

In-Vitro Assessment of Synthesized Hydroxyapatite Specimen in Simulated Body Fluid (SBF)



Istikamah Subuki, Ainina Kamarulzaman, Nor Fadhreen Mohd Dahlan

Abstract: Recently, the patient that requires bone replacement has increase, especially the patients who suffering from bone cancer, trauma and ageing. This attracts attention of researchers related to biomaterial fields to synthesis materials from biomaterials waste for bone tissue replacements. Hydroxyapatite was identified as a suitable source for bone substitution due its excellent bioactivity and biocompatibility. The strategies for tissue engineering include developing those cells to form the required tissue/organ in-vitro before inserting them into the body. This study is aimed to investigate In-Vitro properties of hydroxyapatite (HAp) specimens synthesize from the clamshells via precipitation method. HAp moulded specimen were immersed in the simulated body fluid (SBF). It displayed that the development of apatite layers materialized in the surface of HAp after being immersed for 25 days in the 1.5SBF.

Keywords—Hydroxyapatite, clamshell, In-Vitro, Simulated Body Fluids (SBF)

I. INTRODUCTION

Hydroxyapatite incorporates a chemical composition of $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ known as an artificial biomaterial mimicking to the mineral part of natural bones and tissues in mammals and has the hexagonal crystalline structure. HAp has been wide employed in medical science, dental, drug delivery application [1]. HAp is resembles mineral bone and exhibit good biocompatibility. It is also promoting rapid bone regeneration wherever the bond generates without the necessity of intermediate connective tissues and frequently applied to reconstruct the hard tissue owing to its osteoconductive properties [2]. In current, interest in the synthesis of nanosized HAp with grain size less than 100nm has inflated owing to its high surface activity and increased bioresorption [3]. It is proved that the created HAp powder is a fine particle with nanosized and has purity which will establish as medical demand.

This invention provides an environmentally beneficial and price effective method of manufacturing medical grade HAp biomaterials utilizing clamshell waste.

Recently, HAp has been successfully synthesized from natural waste material sources such as oyster shells [4], corals [5] eggshells and seashell [6]. Owing to high content of CaCO_3 which is the important calcium source for the preparation of HAp [7]. However, the research on HAp from waste material of clamshells is still new and need more exploration. Hence, it has been inspired to investigate the possibility to produce HAp powder from waste material of clamshells since its sources are abundant and easily found in Malaysia.

A revised SBF solution was proposed by Oyane et al. after considering the large concentration of calcium and magnesium which were bounded to proteins and hence inaccessible for apatite precipitation [8]. The composition of revised SBF solution contains 40% less calcium concentration and a 33% less magnesium concentration [9].

The bioactivity testing using SBF has been widely used since 1987 due to ionic concentration similarity to chemical composition of blood [10]. Recently, the development of apatite on an immersed substrate in SBF media shows the potential and indicates its bone bonding ability in vivo [9]. Besides, the use of SBF for in vitro assessment to determine the bioactivity of artificial materials based on their apatite-forming ability, have been conducted worldwide. From the other side, SBF also has been utilized for the preparation of bioactive composites such as the mineralization of bone-like apatite on different substrates [8].

Therefore, the main objective of this research is to stimulate the in vitro behavior of HAp specimen that synthesize from clamshell via precipitation method over the simulated body fluid (1.5 SBF).

II. METHODOLOGY

A. Synthesize the HAP via precipitation methods

The precipitation method was employed to produce the HA powder from clamshells [11]. In order to eliminate any organic materials and to transform into CaO, the clamshell powder (CaCO_3) was calcined for 3 hours at 850°C . The calcined powder (CaO) was then blended with boiled distilled water and cooled to room temperature. During the course, CaOH was developed due to exothermic reaction. The CaOH was then combined with low concentration of phosphoric to regulate the pH of the mixture to 6.5 by means of titration method.



Revised Manuscript Received on February 28, 2020.

