Injectable Bone Substitute Synthesized from Mangrove Snail Shell

Yessie W. Sari[1], Reza Pahlevi Rudianto[1], Nur Aisyah Nuzulia[1], Sulistioso Giat Sukaryo[2]

[1]Biophysics Research Group, Department of Physics, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University, Indonesia
[2]Nuclear Industrial Materials Technology Center, National Nuclear Energy Agency of Indonesia, Indonesia

E-mail: yessie.sari@apps.ipb.ac.id

Received August 31 2017
Accepted for publication September 02 2017
Published September 04 2017

Abstract: The need for biomaterials tends to increase especially in the field of medicine caused by cases of increasing bone damage. The biomaterial that is being developed for bone implants is hydroxyapatite (HAp). The high value of imports of bone-replacement biomaterials in Indonesia is considered as a thoughtful problem because of the high price, therefore the development of biomaterials used as a bone substitute from low cost materials is demanded. In this study, HAp in the form of an injectable bone substitute (IBS) was synthesized from mangrove snail shell which currently is considered as a waste. IBS is a bone-substitute material in the form of a suspension. IBS can be applied by injection to reach deeper areas of bone defects and be able to adjust the shape of bone or tooth defects properly. HAp was synthesized in this study with assistance of microwave irradiation. Mangrove snail shell was used as calcium sources. Furthermore, this study indicated that HAp synthesized from mangrove snail shell had a high potency to be developed as IBS.

***Keywords**: Fourier Transform Infrared Spectroscopy, Hydroxyapatite, Injectability, X-ray Diffraction*

1. Introduction

Bone is stereotyped as an organ with a primary function as a protective and supportive framework for the body. In addition, bone also has a function as a protector for vital organs inside the body, as a place of red blood cell formation, where muscle attachment, calcium storage and drive the body.[1] The trend of bone fracture is increased, mostly due to accidents and illness. The case of bone damage caused by motor accidents in Indonesia reaches 24 million cases per year[2], while one of the causes of bone damage is osteoporosis. Hospital Information System, or Sistem Informasi Rumah Sakit (SIRS), shows the number of bone damage caused by osteoporosis of about 200 out of 100,000 cases.[3]

The need for biomaterials tends to increase especially in the field of medicine caused by cases of increasing bone damage. The biomaterial that is being developed for bone implants is hydroxyapatite. Hydroxyapatite (HAp) which has the chemical formula $Ca_{10}(PO_4)_6(OH)_2$ is one of the biomaterials that has a chemical composition resembling bone constituent components and in common with bone mineral phases.[4] HAp absorbs well enough organic chemicals in the body and has good biocompatibility and bioactivity properties. The bioactive properties of HAp are able to stimulate new bone growth around bone implants. In addition to being bioactive, HAp is also biocompatible, meaning it is able to withstand corrosion and does not cause rejection by body tissues.[5]

The high value of imports of bone-replacement biomaterials in Indonesia is conidered as a thoughtful problem because of the high price, therefore the development of biomaterials used as a bone substitute from low cost materials is demanded. The snail, a species derived from the gastropod class mollusca phylum, produces shell waste and can be utilized as a calcium source for HAp. Utilization of shells as a source of natural calcium is inseparable from the lack of mangrove snail waste management. This unused shell can be utilized because it is rich in minerals including calcium.[6] However hydroxyapatite is fragile and therefore rather difficult to be formed in accordance with the implant materials required in place of bone. The hydroxyapatite powder used for implants in damaged bones has a disadvantage that is not stable planted as implant. Therefore, some researchers have developed a hydroxyapatite composite with a polymer to improve its mechanical properties.[7]

In this study, a composite of hydroxyapatite in the form of an injectable bone substitute (IBS) was synthesized. IBS is a bone-substitute material in the form of a suspension. IBS can be applied by injection to reach deeper areas of bone defects and be able to adjust the shape of bone or tooth defects properly. In this study IBS was synthesized from mangrove snail-based HAp and gelatine. Based on the bone constituent composition to be fixed, the IBS suspension can be prepared from HAp/gelatine using hydroxypropyl methylcellulose (HPMC) as a suspending agent or as a solvent.[8]

2. Materials and methods

Hydroxyapatite was prepared from mangrove snail shell as bio-based calcium sources and phosphoric acid (H_3PO_4, Merck). Commercial microwave oven (Sharp R-230 R) with three power level was used as microwave source. Furnace Nabertherm B180 was used for mangrove snail shell calcination and hydroxyapatite sintering.

