In-Vitro Study of Hydroxyapatite (HAp) Specimen in Simulated Body Fluid (1.5SBF)

Ainina Binti Kamalrulzaman, Dr. Istikamah Binti Subuki

Faculty of Chemical Engineering, Universiti Teknologi Mara

***Abstract*—** This study had been conducted to determine *In-Vitro* properties of hydroxyapatite (Hap) pellets synthesize from the clamshells via precipitation method. HAp pellets were successfully prepared and then immersed in the simulated body fluid (SBF) which has a similar composition to the human blood plasma. Therefore, 1.5SBF concentration has been chosen as fluid for In-Vitro studies. This research also highlights the purpose of In-Vitro studies of HAp powder in order to verify the formation of apatite layer on the surface of HAp pellets after immersed in the simulated body fluid in certain period of time up to 25 days. After immersion, the HAp pellets are then characterized by using Field Scanning Electro Microscopy (FESEM) with Energy Dispersive X-ray Spectroscopy (EDX) and Fourier Transform Infrared Spectroscopy (FTIR). The Fourier Transform Infrared Spectroscopy (FTIR) shows the intensity of functional groups in the Hap such as phosphate, carbonate and OH groups. While, by using Field Scanning Electro Microscopy (FESEM) with Energy Dispersive X-ray Spectroscopy (EDX), the morphology of apatite layer formed in the surface of Hap pellet was being analysed and studied. It is shows the growth of apatite layers formed in the surface of HAp after immersed in the 1.5SBF.

***Keywords*—**Hydroxyapatite, In-Vitro, SBF, Simulated Body Fluids

I. INTRODUCTION

Hydroxyapatite incorporates a chemical composition of $Ca_{10}(PO_4)_6(OH)_2$ which is an artificial biomaterial that is equally 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 [3]. 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]. Within the recent years, interest in the synthesis of nanosized HAp with grain size less than 100nm has inflated owing to its high surface activity and increased bioresoption [9]. 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.

Purpose of In-Vitro studies is used as a basis for clinical applications of the HAp biomaterial as a result of HAp composite biomaterial is suit for In-Vitro applications. Besides that, the aim of the In-Vitro studies is to verify HAp properties before additional experimental is conducted as In-Vivo applications then are be able to applied for medical purposes. Thus, the HAp powder synthesizes via precipitation methodology from clamshell is then applied for In-Vitro application in this research. This can be to characterize the HAp pellets once soaking within the SBF after a certain period and to make sure the formation of the apatite layer on the surface of HAp pellets [4].

Therefore, for additional study on hydroxyapatite properties, the purposes to study the In-Vitro properties of hydroxyapatite (HAp) composite materials was conducted completely to verify the formation of apatite layer on the HAp pellets. It is to study In-Vitro properties of HAp pellets and to characterize HAp pellets after immersion in the simulated body fluid (SBF) at certain periods. The calcination temperatures of 800°C HAp powder is used and the pH of the simulated body fluid (1.5SBF) used is maintained at 7.25. In-Vitro studies is chosen as a result of it is much cheaper and quicker than In-Vivo studies to evaluate the potential bioactivity of the HAp powder. Thus, the characterized of the HAp pellets once immersion in the simulated body fluid is then conducted by using Field Scanning Electro Microscopy (FESEM), Fourier Transform Infrared Spectroscopy (FTIR) and X-ray Diffractometer (XRD). This In-Vitro study is important to verify the formation of apatite layer on the surface of HAp pellets.

II. METHODOLOGY

A. Preparation of small pellet

The preparation of small pellet was started by commixture the HAp powder with starch and milled for 48 hours in ball mill. Then, add drop-wise of Triton-X 100 with the HAp powder to create a paste. After that, injection press method was applied to create small pellets with dimension of 2 cm diameter with this paste. Next, the small pellets were dried at 80°C for a few hours using oven and sintered for two hours by using furnace at 1000°C, [11].

B. Preparation of Simulated Body Fluids (SBF)

Simulated body fluid (SBF) was develop by Kokubo and his colleagues that has inorganic ion concentrations just like the human blood plasma. 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. Therefore, Table 1 shows the simulated body fluid and human blood plasma that evidenced has nearly similar ion concentration and might act in same principal [8].