* Correspondence Author

Istikamah Subuki*, Faculty, Department of Chemical Engineering, University Technology MARA, 40450 Shah Alam, Selangor, Malaysia. E-mail: istikamah@uitm.edu.my

Ainina Kamarulzaman, Faculty, Department of Chemical Engineering, University Technology MARA, 40450 Shah Alam, Selangor, Malaysia. E-mail: aininakamalrulzaman@gmail.com

Nor Fadhreen Mohd Dahlan, Faculty, Department of Chemical Engineering, University Technology MARA, 40450 Shah Alam, Selangor, Malaysia. E-mail: fadhreen.dahlan.94@gmail.com

© The Authors. Published by Blue Eyes Intelligence Engineering and Sciences Publication (BEIESP). This is an [open access](http://creativecommons.org/licenses/by-nc-nd/4.0/) article under the CC-BY-NC-ND license <http://creativecommons.org/licenses/by-nc-nd/4.0/>

In-Vitro Assessment of Synthesized Hydroxyapatite Specimen in Simulated Body Fluid (SBF)

Then, the solution of HAp was sifted and dehydrated in a furnace at 850°C with 5°C/min of heating rate.

B. Moulding specimen

The preparation of specimen was started by commixture the synthesized HAp powder with starch and ball-milled for 48 hours. Then, add drop-wise of Triton-X 100 with the HAp powder for feedstock preparation. After that, injection press method was applied to produce specimen with dimension of 2 cm diameter. Next, the moulded specimen were dried at 80°C for a few hours using oven and sintered for two hours by using furnace at 1000°C [12].

C. Preparation of Simulated Body Fluids (SBF)

Simulated body fluid (SBF) which was developed by Kokubo and his colleagues has similar inorganic ion concentrations to the human blood plasma [10, 13]. This fluid employed in this analysis to boost formation of apatite layer on HAp biomaterial. Besides that, it is used for coating of apatite on varied materials underneath biomimetic conditions.

In this study, the SBF was prepared accordingly to [10, 13]. The pH of SBF was altered to pH 7.25 at 35.6°C by utilizing 50 mmol/dm³ of tris(hydroxymethyl)aminomethane and roughly 45 mmol/dm³ of HCl. 1.5SBF was chosen in this research for immersion of HAp pellets as has greater ion concentrations.

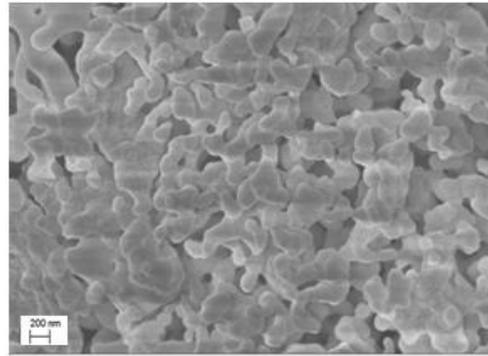
For pH adjustment, the pH were measured as the temperature of solution is at 36.5°C using calibrated pH meter until the pH changed to 7.25. Therefore, the solution should be titrated with 1 kmol/m³ HCl solution with pipette and transferred to 1000 mL volumetric flask. The inside of the beaker was washed with ultra-pure water for a few times and the solution was added to the flask, before further addition of ultra-pure water to keep the total volume of 1000 mL solution. The flask was kept at room temperature until its temperature should be just about 20°C. Then, the flask was shaken well. Finally, the polyethylene bottle of 1000 mL was rinsed with some of the prepared solution (SBF) for at least three times. Then, the solution should be transferred from flask to the polyethylene bottle. After that, the bottle of the solution was stored in the refrigerator at 5°C– 10°C.