2.1. Mangrove snail shell calcination

Mangrove snail shell was washed under flowing water to remove the surface contaminant. Mangrove snail shell was sun-dried and ground before calcination. Calcination was conducted at 1000 °C using Nabertherm B180 for 8 h. Mass of eggshell before and after calcination was weighted.

2.2. HAp synthesized with microwave irradiation assistance

HAp was synthesized modifying methods developed in previous study.[9] A stoichiometric amount of calcined mangrove snail shell was dispersed in distilled water. Under rigorous stirring (300 rpm), analytical grade phosphoric acid solution was added drop wisely at a controlled rate to the suspension at room temperature. After the completion of addition, the precipitate formed was mirowave irradiated at 400 W for 45 mins using a household microwave (Sharp, R-230R) equipped with magnetic microwave stirrer. Following microwave irradiation, the precipitate was then subjected to sintering at 900 °C for 5 h using Nabertherm B180 A.

2.3. IBS synthesized

The synthesis of the IBS suspension was prepared by dissolving the gelatine powder into a 15 ml aquades stirred using a magnetic stirrer to homogeneous at 40 °C. Then the HAP powder was added to gelatine solution with a variation of HAp / gelatine composition 100/0, 70/30, 30/70, and 0/100. The samples were then stirred using a magnetic stirrer for 60 minutes at 40 °C until homogeneous. Furthermore, hydroxyl propyl methyl cellulose (HPMC) solution was prepared by dissolving HPMC powder into 85 ml of aquades at 80 °C until homogeneous, then coolled to room temperature. The HPMC solution was added to a mixture of HAp and gelatine with a HPMC: HAp / gelatine ratio of 3:1 drop wisely at 40 °C and then stirred for 6 h to finalize the IBS suspension synthesized. In addition, part of IBS suspension was dried using a freeze dryer for XRD, and FTIR analysis.

2.4. Analysis

Calcium content in calcined snail shell was measured using Shimadzu AA-7000 Atomic Absorption Spectrometer. The X-ray diffraction (XRD) pattern of snail shell powder and synthesized samples were obtained using Pananalitical Empyrean XRD diffractometer in the range between $20 \leq \theta \leq 80$ using Cu Kα radiation. Crystallite size of hydroxyapatite at (211) was calculated using Scherrer equation:

$$t_{(hkl)} = \frac{0.9\lambda}{\beta \cos\theta_{(hkl)}} \quad (1)$$

where $t_{(hkl)}$ is the crystallite size, λ the wavelength of the monochromatic X-ray beam (λCu = 0.154056 nm), β the full width at half maximum (FWHM), $\theta_{(hkl)}$ the peak diffraction angle and satisfies the Bragg's law for the (hkl) Miller's plane. Miller's plane (211) was selected to analyse the crystallite size since it is sharp and isolated from others. A Fourier Transform Infrared spectrometer (Bruker) was used in the wavenumber range from 4000 – 400 cm^{-1}

to conform the presence of synthesized hydroxyapatite. KBr pellet method was applied with ratio KBr: hydroxyapatite = 1:10. Viscosity was measured following Ostwald method. Injectability of the IBS suspension was analysed to measure the ease of by which a formulation can be dispensed out of a syringe. In this study, injectability was measured by modifying previous method.[10] The injectability percentage was determined as the percentage by weight of that part of the amount of the IBS suspension that could be extruded from the syringe with respect to the total mass of the IBS suspension introduced in the syringe.

3. Results and discussion

3.1 HAp synthesized with microwave irradiation assistance

Mangrove snail shell was used as the source of calcium. Calcination at 1000 °C for 8 h resulted a calcined shell with calcium concentration of 42%. This datum was further used for stoichiometric calculation of calcium and phosphate solution used for HAp synthesized. The XRD specra (Fig. 1) and FTIR spectra (Fig. 2) indicated the presence of HAp in the sample.

The x-ray diffraction pattern of HAp at 2θ = 20 ° - 60 ° was depicted in Fig 1. JCPDS database (Joint Committee on Powder Diffraction Standards) with no. 09-0432 was used in determining the presence of HAp in the sample. Peak identification strongly indicated the presence of HAp with characteristic peaks at 2θ = 31.79 °, 32.2 °, 32.93 °, and 34.08 °. In addition to HAp, CaO also presence in the sample which was indicated by the presence of peak at 2θ = 37.39 ° that was in accordance with JCPDS no.37-1497. The presence of CaO might be due to the incomplete transformation of $CaCO_3$ into Ca^+ during calcination of mangrove snail shell. Moreover, calculation of latice parametter indicated that the accuracy of lattice parameter obtained in this study is very high (> 99%), strongly indicating the presence of HAp following the synthesize conducted in this study (Table 1).