Table 1: Ion concentrations of the simulated body fluid and human blood plasma

Ion	Concentration (mmol/dm^3)	
	Simulated body fluid (SBF)	Human blood plasma
Na^+	142.0	142.0
K^+	5.0	5.0
Mg^{2+}	1.5	1.5
Ca^{2+}	2.5	2.5
Cl^-	147.8	103.0
HCO_3^-	4.2	27.0
HPO_4^{2-}	1.0	1.0
SO_4^{2-}	0.5	0.5

The pH of SBF is adjusted to pH 7.25 at 35.6°C by utilizing 50 mmol/dm^3 of tris(hydroxymethyl)aminomethane and roughly 45 mmol/dm^3 of HCl. Modification of the ion concentrations in simulated body fluid is accessible and some of the modification is shown in Table 2 [8].

Table 2: Modification of the ion concentrations in SBF

Solution		1.5SBF	SBF(7.5)	SBF
Concentration (mol/dm^3)	Na^+	213.0	142.0	142.0
	K^+	7.5	5.0	5.0
	Mg^{2+}	2.3	1.5	1.5
	Ca^{2+}	3.8	2.5	2.5
	Cl^-	221.7	147.8	147.8
	HCO_3^-	6.3	4.2	4.2
	HPO_4^2	1.5	1.0	1.0
	SO_4^{2-}	0.8	0.5	0.5
pH		7.25	7.50	7.25
Buffering agent		A	B	B

Note :
Buffer A : $(CH_2OH)_3(CNH)_2$ 75 mol/m^3, appropriate amount (app. 67.5 mol/m^3) of HCl.
Buffer B : $(CH_2OH)_3(CNH)_2$ 50 mol/m^3, appropriate amount (app. 45 mol/m^3) of HCl.

As mentioned before, there are a few types of SBF such as 1.5SBF where the solutions were contained with ion concentrations 1.5 times of SBF to encourage acceleration of apatite formation. Therefore, the 1.5SBF is chosen in this research for immersion of HAp pellets as has greater ion concentrations. Thus, the amount of reagents for preparation of 1.5SBF shown in Table 3 is used. The preparation of SBF and 1.5SBF can be referred to the table below.

Table 3: Amount of Reagent for preparation of SBF and 1.5 SBF

Order	Reagent	Amount	
		SBF	1.5 SBF
		1000 mL	1000 mL
0	Ultra-pure water	750 mL	750 mL
1	NaCl	7.996 g	11.994 g
2	$NaHCO_3$	0.350 g	0.525 g
3	KCl	0.224 g	0.336 g
4	$K_2HPO_4 \cdot 3H_2O$	0.228 g	0.342 g
5	$MgCl_2 \cdot 6H_2O$	0.305 g	0.458 g
6	1 kmol/m^3 HCl	40 cm^3	60 cm^3
7	$CaCl_2$	0.278 g	0.417 g
8	Na_2SO_4	0.071 g	0.107 g
9	$(CH_2OH)_3CNH_2$	6.057 g	9.086 g
10	1 kmol/m^3 HCl	Appropriate amount for adjusting pH	

First step is to prepare simulated body fluid is cleansing. All the bottles as well as flasks and beaker are cleaned with dilute hydrochloric acid solution, sterilizing agent and ultra-pure water with this order. After that, all the bottles were immersed in dilute hydrochloric acid solution for several hours. Then, all the bottles were removed from the solution and washed well with tap water. Next, all the bottles once more were immersed in sterilizing liquid for nightlong. Then, all the bottles are removed from the liquid and washed well with ultra-pure water. Last step in cleansing, the bottles were washed with ion-exchanged water for several times and coated their mouths with wrapping film.

Secondly, the dissolution of chemicals step. The 750 mL of ultra-pure water were placed into 1000 mL beaker and stirred by exploitation magnetic stirrer with heater and also the temperature is maintained at 36.5°C. The beaker should be placed in clean bench to avoid dirt. Next, every chemical in Table 4 as shown below were added into the water one by one in order given from 1 to 8, once the reagent was fully dissolved. Then, Reagent 9 - $(CH_2OH)_3CNH_2$ was added in the solution little by little to avoid local increase in pH of the solution.