D. In-Vitro Analysis

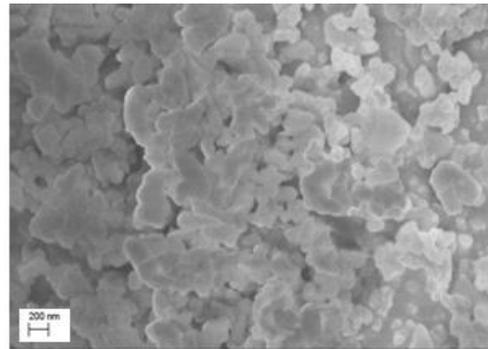
The HAp specimen were immersed in 50 mL SBF at 37°C for a period up to 25 days [1]. When the immersion periods finish, the sample were removed from the SBF solution by filtration then gently rinsed with 50 mL distilled water and followed by 20 mL ethanol [3]. Then, the sample were dried and characterised using Field Scanning Electron Microscopy (FESEM) and Fourier Transform Infrared Spectroscopy (FTIR).

III. RESULTS AND DISCUSSION

Fig. 1 shows the FESEM image for HAp powder synthesized from clamshell via precipitation methods. The particle size of synthesized HAp was smaller than commercial HAp and therefore has greater surface area. The increment of surface area rises the friction in powder mass, providing less efficient flow and packing. The surface texture of synthesized HAp appeared in rough agglomeration and irregular size distribution, due to the powder form which impact the force resisting among particles [14].



(a)



(b)

Fig. 1: FESEM of a) commercial HAp, b) synthesized HAp at 850°C

Fourier transform infrared spectroscopy (FTIR) was utilized to characterize the functional groups of hydroxyapatite (HAp) Ca₁₀(PO₄)₆(OH)₂ obtained from natural resources. Fig. 1 and Fig. 2 show the FTIR spectrum of synthesized HAp from clamshells before and after immersion in 1.5SBF respectively. The spectrum was recorded within the wavenumbers of 515 - 4000 cm⁻¹. The representative FTIR spectrum shows all characteristic absorption peaks of HAp. The primary indication for formation of HAp is within the form of a strong complex broad FTIR band targeted at about 1000 - 1300 cm⁻¹ as a result of uneven stretching mode of vibration for PO₄³⁻ group [15]. In Fig. 1, the FTIR spectrum of HAp can be identified by its characteristic band of phosphate group, PO₄³⁻ at 1029, 1053, 1109 and 1159 cm⁻¹. While, in Fig. 2 the phosphate group was found in HAp at 1047, 1107, 1160, and 1239 cm⁻¹. The crystalline HAp powder generates two characteristic stretching modes of O-H bands which is notice in FTIR spectrum of HAp at 3336 cm⁻¹ and 2900 cm⁻¹ by referring to the Fig. 1. While, in Fig. 2 O-H bands found at 3336, 2945 and 2916 cm⁻¹. The carbonate group can be found at 1314 cm⁻¹ at Fig. 1 while in Fig. 2 at 1365 and 1397 cm⁻¹. Therefore, the intensity in HAp spectrum increased from 104.7 %T to 113.4 %T after immersion in 1.5SBF compared to before immersion is proved the formation of apatite layer.

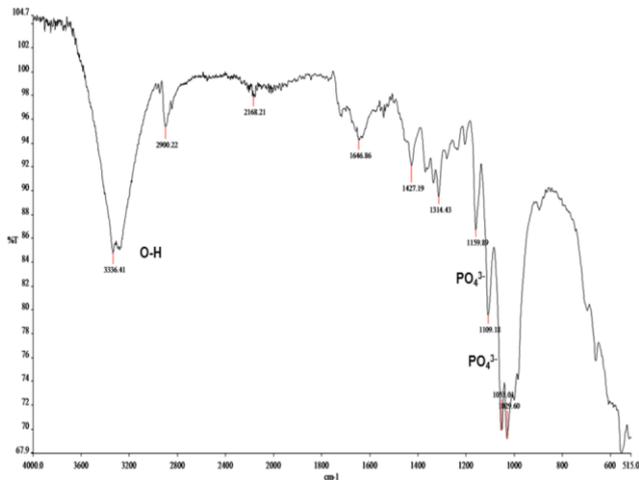


Fig. 2: FTIR spectrum of synthesized HAp from clamshells before immersion in 1.5SBF