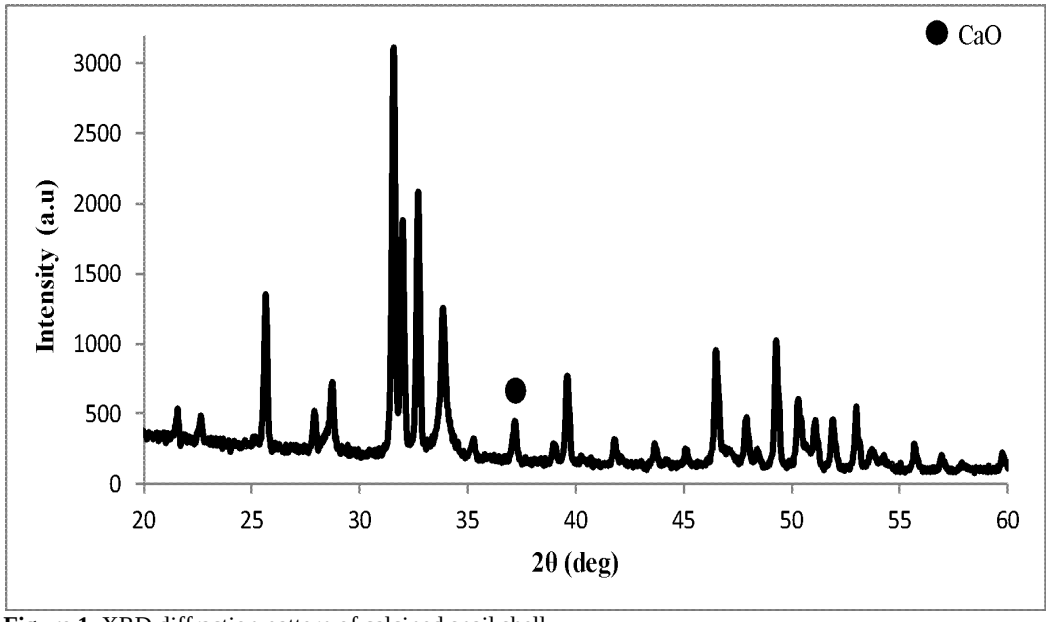

Figure 1. XRD diffraction pattern of calcined snail shell.

Table 1. Lattice parameter calculated by Cohen method.

Sample	Lattice Parameter (Å)		Accuracy (%)	
	a=b	c	a=b	c
HAp synthesized in this study	9.384	6.860	99.65	99.66
HAp from references (JCPDS no. 09-0432)	9.418	6.884		

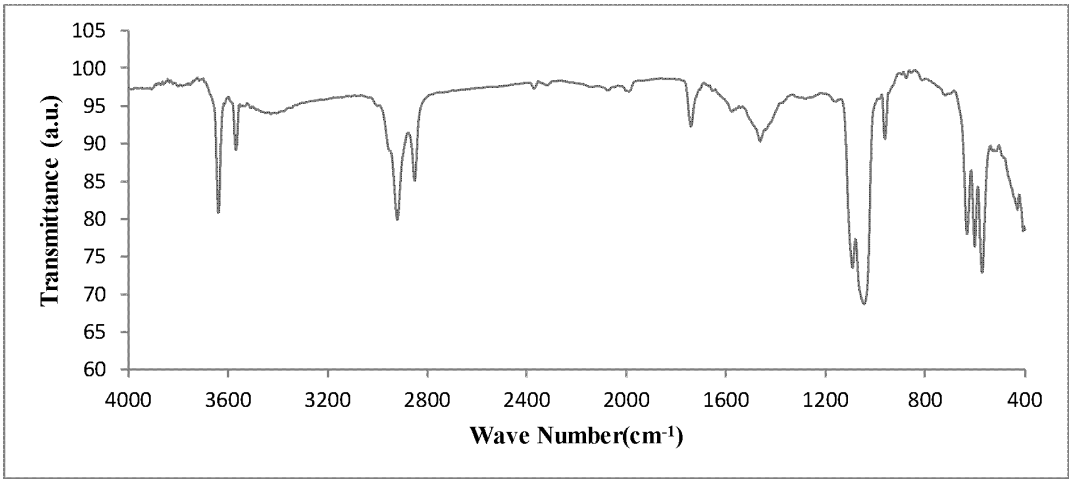

Figure 2. FTIR of HAp synthesized from mangrove snail shell.

