

SYNTHESIS OF HAp-CHITOSAN-PVA COMPOSITE AS INJECTABLE BONE SUBSTITUTE MATERIAL

Firnanelty¹, S. Sugiarti^{1,*} and Charlena¹

¹Department of Chemistry, Bogor Agricultural University, Bogor, West Java, 16680, Indonesia

*E-mail : sri.sw07@gmail.com

ABSTRACT

The main objective of this study is to synthesize a composite of hydroxyapatite (HAp)-chitosan-polyvinyl alcohol (PVA) and to evaluate its uses as bone filler. An injectable bone substitute (IBS) material is a suspension of bone filler which applied by injection to fill the gaps caused by osteoporosis. A hydroxyapatite used as the composite's component was synthesized from *Tutut* shell. The composite was made by mixing hydroxyapatite, chitosan and PVA to homogeneous followed by irradiation with gamma rays at 20 kGy. The composite was characterized by FTIR analysis, XRD, SEM, viscosity test, and cytotoxicity assay. The FTIR analysis showed the bond formation between hydroxyapatite and chitosan, which later formed cross linkages by the addition of PVA. The viscosity of the composite was 36dPa. The cytotoxicity assay (MTT assay) showed that the suspension is not toxic because the cell viability value was obtained at more than 50%. As a conclusion, the composite of HAp from *tutut* shell-chitosan-PVA is suitable to be used as an injectable bone substitute.

Keywords: Hydroxyapatite, Injectable bone substitute, Osteoporosis, *Tutut* shell

© RASĀYAN. All rights reserved

INTRODUCTION

Bone filler is a suspension applied by injection to fill in the gaps caused by osteoporotic. This bone filler is also called an Injectable Bone Substitute (IBS). The advantage of using injectable system to prevent the loss of bone density, is that it can be formed according to the shape of the bone cavity, and it is polymerized in situ after being injected. Injectable materials are also sterile and ready to use¹. To be used as IBS, a material should be osteoconductive and have a good mechanical strength².

One material that has osteoconductive property is hydroxyapatite. Hydroxyapatite (HAp) is a group of minerals apatite compound with chemical formula of $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$. HAp is a bioceramics that are frequently used as an element of the main in anorganic constituent of bones. Approximately 65% of bone mineral is hydroxyapatite³. Other properties of HAp are bioactive, biocompatible, non-toxic and non-immunogenic⁴. However, HAp has some weakness as it is fragile, brittle, low resistance, and has a very slow resorption rate. When used alone, hydroxyapatite does not have a mechanical strength and is not resistant to pressure. Manipulation is needed to produce an ideal bone graft. A common strategy is by composited HAp with other materials such as chitosan to address its nature fragility. Its biocompatible, low toxicity, and anti-bacterial properties⁵ makes chitosan eligible for use as a bone substitute. The addition of chitosan causes a decrease of degradation properties and bond strength of bones⁶.

Polyvinyl alcohol (PVA), which has the mechanical characteristics and good biocompatibility can be used to enhance the performance of a composite⁷. PVA is widely used to replace a damaged body tissue because it possesses a physic-chemical property, particularly it is an excellent bio-tribiological due to its slick surface, resistant to friction and wear⁸. Furthermore, PVA has good biocompatibility⁹. In composite, PVA is also acting as cross-linking agent. Composites of hydroxyapatite-chitosan-PVA is suitable to be used as a bone substitute material for tissue engineering applications in the form of IBS, and in the presence of hydroxypropylmethyl cellulose (HPMC) as a suspending agent¹⁰.

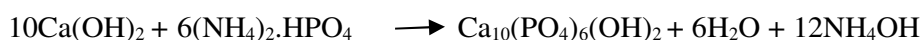
This conducted study is not only an attempt to correct the flaws on the nature of HAp, but also to select the most economical starting material in the manufacture of calcium phosphate. Tutut shell is an abundant waste and has not been used commercially. This waste is rich in various minerals including calcium¹¹. Tutut shell contains mostly calcium carbonate with calcium content about 64.73%¹².