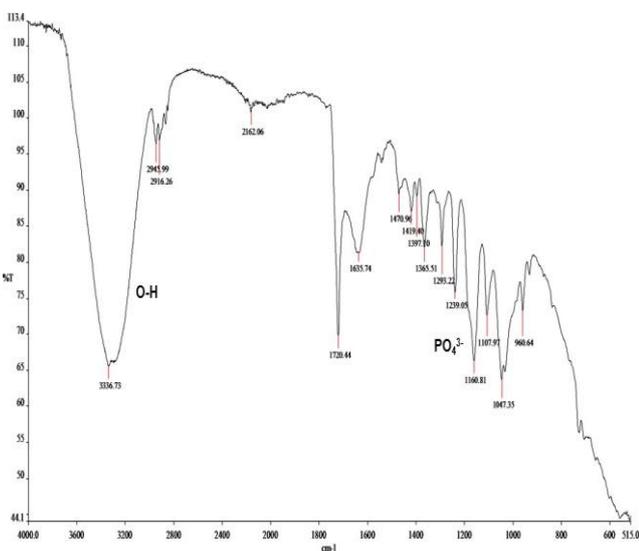
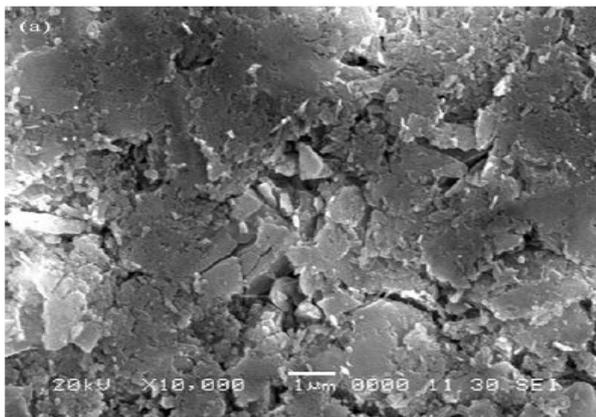
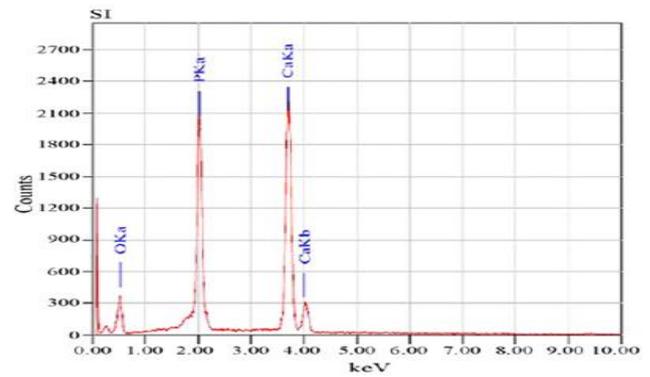


Fig. 3: FTIR spectrum of synthesized HAp from clamshells after immersion in 1.5SBF for 25 days.

FESEM was performed in order to observe the surface morphology of HAp specimen after 25 days of SBF immersion. The comparison between moulded HAp specimen before and after immersion into SBF was shown in Fig. 4 and 5.



(a)

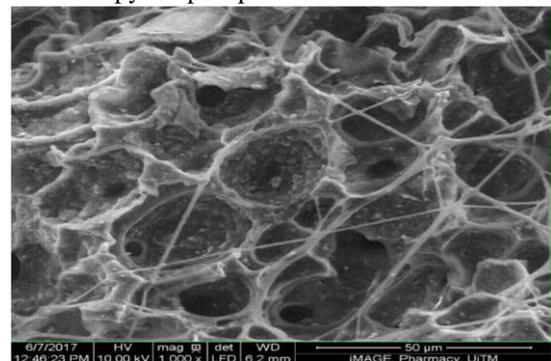


(b)

