + OD_{\text{media}})/(OD_{\text{cell}} + OD_{\text{media}})) \times 100\% \quad (2)$$

Result and Discussion

Functional Group Test. The IBS was synthesized in four variations, the hydroxyapatite-chitosan ratio in 75:25 ratio was used in this test. The FTIR test was performed at several variations shown in Fig. 1.

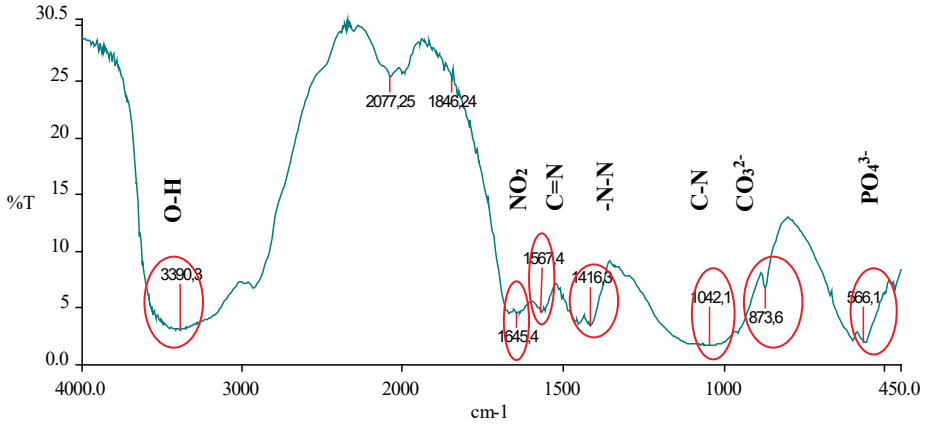

Fig. 1. The FTIR Result of IBS Sample with a Ratio of HA:chitosan (75:25)

The results show that there are several absorbance peaks related to some specific functional groups. The peak at wavenumber of 3390.3 cm^{-1} resembled the stretching vibration of hydroxyl group from the material used in this study; HA, chitosan, HPMC and streptomycin. The absorbance at wavenumber of 2077.25 cm^{-1} was the characteristic of stretching vibration C-C which was the specific functional group from HPMC and streptomycin. The peak at 1645.4 cm^{-1} showed the bonding between the carbonyl functional group originated from chitosan and the amine group from the streptomycin. Furthermore, the stretching vibration of carbonate (CO_3^{2-}) and phosphate (PO_4^{3-}) functional group which was the specific functional groups from HA showed at wavenumber of 873.6 cm^{-1} and 566.1 cm^{-1} [12].

Injectability Test. The injectability test was performed by using a 10 cc syringe. The four samples of IBS pasta show the good result which had percentage of injectability near to 100%. The best result was chosen in HA:chitosan ratio of 75:25 that had 96.24% in 15 seconds. This result was correlated with Shen et al. in 2014 who synthesized IBS based on calcium phosphate and alendronate 3% which has injectability result 96.88 % [4].

Setting Time Test. The setting time test was performed by using a freeze-dried HA scaffold as a model of human bone. The substrates had the same main component of the sample as the natural bone, such as the composition (hydroxyapatite) and the structure. The result of this test was shown

in Fig. 2. From the result of this result, all samples have long setting time over 1 hour. The fastest setting time occurred in nano HA: chitosan ratio on 75:25, it needs 72 minutes. The data mentioned below so fluctuating. By adding chitosan, the setting time should be decreased because chitosan was produced gel-like matrix which reduced the setting time of IBS based on their viscosity [13]. The fluctuating data may be caused by handling factor of IBS pasta into their injectability. Pasta injection through the needle based on the driving force that will be applied on bone will affect the setting time [13]. Based on the study of Thai in 2010, they mentioned that the setting time of the IBS sample with calcium phosphate, calcium sulphate, HPMC, and citric acid was 30 minutes with 20% of citric acid and less than 10 minutes with 40% of citric acid [14]. The setting time of IBS less than 10 minutes could be applied in the defect of the small bone, such as carpal bone, while the other one could be used in the bigger bone, such as clavicle bone.

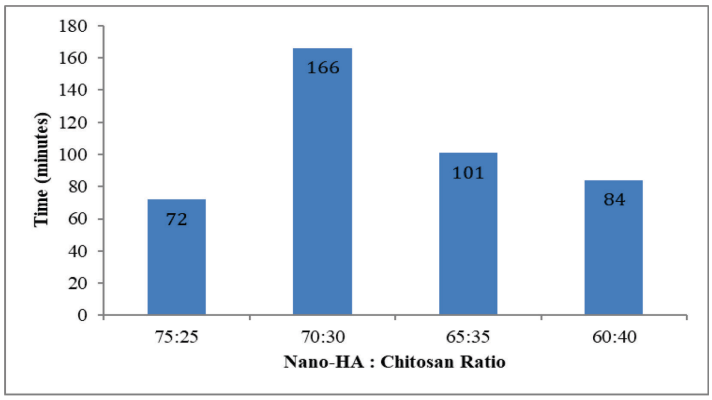

Fig. 2. The Setting time test results of IBS

After the result above, the four variations was tested with the change in the mass due to the presence of the IBS in the HA-collagen scaffold as a human bone model in dry condition comparing HA-collagen scaffold without IBS pasta. IBS pasta variations on 75:25, 70:30, 65:35, and 60:40 increasing the weight 0.0292 gr, 0.0463 gr, 0.0518 gr, and 0.0377 gr. This showed the ability of IBS pasta attached the HA-collagen scaffold. The morphology of human bone scaffold model that covered before and after injected IBS pasta was shown in Fig. 3 with the ratio of HA:chitosan on 60:40. The pore size of human bone scaffold model before injected IBS pasta about 780.8-835.4 μm and after injected become 99.76-134.2 μm. From the SEM imaging, it might be conclude that IBS pasta could spread into the human bone scaffold model and bounded the hydroxyapatite [12, 15]. Increasing of the weight and decreasing of the pore size diameter caused by apatite mineral of IBS pasta would imply the density of bone become higher. Apatite and chitosan formation would promote the osteoblast cell proliferation around spinal bone and then could heal the osteoporotic spinal bone.