Table 2. Functional groups present in the HAp as detected by FTIR spectroscopy.

Functional Groups	Wave Number (cm^{-1})	
	HAp obtained in this study	HAp from references[11,12]
OH-	2852.72 2922.16 3572.16 3641.6	(2600-3600)
PO_4^{3-}	570.93	(374-603)
	1047.35	(950-1100)
CO_3^{2-}	1463.97	(1350-1550)
	1741.72	(1700-1750)

FTIR spectra (Fig. 2) indicated the presence of functional groups of phosphate and carboxyl groups which are belongs to HAp. In addition, carbonate groups also presence which may be due to incomplete transformation of $CaCO_3$ into Ca^+ during calcination of mangrove snail shell leaving carbonate groups in the calcined mangrove snail shell. However, the carbonate groups did not incorporate into calcium phosphate lattice to yield apatitic carbonate. If apatitic carbonate were formed, then the accuracy of lattice parameter would not yield > 99% (Table 1). Table 2 listed functional groups detected by FTIR spectroscopy.

3.2 IBS suspension synthesized

The x-ray diffraction patterns of HAp (powder) and IBS consist of HAp/gelatine 100/0, HAp/gelatine 70/30, HAp/gelatine 30/70 samples are shown in Fig. 3. The phase identification was performed at an angle of 2θ = 20 - 60°. Contrasting Fig.3a and Fig.3b, it can be inferred that the suspension of HAp in HPMC had a decreased crystallinity, indicated by the broadening of Full Width at Half Maximum (FWHM) at 2θ in the range of 30-35° and other minor peaks. Furthermore, the addition of gelatine to the IBS suspension had an effect on the degree of crystallinity of the sample which can be inffered by contrasting Fig 3b, 3c, and 3d. Suspensing HAp in gelatine containing HPMC also affected in reducing its crystallite size (Table 3).

3.3 IBS Viscosity and Injectability Test

The presence of HAp at higher concentration followed by the increase the viscosity (Fig. 4). This is supported by the study on the effect of nanoparticle concentration on the viscosity indicating the increase of nanoparticle concentration result in an increased viscosity.[13-15]

Injectability is one of the important aspect for drug during its administration to the patients. The injectability is considered not only as a function of the rheological properties of the drug, but also device and human factors. Limited data are available that represent the correlation of these 3 factors.[16] In this study, injectability is depicted by the percentage of injectability. Measurement of injectability using two different size of syringe indicated the similar trends yet different in the injectability (Fig. 5). Injectability test on 1 and 5 ml syringe indicated that injectability has inverse linear correlation with the HAp concentration, the higher HAp concentration, the lower injectability percentage. This phenomenon correlates with the viscosity of the suspension, which some researchers indicated that viscosity can be used to indicate the injectability. More viscouse suspension has lower injectability.[10]