Table 4: Reagent for properties of 1.5SBF (pH 7.25, 1 L)

Order	Reagent	Amount
1	NaCl	11.994 g
2	$NaHCO_3$	0.525 g
3	KCl	0.336 g
4	$K_2HPO_4 \cdot 3H_2O$	0.342 g
5	$MgCl_2 \cdot 6H_2O$	0.458 g
6	1 kmol/m^3 HCl	60 cm^3
7	$CaCl_2$	0.417 g
8	Na_2SO_4	0.107 g
9	$(CH_2OH)_3CNH_2$	9.086 g
10	1 kmol/m^3 HCl	Appropriate amount for adjusting pH

Next step is for the adjustment of pH of the solution. The pH meter should be calibrated with fresh standard buffer solution. Then, the temperature of the solution should be checked after the Reagent 9 was already added and the electrode of pH meter was immersed into the solution. The pH should be measured while the temperature of solution is at 36.5°C. At this point, the pH of the solution is approximately 7.5 and need to adjust the pH at 7.25. Therefore, the solution should be titrated with 1 kmol/dm3 HCl solution with pipette. Then, the solution should be transferred from beaker to volumetric flask of 1000 mL. The inside of the beaker was washed with ultra-pure water for a few times and the solution was added to the flask. After that, ultra-pure water was added to the solution to adjust the total volume of the solution to 1000 mL and the flask should be shake well. The flask was kept at room temperature until its temperature should be just about 20°C. After cooling, the ultra-pure water was added to the solution until the total volume of the solution is 1000 mL. Then, shake the flask well.

Finally, the polyethylene bottle of 1000 mL was rinsed with a 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.

C. In-Vitro Analysis

Simulated body fluid (1.5SBF) was prepared as stated in the method reported by Ohtsuki. The HAp powder that has been pressed into small pellet were immersed in 50 mL SBF at 37°C for a period up to 25 days [4]. 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 [1]. Then, dried the sample at room temperature.

D. Characterization

The dried sample were characterized by totally different technique to determine the impact of immersion in 1.5SBF on the characteristic properties of HAp like using Field Scanning Electro Microscopy (FESEM) and Fourier Transform Infrared Spectroscopy (FTIR). Thus, the morphology of mineral layer formed on the HAp pellets surface and chemical composition of newly formed apatite layer was analysed respectively by Field Scanning Electro Microscopy (FESEM) with Energy Dispersive X-ray Spectroscopy (EDX) [2][9]. Lastly, the intensity of bands functional groups in the HAp like phosphate, carbonate and OH groups were detected by Fourier Transform Infrared Spectroscopy (FTIR). It is shows that the research was done completely.

III. RESULTS AND DISCUSSION

A. Fourier Transform Infrared Spectroscopy (FTIR)

Fourier transform infrared spectroscopy (FTIR) was utilized to characterized the functional groups of hydroxyapatite (HAp) $Ca_{10}(PO_4)_6(OH)_2$ obtained from natural resources. Figure 1 and Figure 2 show the FTIR spectrum of synthesized HAp from clamshells before and after immersion in 1.5SBF respectively. The spectrum was recorded within the vary 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 PO_4^{3-} group [6]. In Figure 1, the FTIR spectrum of HAp can be identified by its characteristic band of phosphate group, PO_4^{3-} at 1029, 1053, 1109 and 1159 cm^{-1}. While, in Figure 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 Figure 1. While, in Figure 2 O-H bands found at 3336, 2945 and 2916 cm^{-1}. The carbonate group can be found at 1314 cm^{-1} at Figure 1 while in Figure 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[4].