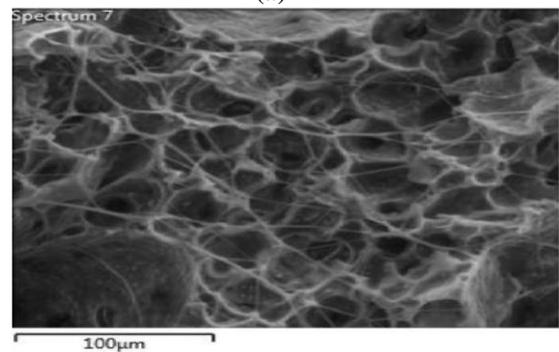
Fig. 4: (a) SEM image of moulded HAp specimen before immersion in SBF, (b) EDX Spectrum of HAp specimen before immersion in SBF

From Fig. 4 (a) and (b), the HAp specimen showing smooth and dense surface, while the existence of compositional elements of Hap were detected by EDX. Fig. 5 (a-b) show the morphology from the FESEM image and 5 (c) shows the EDX analysis of HAp after immersion in SBF solution for 25 days. After 25 days of incubation in SBF, the HAp specimen was observed to have highly porous hemispherical globules. Results suggested that the deposition of porous interconnected dense apatite layer using SBF was formed in this study.

The composition and percentage of each elements is shows in the Table1. It shows that the ratio of Ca/P is 2.43 which is higher that the theoretical value which is 1.67. According to Omer et. al. [16] reported that the Ca/P molar ratio higher than the value of 1.67 is B-type carbonated apatite which is carbonates occupy the phosphate sites.

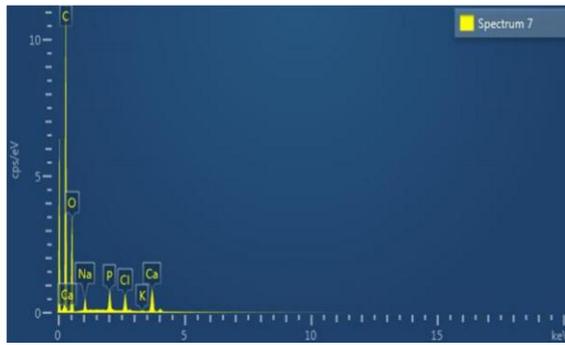


(a)



(b)

In-Vitro Assessment of Synthesized Hydroxyapatite Specimen in Simulated Body Fluid (SBF)



(c)

Fig. 5: SEM image of moulded HAp specimen before immersion in SBF at (a) high magnification, (b) low magnification and (c) EDX Spectrum

Table I: EDX micrograph of HAP specimen after immerse in SBF

Element	Apparent Concentration	k Ratio	Wt%	Wt% Sigma	Standard Label
C	123.79	1.2379 2	64.01	0.17	C Vit
O	60.38	0.2031 8	27.08	0.16	SiO2
Na	4.78	0.0201 6	1.08	0.03	Albite
P	9.5	0.0531 4	1.71	0.03	GaP
Cl	6.81	0.0594 7	1.88	0.03	NaCl
K	0.3	0.0025 6	0.08	0.02	KBr
Ca	15.11	0.1349 9	4.15	0.05	Wollastonite
Total:			100		

After gestation in SBF, HAp specimen hastens the course of mineralization in the area for genuine bone material on useful investigations. Energy Dispersive X-ray examination of the HAp specimen in SBF after 25 days exhibited the existence of elements Ca, P, O as well as C, Na and Cl. The major peaks due to Ca, P, and O corroborated the growth of calcium phosphate layer on the HAp surface, as presented in Fig. 5(c).

When a substance is gestated in SBF solution, the creation of apatite layer on the surface of pellet progresses through a sequence of chemical reactions like spontaneous precipitation, nucleation and growth of calcium phosphate [12]. It has been advocated that surface chemistry performs a crucial role in this course and even the working groups of materials have a big effect on the bone bonding property. As stated earlier, the HAp structure entails of Ca, PO₄, and OH groups tightly packed together.