Synthesis of hydroxyapatite and the composites are done with wet precipitation method. To fit its purpose to be used in a human body, the composites received a high-intensity gamma irradiation to kill harmful organisms and simultaneously sterilizes the material. Visually, a composite which underwent irradiation showed a thick paste and a homogeneous gel consistency¹³. In this research, HAp synthesized from Tutut composited with chitosan and PVA to be used as IBS material. The resulting composite can be used in a variety of clinical purposes in the field periodontal, orthopedic and plastic surgery and has met some of the qualities required in lieu of bone (bone substitute) corresponding in vitro test result, physical and chemical¹⁴.

EXPERIMENTAL

General Procedures

Cleaned shells of Tutut were boiled for 1 hour, the sundried, grounded, and shieved to give a 100 mesh of powder. The Ca level of the powder was determined before undergo calcinations process to give CaO compound. Ca(OH)₂ was obtained by allowing the CaO to interact with air (hydrated) for one week period¹⁵. Hydroxyapatite was synthesized through wet precipitation method from Ca(OH)₂ 0,5M by a reaction with (NH₄)₂.HPO₄ 0,3 M according to the following reaction:



Composite of HAp-chitosan-PVA was synthesized trough sol method. 0.5% solution of chitosan in 3% of acetic acid was mixed with 5% which previously dissolved in water. Solution of hydroxyapatite is the added into the mixture, followed by a solution of HMPC 2%. The mixture then stirred at room temperature for 6 hours to give a white suspension. The composite the irradiated with a dose of 20 kGy. The synthesized composites were analyzed using XRD, FTIR and SEM. Since the composites are addressed as injectable material, a suspension made from the composites were tested for its viscosity value. The viscosity of the suspension was measured using Viscotester TV-10.

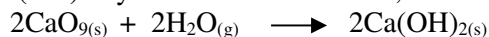
Cytotoxicity test of the composite

Cytotoxicity of the composite was tested by the MTT (3-(4,5-dimetiltiazol-2-yl)2,5-difeniltetrazolium bromide) performed on CPAE cells. CPAE cells were grown in DMEM medium. Cells used inoculated plates comprising 96 wells by the number of media grower 100mL/well containing 2200 cells/well. A total of 100 mL sample with a sequence specific concentration was added to the inoculants, then incubated for 48 hours in a 5% CO₂ incubator at 37 °C. Further into each well added 10 mL of MTT and incubated back for 4 hours in a 5% CO₂ incubator at 37 °C. Living cells that react with MTT to form formazan become blue. Formazan formed was dissolved in 96% ethanol. Uptake is read by a spectrophotometer ELISA microplate reader at λ 595 nm.

RESULTS AND DISCUSSION

Shells of Tutut, Figure-1, is a source of calcium, in the form CaCO₃. XRD analysis of Tutut Shell powder showed peaks for aragonite CaCO₃ as the main component, as shown by Figure-2a.

The transformation of CaCO₃ to CaO was done by calcinations at temperature between 1000 to 1200 °C for 3 hours. This high temperature is chosen to prevent the high formation of CO₂. The obtained CaO compound was converted into Ca(OH)₂ by direct contact with air, following the equation:



The XRD pattern of Tutut shell after calcinations indicates that the powder mainly consisted of portlandite (Ca(OH)₂) as shown by the presence of peaks at 2θ (°) of 18.18, 28.68, 34.30, 47.40, 50.92, 54.16, 62.62, 64.16, 67.46 (Figure-2b).

The Calcium content of Tutut shell powder was 55.37% which has lower than the results reported who found the value to be 64.73%¹⁶. However, this calcium content is higher than that of rice fields nail shell which was found to be 52%¹⁷. This high content of calcium showed that Tutut shell can be used as precursor in the synthesis of HAp.