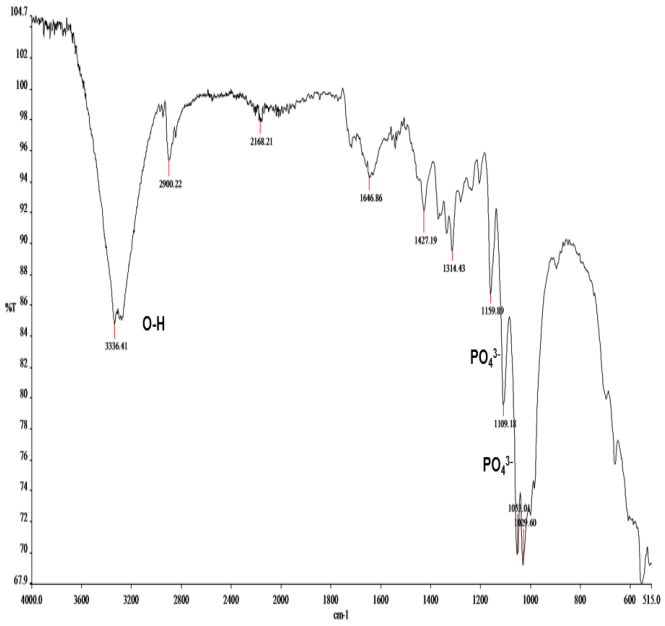

Figure 1: FTIR spectrum of synthesized HAp from clamshells before immersion in 1.5SBF

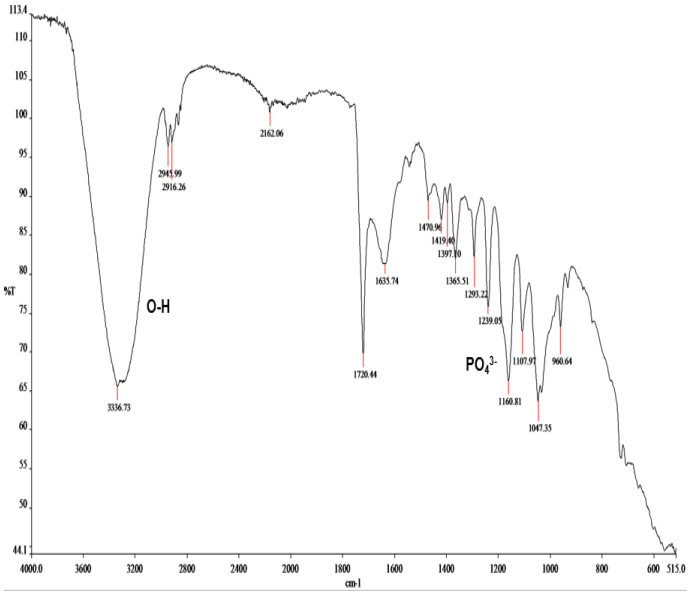

Figure 2: FTIR spectrum of synthesized HAp from clamshells after immersion in 1.5SBF for 25 days.

B. Field Scanning Electro Microscopy (FESEM) with Energy Dispersive X-ray Spectroscopy (EDX)

Field Scanning Electro Microscopy (FESEM) with Energy Dispersive X-ray Spectroscopy (EDX) was used to analyzed the morphology of mineral layer formed on the HAp pellet surface and chemical composition of newly formed apatite layer was. Figure 3 to 6 show the morphologies of the HAp pellet where the phase showed the formation of new apatite layer composed by dense agglomerates microstructure and composed of the asymmetrical shaped particles.