The negativity of HAp surface is highly depend on the OH⁻ and PO₄³⁻ groups while the positivity of HAp is depend on Ca²⁺ ions. The procedure of apatite formation mostly rests on negative group, which in turn hinge on the large number of negative ions (i.e. OH⁻ and PO₄³⁻) on the surface. During gestation period, the positive Ca²⁺ ions from SBF are drawn by the OH⁻ and PO₄³⁻ ions present on HAp surface. Consequently, the surface acquires positive charge with respective to the surrounding SBF and further entices the

negatively charged OH⁻ and PO₄³⁻ ions from the SBF. This directs to the establishment of the apatite layer [12].

After the immersion periods, the solution was analysed by spectrophotometer to detect calcium ions at λ = 570 nm and phosphorus ions concentration at λ = 675 nm. To confirm the value the average value was taken. Therefore, each test was repeated three times. Thus, the average value obtained before immersion for calcium ions is 0.199 and phosphorus ions is 0.134. While, the average value obtained after immersion for calcium ions is 0.012 and phosphorus ions is 0.009. Thus, it shows that the concentration after the immersion is decreased.

IV. CONCLUSIONS

Characteristic of the absorption bands registered and outlined by FTIR spectroscopy ensure the formation of new apatite layer throughout the bone repair process of HAp composite in the simulated body fluid (1.5SBF) by showing the intensity of HAp bands such O-H, phosphate and carbonate groups. The formation of apatite layer on the surface of HAp specimen after immersion in the 1.5SBF solution.

ACKNOWLEDGMENT

The researchers would like to thank Universiti Teknologi MARA (UiTM), Malaysia for the financial support given through the Fundamental Research Grant Scheme (FRGS, 600-RMI/FRGS 5/3 (457/2019).

REFERENCES

- Mohamed, K. R., El-Rashidy, Z. M., & Salama, A, *In Vitro properties of nano-hydroxyapatite/chitosan biocomposites*, Ceramics International, 2011, pp. 3265-3271.
- Alshemary, A. Z., Akram, M., Goh, Y.-F., Tariq, U., Butt, F. K., Abdolahi, A., & Hussain, R., *Structural characterization, optical properties and in vitro bioactivity of mesoporous erbium-doped hydroxyapatite*, Alloys and Compounds, 2015, pp. 478-486.
- Alshemary, A. Z., Akram, M., Goh, Y.-F., Tariq, U., Butt, F. K., Abdolahi, A., & Hussain, R., *Synthesis, characterization, in vitro bioactivity and antimicrobial activity of magnesium and nickel doped silicate hydroxyapatite*, Ceramics International, 2015, pp. 11886-11898.
- Wu, S. C., Hsu, H. C., Wu, Y. N., Ho, W. F., *Hydrothermal synthesized from oyster shell powders by ball milling and heat treatment*, Material Characterisation, 62, 2011, pp. 1180-1187.
- Ripamonti, U., Crooks, J., Khoali, L., Roden, I., *The induction of bone formation by coral derived calcium carbonate/hydroxyapatite constructs*, Biomaterials, 30, 2009, pp. 1428-1439.
- Kumar, G.S., Thamizhavel, A., Girija, E. K., *Microwave conversion of eggshell into flower-like hydroxyapatite nanostructure for biomedical applications*, Materials Letters, 76, 2012, pp. 198-200.
- Ho, W. F., Hsu, H. C., Hsu, S. K., Hung, C. W., Wu, S. C., *Calcium phosphate bioceramics synthesized from eggshell powders through a solid state reaction*, Ceramic International, 39, 2013, pp. 8183-8188.
- Oyane, A., Kim, H.-M., Furuya, T., Kokubu, T., Miyazaki, T., Nakamura, T., *Preparation and Assesment of Revised Simulated Body Fluids*, J Biomed Matter Res A, 2003, pp. 188-195.
- Marc Bohner, Jacques. L. *Can Bioactivity be Tested In Vitro with SBF Solution?*, Biomaterials, 2009, pp. 1-5.
- Kokubo, T., H. Takadama. *How Useful is SBF in predicting In Vivo Bone Bioactivity?*, Biomaterials, 2006, pp. 2907-2915.
- Ali, N. H., Subuki, I., Ismail, *Synthesized Hydroxyapatite Powder from Clamshell via Chemical Precipitation Method*, Advance Materials Research, 2014, pp. 72-76.
- Mondal, S., Mondal, B., Dey, A., Mukhopadhyay, S. S., *Studies on Processing and Characterization of Hydroxyapatite Biomaterials from Different Bio Wastes*, Minerals & Materials Characterization & Engineering, 11, 2012, pp. 55-67.