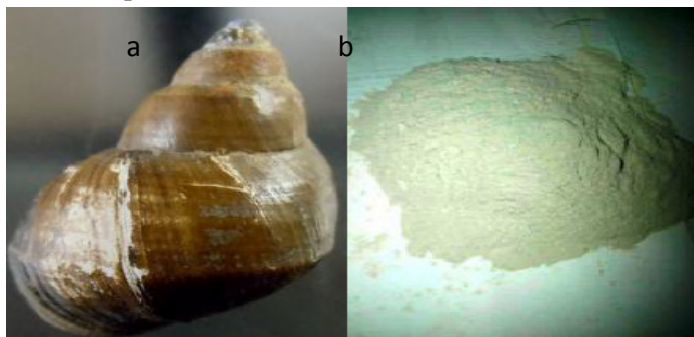


Fig.-1: Shells of Tutut (*Bellamyja javanica*) (a) Dry, (b) Powder

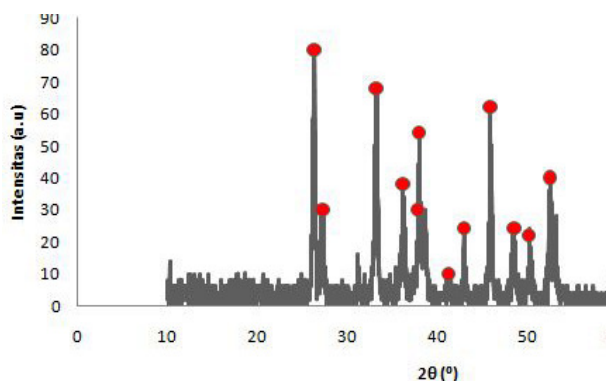


Fig.-2a: Diffractogram x-ray Tutut shell powder

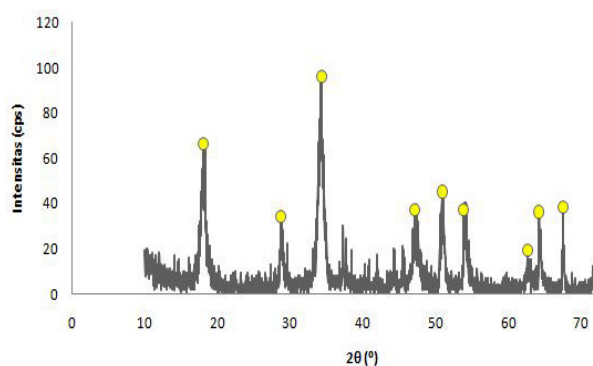


Fig.-2b: Diffractogram x-ray of calcined Tutut shell powder

Hydroxyapatite (HAp) was synthesized using wet precipitation method. This method does not need organic solvent and the yield is usually higher¹⁸. $\text{Ca}(\text{OH})_2$ is used as the source of calcium, while solution of $(\text{NH}_4)_2\text{HPO}_4$ is used as the source of phosphate. In the synthesis process it is necessary to monitor and adjust the pH level of the solution. If the pH of solution is less than 10 or more than 10. It will cause the formation of calcium monophosphate and calcium dehydrated, two compounds that soluble in water¹⁹. Therefore, this process must be carried out at pH 10 which is the optimum pH for the formation of HAp²⁰. the formation of HAp was indicated by the presence of peaks at 2θ (°) angle of 31.98, 32.96, and 33.08 (Figure-3a).

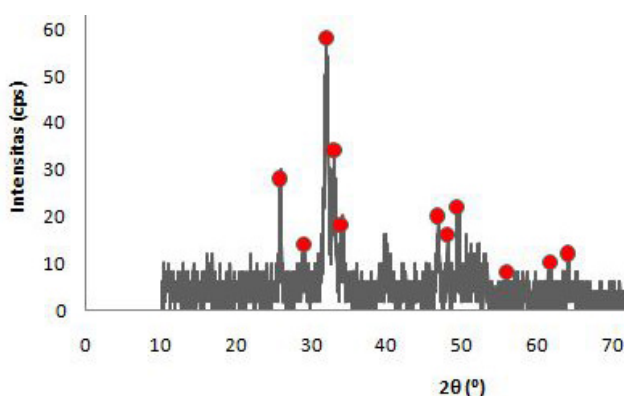


Fig.-3a: Diffractogram of HAp from Tutut shell

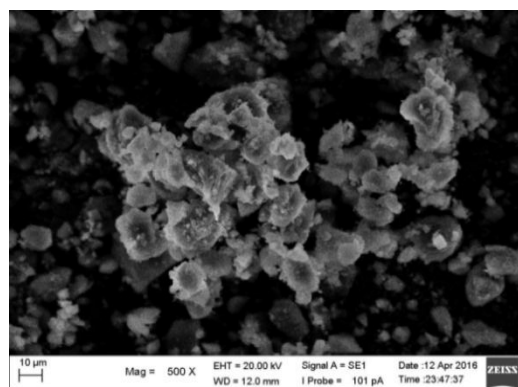


Fig.-3b: Morphology of synthesized of HAp

Morphological observation using SEM showed the formation of an agglomeration (Figure-3b) and makes it difficult to determine the crystal size of the synthesized HAp, a similar result with synthesized HAp that the size of HAp crystal grains was reported to be around 150-300 nm^{21,22}. The SEM also showed that the single particle of HAp crystals tend to form a round shape.