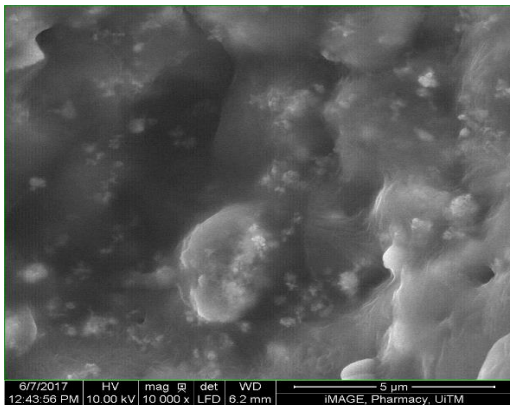

Figure 3 FESEM image of HAp 30N at 5µm

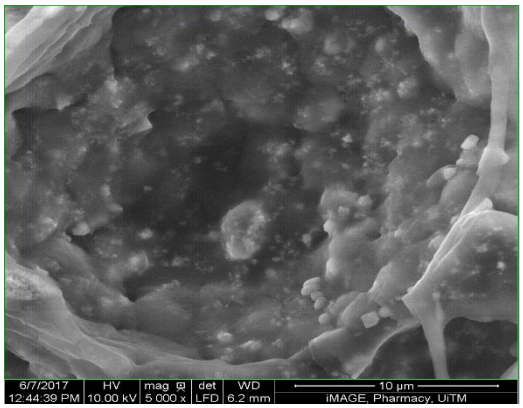

Figure 4 FESEM image of HAp 30N at 10µm

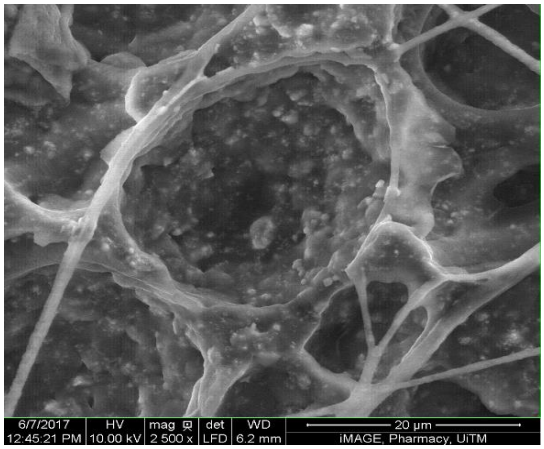

Figure 5 FESEM image of HAp 30N at 20µm

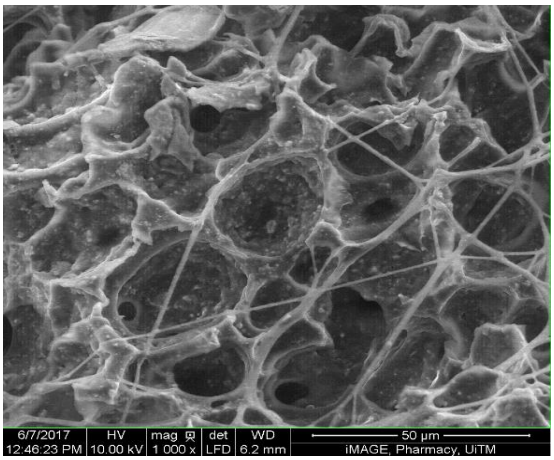

Figure 6 FESEM image of HAp 30N at 50µm

C. *Energy Dispersive X-ray Spectroscopy (EDX)*

Based on Figure 8, it shows the EDX spectra respectively to the Figure 7 for HAp. The composition and percentage of each elements is shows in the Table 5. 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. [9] reported that the Ca/P molar ratio higher than the value of 1.67 is an B-type carbonated apatite which is carbonates occupy the phosphate sites.