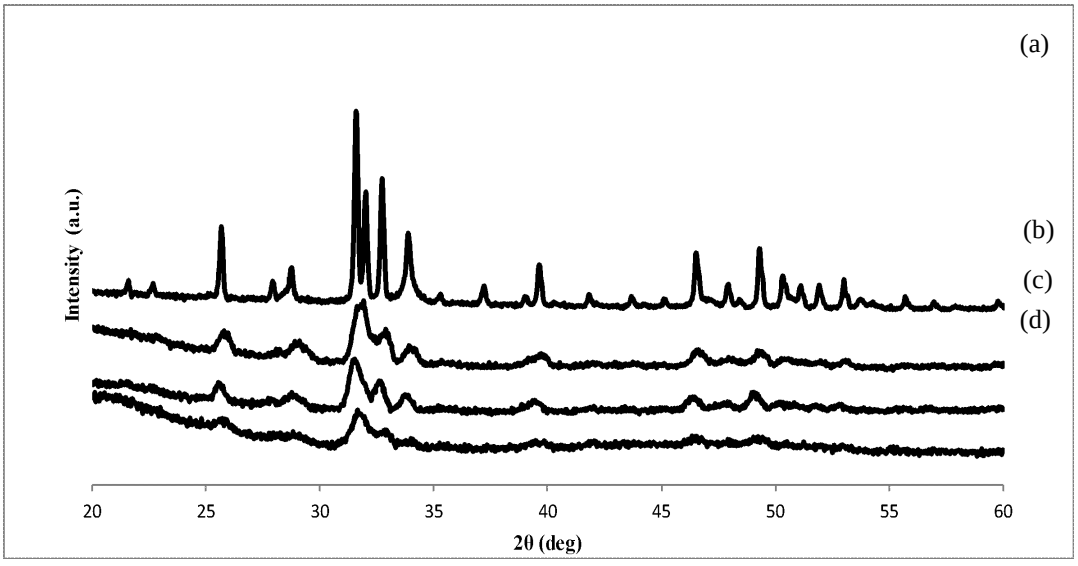

Figure 3. XRD spectra of HAp powder synthesized from mangrove snail shell (a) and IBS suspension of HAp / gelatin 100/0 (b), HAp /gelatine 70/30 (c), HAp/gelatine 30/70 (d).

Table 3. Crystallite size of HAp at hkl (002).

Sampel	Crystallite size (nm)
HAp (powder)	55.51
HAp/gelatine 100/0	16.13
HAp/gelatine 70/30	19.97
HAp/gelatine 30/70	26.29

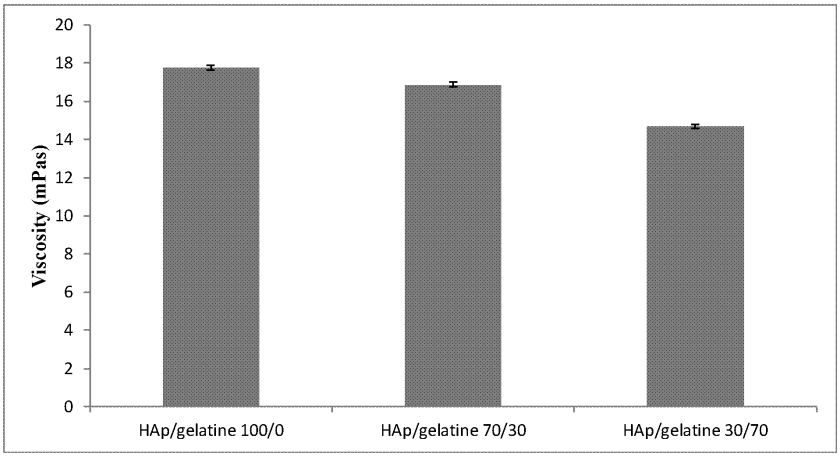

Figure 4. Viscosity of IBS suspension with various HAp concentration.

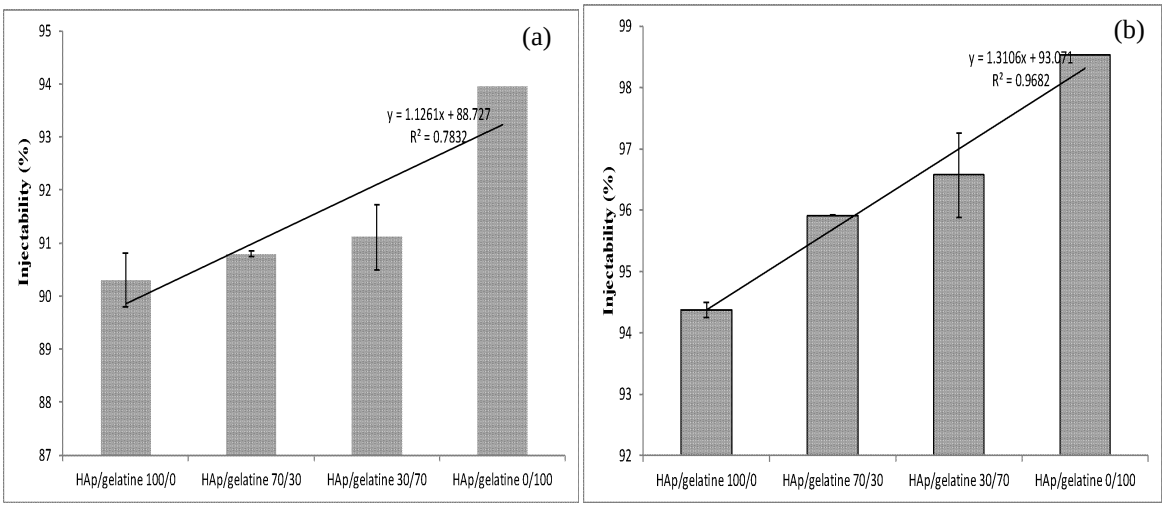

Figure 5. Injectability of IBS suspension with variety of HAp concentration conducted with 1 ml (a) and 5 ml (b) syringe.