FTIR spectrum also confirmed the formation of HAp by the presence of absorption peak at 604 and 565 cm⁻¹ showing the bending mode of phosphate (PO₄³⁻) cluster, and the absorption at 1033 cm⁻¹ showing the stretching mode of phosphate group (Figure-4). The absorption peak at 1421 cm⁻¹ is an indication of a carbonate groups (CO₃²⁻). The hydroxyl group (OH) of HAp appeared at 3431 cm⁻¹. The FTIR results showed that the phosphate groups are the dominant compounds presence of a very small amount of calcium carbonate²³.

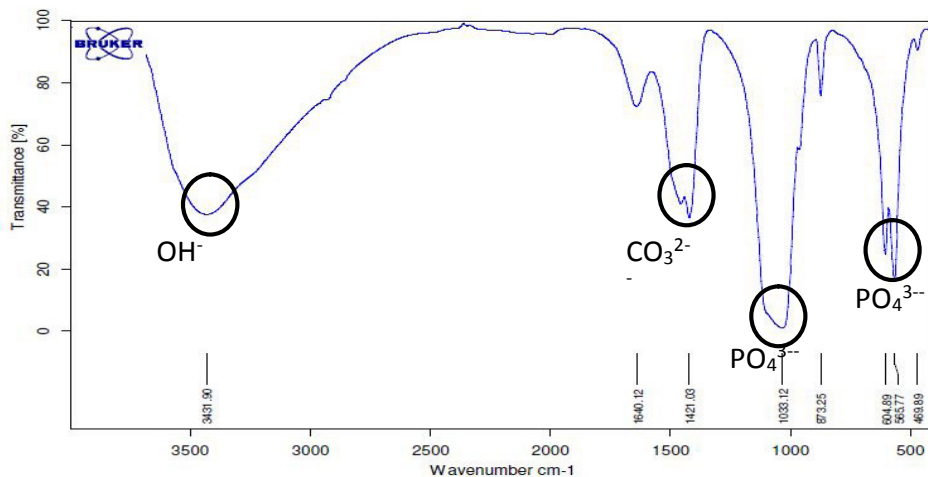


Fig.-4: FTIR spectrum of synthesized hydroxyapatite

The synthesized HAp was mixed physically with chitosan and PVA to give a composite that later used as IBS material. Gamma rays were used to sterilize and to jellify the composite, at a dose of 20 kGy based on the Association for the Advancement of Medical Instrumentation (AAMI) TIR33: 2005 which states that the dose for sterilization is in range of 15 to 35 kGy. Gamma irradiation is used for the sterilization of materials to be used in the health field because it left no radioactive residue. Visually, after the irradiation, the IBS sample showed a colorless and homogeneous clear gel.

Interaction between materials used in the formation of composite was observed using FTIR, and the results is given on Figure-5. The polyvinyl alcohol (PVA) is showed by the absorption at 3438 cm⁻¹ (OH stretch vibration), 2924 cm⁻¹ (CH stretch vibration/CH₂), 1701 cm⁻¹ (strain vibration -C=O), 1381 cm⁻¹ (strain -CH vibration), 1055 cm⁻¹ (vibration -C-C-). In addition, the hydrogen bond on PVA (HOH bond) was observed at 1649 cm⁻¹. Meanwhile, the chitosan gave the absorption peaks at 3730 cm⁻¹ (strain vibration H-NH), and at 1654 cm⁻¹ for the functional group of amide I. A sharp peak at 1421 cm⁻¹ indicated the mode changes of CH₃ symmetrical shape. Chitosan also showed absorption peaks at 3432 cm⁻¹ (OH stretch vibration). The three materials was interacted by a hydrogen bonding which was showed by the presence of the absorption peaks at 3000-3500 cm⁻¹, an indication of a merge vibrations of the -OH of hydroxyapatite, chitosan and PVA. In the FTIR spectrum composite, the absorption peaks of each material are still observable but with a decrease in intensity, as shown by the peak at 2874 cm⁻¹ (vibration -CH aliphatic of chitosan). The absorption peaks of Tutut shell still appear at 3431 cm⁻¹ (OH stretch vibration), 1640 cm⁻¹ (H-OH bending vibration), and at 1033 cm⁻¹, 604 cm⁻¹ and 565 cm⁻¹ for the phosphate group. A broadening peak around 300-3500 cm⁻¹ is due to the interaction between the constituent elements of the composite, such as hydrogen bonding between chitosan-hydroxyapatite. The -OH and NH₂ groups of chitosan bind with the -OH group of hydroxyapatite²⁴.