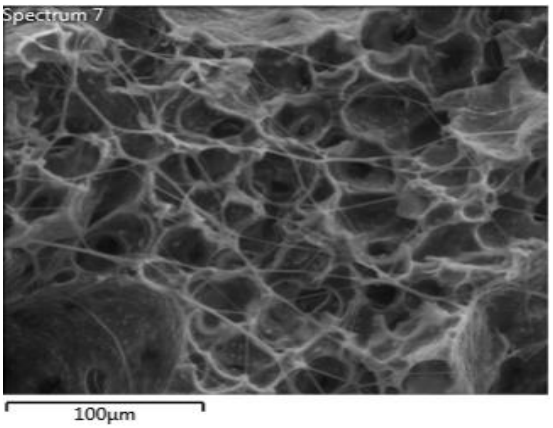

Figure 7 FESEM image of HAp 30N at 100µm

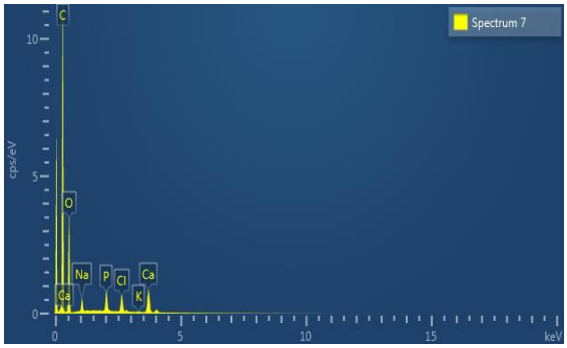

Figure 8 EDX spectra image of HAp 30N at 100µm

Table 4: EDX micrograph of HAp 30N at 100µm

Element	Line Type	Apparent Concentration	k Ratio	Wt%	Wt% Sigma	Standard Label
C	K series	123.79	1.23792	64.01	0.17	C Vit
O	K series	60.38	0.20318	27.08	0.16	SiO2
Na	K series	4.78	0.02016	1.08	0.03	Albite
P	K series	9.50	0.05314	1.71	0.03	GaP
Cl	K series	6.81	0.05947	1.88	0.03	NaCl
K	K series	0.30	0.00256	0.08	0.02	KBr
Ca	K series	15.11	0.13499	4.15	0.05	Wollastonite
Total:				100.00		

D. *Solution analysis*

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. CONCLUSION

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. Besides that, HAp growth is characterized by a decrease of the absorption bands at 1047 cm^{-1} (Figure 2) from 1053 cm^{-1} (Figure 1). While, morphology of the HAp is successfully studied based on the image of FESEM. By referring the result obtained, we can conclude that the formation of apatite layer on the surface of HAp after immersion in the 1.5SBF solution is proven. But, the HAp used was needle shape with different composition HAp which is 20N to utilized for FTIR and spectrophotometer while the HAp 30N was used for FESEM. This is due limitation of HAp pellet with same composition of the needle shape HAp. Even though, the results of the analysis done by FTIR and FESEM show the formation of apatite layer but it is cannot be compared. Therefore, we need to use the same composition of HAp needle shape in order to justify the similarity of the result that obtain. Besides that, the research could be done by varying the time periods of the HAp pellets immersion in the 1.5SBF.

ACKNOWLEDGMENT

Thank you to my supervisor Dr Istikamah Binti Subuki and Universiti Teknologi Mara.

References

[1] Ammar Z. Alshemary, M. A.-F. (2015). Synthesis, characterization, in vitro bioactivity and antimicrobial activity of magnesium and nickel doped silicate hydroxyapatite. *Ceramics International*, 11886-11898.

[2] Ammar Z. Alshemary. Muhammed Akram, Y.-F. G. (2015). Structural characterization, optical properties and in vitro bioactivity of mesoporous erbium-doped hydroxyapatite. *Alloys and Compounds*, 478-486.

[3] Hadeel Alobeedallah, j. L. (2010). Preparation of Nanostructured Hydroxyapatite in Organis Solvents for Clinical Applications.

[4] Khaled R. Mohamed, Z. M.-R. (2011). In Vitro properties of nano-hydroxyapatite/chitosan biocomposites. *Ceramics International*, 3265-3271.

[5] M. Enamul Hoque, N. S. (2014). Synthesis and Characterization of Hydroxyapatite Bioceramic. *International Journal of Scientific Engineering and Technology*, 458-462.

[6] Mohammad Shakir, R. J. (2015). Nano-hydroxyapatite/chitosan-starch nanocomposite as a novel bone construct: Synthesis and in vitro studies. *International journal of Biological Macromolecules*, 282-292.

[7] Muhammad Akram, R. A. (2014). Extracting hydroxyapatite and its precursors form natural resources. 1461-1475.

[8] Ohtsuki, C. (2006). *How to prepare the simulated body fluid (SBF) and its related solutions*. Retrieved from Nara Institute of Science and Technology.

[9] Omer Kaygili, S. K. (2015). Strontium subtituted hydroxyapatite: Synthesis and determination of their structural properties, in vitro and in vivo performance. *Material Science and Engineering C*, 538-546.

[10] S. Ramesh, A. N. (2015). Characteristics and properties of hydroxyapatite derived by sol-gel and wet precipitation methods. *Ceramics International*, 10434-10441.

[11] Sudip Mondal, B. M. (2012). Studies on Processing and Characterization of Hydroxyapatite Biomaterials from Different Bio Wastes. *Minerals & Materials Characterization & Engineering, 11*, 55-67.