4. Conclusions

Hydroxyapatite can be synthesized from mangrove snail shell serving as calcium source. Based on XRD pattern analysis, the most dominant phase formed was HAp with the lattice parameter accuracy was above 99%. FTIR of HAp shows the presence of hydroxyl, phosphate and carbonate functional groups belonging to functional group of HAp. Suspensing HAp in gelatine containing HPMC solution resulted in the decrease of HAp crystallinity. Furthermore, the higher gelatine concentration, the lower crystallinity of the HAp in the suspension. IBS suspensions were successfully synthesized in this study, as indicated by the high injectability percentage of HAp/gelatine 70/30 and HAp gelatine 30/70.

References

1 Sefrina A. *Osteoporosis The Silent Disease.* Jakarta Andi Publisher; 2016.

2 Imron S. *Peneliti Indonesia Kembangkan Cangkok Tulang dari Jaringan Sapi*. http://tekno.liputan6.com/read/2535505/peneliti-indonesia-kembangkan-cangkok-tulang-dari-jaringan-sapi, 2016.

3 Info DATIN Pusat Data dan Informasi Kementrian Kesehatan RI. *Data & Kondisi Kenyakit Osteoporosis di Indonesia.* 2015.

[4] Ivankovic H, Orlic S, Kranzelic D, and Tkalcec E, "Highly Porous Hydroxyapatite Ceramics for Engineering Applications," *Advances in Science and Technology*, **63**, 408-413 (2010).

[5] Tazaki J, Murata M, Akazawa T, et al., "BMP-2 release and dose-response studies in hydroxyapatite and β-tricalcium phosphate," *Bio-Med. Mat. & Eng*, **19**, 141-146 (2009).

[6] Baby R, Hasan I, Kabir KA, and Naser MN, "Nutrient analysis of some commercially important molluscs of Bangladesh," *Journal of Scientific Research*, **2**, 390-396, 2010.

[7] Kashiwazaki H, Yusuke K, Atsushi M, Keisuke Y, and Tadashi I, "Fabrication of porous chitosan/hydroxyapatite nanocomposites: their mechanical and biological properties," *Bio-Med. Mat. & Eng.*, **19,** 133-140 (2009).

[8] Weiss P, Gauthier O, Bouler JM, Grimandi G, and Daculsi G, "Injectable Bone Substitute Using a Hydrophilic Polymer," *Bone*, **25,** 67S-70S (1999).

[9] Sari YW, Saputra A, Maddu A. "Bioinspired hydroxyapatite formation using microwave irradiation at different irradiation power and time," *Submitted*, (2017).

[10] Ginebra MP, Rilliard A, Fernández E, Elvira C, San Román J, and Planell JA, "Mechanical and rheological improvement of a calcium phosphate cement by the addition of a polymeric drug," *J. Biomed. Mater. Res.* **57,** 113–118 (2001).

[11] Gergely G, Weber F, Lukacs I, et al., "Preparation and caracterization of hydroxyapatite from eggshell," *Ceramic International.* **36**, 803-806 (2010).

[12] Mujahid M, Sarfraz S, and Amin S, "On the formation on hydroxyapatite nano crystals prepared using cationic surfactant," *Material Research.***18,** 468-472 (2015).

[13] Bahiraei M, Hosseinalipour SM, Zabihi K, and Taheran E, "Using Neural Network for Determination of Viscosity in Water-TiO2 Nanofluid," *Advances in Mechanical Engineering.* **4,** 742680 (2012).

[14] Koca HD, Doganay S, Turgut A, Tavman IH, Saidur R, and Mahbubul IM, "Effect of particle size on the viscosity of nanofluids: A review," *Renewable and Sustainable Energy Reviews.* **82**(Part 1), 1664-1674 (2018).

[15] Sundar LS, Sharma KV, Naik MT, and Singh MK. "Empirical and theoretical correlations on viscosity of nanofluids: A review," *Renewable and Sustainable Energy Reviews.* **25**(Supplement C), 670-686 (2013).

[16] Cilurzo F, Selmin F, Minghetti P, et al., "Injectability Evaluation: An Open Issue," *AAPS Pharm. Sci. Tech.* **12**(2), 604-609 (2011).