Figure-6a showed the XRD pattern for the composite, and showed that the composite consisted of mostly amorphous materials. The major content of the composite is the semicrystalline PVA, and appeared as a

broad peak at 2θ angle of 19.84° . The hydroxyapatite peaks can be seen at 2θ ($^\circ$) 31.98 , 32.96 , 33.08 , while the amorphous chitosan appeared at 20.42° . Interaction between chitosan and PVA caused a disappearance of peak at 19.84° for PVA and at 21.42° for chitosan to give a single peak 19.57° . The XRD pattern is also tell that the mixture of the three elements do not form a new compound, as no new diffraction peaks appeared in the composite diffraction pattern.

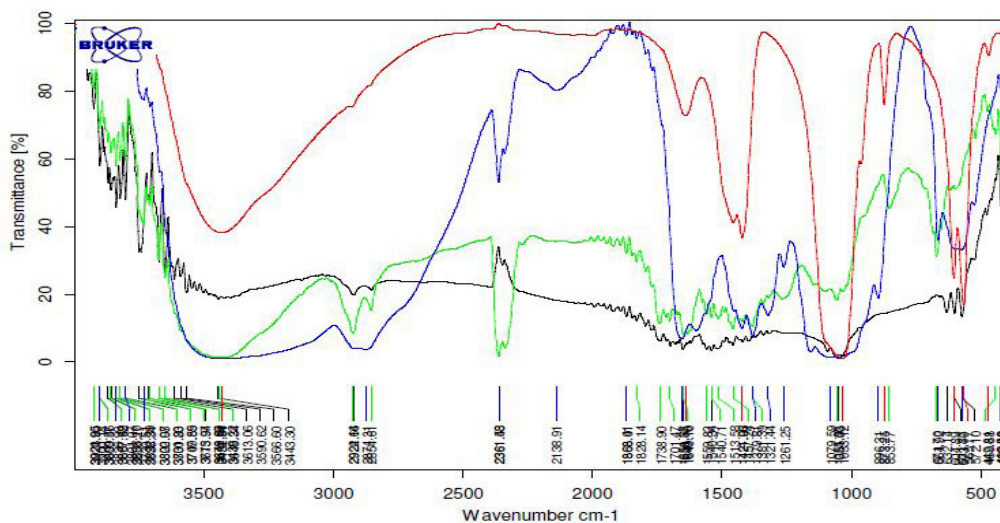


Fig.-5: Spectrum of FTIR PVA (—), chitosan (—), hydroxyapatite (—), and composite HAp-Chitosan-PVA (—)