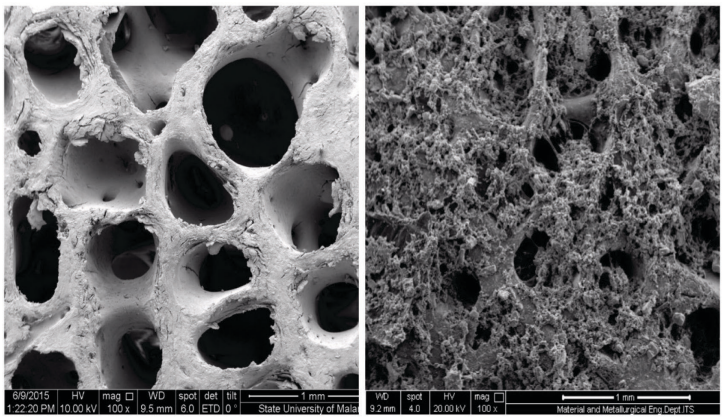

Fig. 3. Morphology of human bone scaffold model **(a)** Before injected IBS pasta (100X) **(b)** After injected IBS pasta (100X) using SEM

Acidity (pH) Test. The acidity of the sample is one of the important points in the evaluation of the performance of IBS. The result showed that the average pH of sample with HA-chitosan ratio of 75:25, 70:30, 65:35 and 60:40 (w/w) were 7.29, 6.91, 7.19, and 6.99, respectively. The IBS needed pH more than 6 to be set in the bone. This result was tolerable for the body and could give no pain effect.

Repasta Test. Repasta test was aimed to know the freeze-dried IBS pasta behaviour in human's body fluid using Simulated Body Fluid (SBF) solution as human's body fluid. Freeze dried IBS pasta used as the setting model of IBS pasta that harden in human's spinal. The other purpose of repasta test is for knowing the release function of streptomycin as anti-TB drug. The result could be seen on Fig. 4. Increasing the nano-HA concentration would increase the needed time to become IBS pasta again. The result of pH compound between 6.88 and 7.03 which was still preferable to be injected in the human's body. Streptomycin works on pH 3 – 7 to attack *Myobacterium tuberculosis*, so the pH compound in repasta test of all samples preferable place for streptomycin doing its function [8].

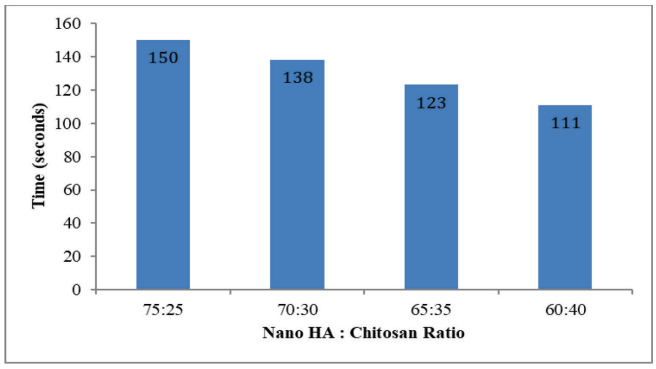

Fig. 4. Repasta time of the IBS sample with Several HA:Chitosan ratio

Cytotoxicity Test. The cytotoxicity test was conducted by using fibroblast cells from Baby Hamster Kidney (BHK-21). The MTT assay function is to quantify mitochondrial activity that would detect living cells [16]. From this test, the result depicted that the IBS samples were non-toxic, because the cell viability was more than 50% shown in Fig. 5. This result also showed the percentage which was more than 100%. That result meant that the IBS could be the place for the osteoblast cells to grow and promote proliferation of the cell [11].

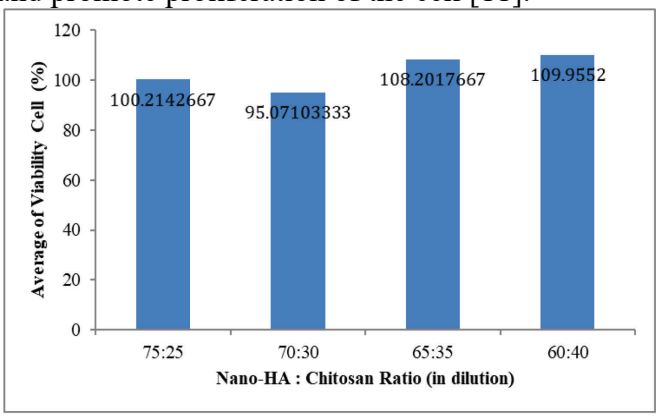

Fig. 5. The Cytotoxicity Test Result of the IBS sample with Several HA:Chitosan ratio

Summary

Injectable bone substitute based on nano-HA – chitosan – streptomycin with several concentration variation showed the best results in injectability test, acidity test, repasta test, and cytotoxicity test. In setting time test, there was a problem with the time when IBS paste was injected to the HA-collagen scaffold model. The failure could be reduced by considering the handling factor with some precision tools like extruder. FTIR result detected any functional group in the composition of IBS pasta. Sample with the ratio 60:40 shows the best result of all samples by considering cytotoxicity

test which has higher cell viability about 109.9552±6.6395% that would provide osteoblast proliferate faster to improve osteoporosis healing in spinal tuberculosis.

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