13. Ohtsuki, C. *How to prepare the simulated body fluid (SBF) and its related solutions*. Retrieved from Biocompatible Materials Science Laboratory, Nara Institute of Science and Technology, 2006.
14. Subuki, I, Ali, N. H., Ismail, M. H., *Rheological Properties of Hydroxyapatite Synthesized Clamshell mix with Palm Stearin Binder System*, Advance Materials Research, 2015, pp. 366-370
15. Shakir, M., Jolly, R., Khan, M. S., Iram, N., Khan, H.M., *Nano-hydroxyapatite/chitosan starch nanocomposite as a novel bone construct: Synthesis and in vitro studies*, International journal of Biological Macromolecules, 2015, pp. 282-292.
16. Omer Kaygili, Keser, S., Kom, M., Eroksuz, Y., Dorozhkin, S.V., Ates, T., Ozercan, I. H., *Strontium substituted hydroxyapatite: Synthesis and determination of their structural properties, in vitro and in vivo performance*, Material Science and Engineering C. 2015, pp. 538-546.
17. Albrektsson, T., Johansson, C., *Osteoinduction, osteoconduction and osseointegration*, Eur Spine J, 2001, pp. 96-101.

AUTHORS PROFILE



Istikamah Subuki, received Bachelor Degree in Chemical Engineering and PhD degree in Mechanical Engineering from Universiti Teknologi MARA (UiTM) Shah Alam, Malaysia in 2004 and 2010 respectively. From 2007 to 2010, she was a researcher of the Advanced Materials Research Center (AMREC), SIRIM Berhad. She is currently a Senior Lecturer in the Faculty of Chemical Engineering, UiTM Shah Alam. Her current research interests are in advanced materials of synthesized hydroxyapatite powder, synthesized zirconia powder, injection moulding process, new binder system for injection moulding process, plasma spray coating of hydroxyapatite, metal and ceramic composite and polymer composite foam.



Nor Fadhreen Mohd Dahlan, pursues her study in Science foundation at Kedah Matriculation College. She next obtained her degree in Chemical Engineering from Universiti Teknologi Mara (UiTM) Shah Alam. Now she is working as Operation Executive in manufacturing solar industry. In her final year project, she has experimented on In-Vitro Study of Hydroxyapatite Powder Synthesized from Clamshell under supervision of Dr. Istikamah Subuki. Her main interest in her study is to verify the formation of apatite layer onto the composite surfaces and to assess the bioactivity of the pure and ion exchanged Hap when immersed in simulated body fluid (SBF).



Ainina Binti Kamalrulzaman, obtained her Foundation in Science at Universiti Teknologi Mara (UiTM) Puncak Alam. She next pursues her degree in Bachelor of Engineering (Hons) Chemical from Universiti Teknologi Mara (UiTM) Shah Alam. She is currently working as QA Engineer in filter manufacturing industry. During her final year project, she has experimented a project which entitled In-Vitro Study of Hydroxyapatite (HAp) Powder Synthesized from Clamshells via precipitation method under supervision of Dr. Istikamah Subuki. Her main research interest is to verify the growth of apatite layer on the surface of specimen after immersed in the simulated body fluid.