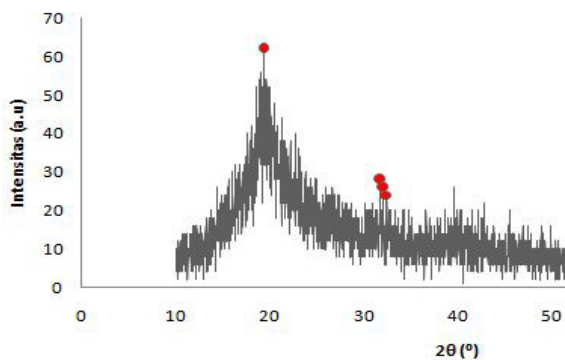


Fig.-6a: Diffractogram X-ray of composite HAp-chitosan-PVA

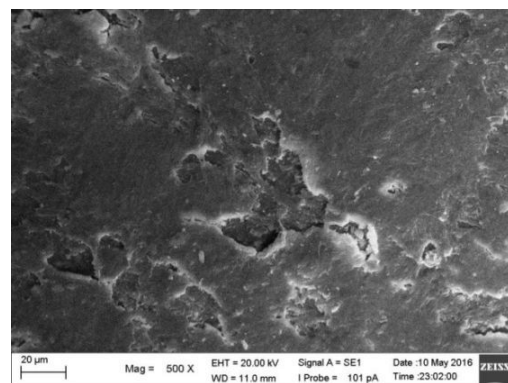


Fig.-6b: Morphology of composite HAp-chitosan-PVA

The morphology of the IBS composite is shown in Figure 6b. The surface of the composite is seen to be porous, and coated by a membrane of microporous hydroxyapatite. This porous membrane enables the circulation of bodily fluids and blood²⁵. The composite showed a viscosity level 36 dPa.s. The viscosity values possessed by this composite is very close to the standard values of injectable bone substitute which is 40 dPa.s²⁶. Qualitatively, the suspension made from the composite can be applied as an injectable bone substitute.

The cytotoxicity test of the composite was done in vitro using an endothelial culture media. This test aims to determine cell viability in the event of direct contact with the sample. Endothelial cells was chosen because they are the main cells involved in blood vessel formation. Hydroxyapatite is known to increase the proliferation of endothelial cells, a process that is required for the formation of new blood vessels

(angiogenesis) which is crucial in the repair process of damaged tissue, tissue growth, and the healing process²⁷. The concentration (%) of the composite used in the MTT test was 100, 75, 50, 25, and 12.5. The test results indicated that the limit of the composite concentration considered to be safe or not toxic is 12.5%, with a percentage of inhibition of 13.13%, which means that more than 50% of the CPAE cells remain alive. If percent inhibition exceeds 50%, the composite is classified as a toxic material²⁸. Cytotoxicity test results can be seen in Figure-7, as more white lines of live CPAE cells can be seen on Figure-7e, in the presence of the composite test solution.

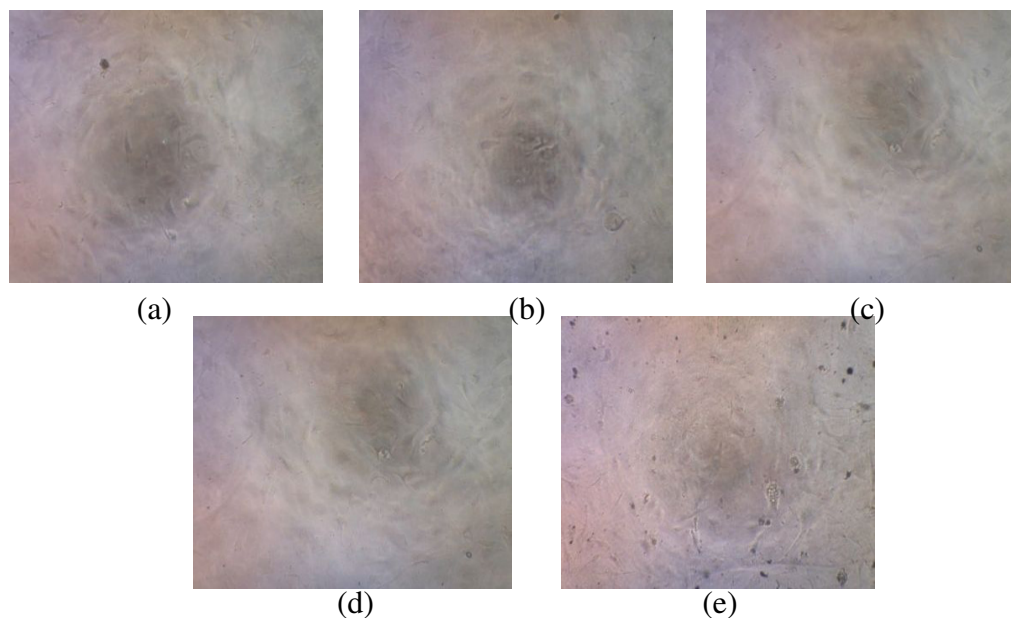


Fig.-7: The MTT test of the composite against CPAE cells with a series of concentration (a)100% (b)75% (c)50% (d)25% (e)12.5%

CONCLUSION

A hydroxyapatite has been successfully synthesized from Tutut shell using wet precipitation method. The composite made from the Tutut shell Hap-chitosan-PVA can be used as injectable bone substitute. The IBS made from the composites has a viscosity value of 36 dPa.s, fairly close to the standard value for injectable bone substitute material. The results of the cytotoxicity assay (MTT assay) indicate that the composite is not toxic as the value of the cell viability is more than 50%.

REFERENCES

1. Y. Warastuti and B. Abbas, *A Scientific Journal for the Applications of Isotope and Radiations*, 74 (2011)
2. W. Pierre, P. Layrolle, P.L. Clergeau, B. Enckel, P. Pilet, Y. Amouriq, G. Daculsi, and B. Giumelli, *Biomaterials*, **28**, 3295(2007)
3. R. Petit, *European Journal of Orthopedic surgery & Traumatology*, **9**, 71(1999)
4. K.A. Nayak, *International Journal of Chemical Technology Research*, **2(2)**, 903(2010)
5. P. Sugita, T. Wukirsari, A. Sjahriza, and D. Wahyono, *Chitosan of Sources Biomaterials for Future*, Bogor, p 148 (2009)
6. K.R. Mohamed, Z.M. El-Rashidy, and A.A. Salama, *Journal Ceramic International*, **37**, 3265 (2011)
7. Ma'ruf, W. Siswomihardjo, M.H.N.E. Soesatyo, and A.E. Tontowi, *Journal of Teknosains*, **3**, 1(2013)

8. A. N. Suciu, T. Iwatsubo, and M. Matsuda, *The Japan Society of Mechanical Engineers* **47(1)**, 199(2004)
9. Y. S. Pan, D. S. Xiong, and R. Y. Ma, *Wear*, **262**,1021(2007)
10. P. Weiss, O. Gauthier, J. M. Boulter, G. Grimandi, and G. Daculsi, *Equipe INSERM Materiaux*, **1**, 1(2007)
11. R. L. .Baby, I. Hasan, K. A. .Kabir, and M. N. .Naser. *Journal of Scientific Research*. **2(2)**, 390(2010)
12. L. Herawaty, Thesis, Department Chemistry, Bogor Agricultural University, Bogor, Indonesia (2014)
13. M. Azami, S. Tavakol, A. Samadikuchaksaraei, M. S. Hashjin, N. Baheiraei, M. Kamali, and M. R. Nourani, *In Vitro and In Vivo of Biomaterials Science* (2012)
14. C. Soído, M. C. Vasconcellos, A. G. Diniz, and J. Pinheiro, *Brazilian archives of Bio Tech* **52(1)**, 93(2009)
15. Winata, Minithesis, Department of Chmeistry, Bogor Agricultural, Bogor, Indonesia
16. G. M. Cunniffe, F. J. O'Brian, S. Partap, T. J. Levingstone, K. T. Stanton, and G. R .Dickson, *Journal Biomedical Material Research*, **95 (4)**, 1142(2010)
17. A. Afshar, M. Ghorbani, N. Ehsani, M. R. Saeri, and C. C. Sorrell, *Materials and design*. **24**, 197(2003)
18. K. Dahlan, F. Prasetyanti, and Y. W. Sari, *Biophysics journal*, **5(2)**, 71(2009)
19. H. Elsami, M. Solati-Hashjir, M. Tahriri, and F. Bakhshi, *Materials Science-Poland*, **28** (2010)
20. S. U. Maheshwari, V. K. Samuel, and N. Nagiah, *Ceramics International*, **40**, 8469(2014)
21. D. Darwis and Y. Warastuti , *Scientific Journal of Isotope and Radiation Applications*, **4(2)**, 143(2008)
22. L. Pighinelli and Kucharska, *Carbohydrate Polymers*, **93**, 256(2013).
23. Teng and Shu-hua, *Journal Biomedical Material Research*, **88A**, 569(2008)
24. A. Putra, A. Ifada, A. Rahmah, F. Rahmawati, F. Qulub, and D. Hikmawati, I n Proceedings of the Seminar National Applied Physics, Surabaya, East Java (2014)
25. M. Rucker, M. W. Laschke, D. Junker, C. Carvalho, A. Schramm, R. Mulhaupt, N. C. Gellrich, and M. D. Menger. *Biomaterials*, **27**, 5027(2006)
26. T. Matsuura, R. Hosokawa , K. Okamoto, T. Kimoto, and Y. Akagawa. *Biomaterials*, **21(11)**, 1121(2000)

[RJC-1465/2016]