In-vitro Study of Hydroxyapatite (HAP) Specimen in Simulated Body Fluid (1SBF)

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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. To examine the bone-bonding capability of a material, in vitro technique is necessary to be performed over SBF to justify the formation of apatite layer on the composite surfaces. Hence, this research aims to study in-vitro of hydroxyapatite specimen to inspect the capability of apatite to form on its surface. The HAp specimens were immersed into SBF for 25 days. The SBF was prepared based on Kokubo method. According to Kokubo, the SBF has inorganic ion concentrations similar to those of human extracellular fluid, in order to reproduce formation of apatite on bioactive materials in-vitro. The simulated body fluid was prepared using the reagents listed in Table 2. These reagents were added to 750 ml ultra-pure water in order given in Table 2, one by one, after each reagent was completely dissolved. After 25 days of immersion, the specimen then will describe utilizing using Fourier Transform Infrared (FTIR), Scanning Electron Microscope (SEM) and Energy Dispersive X-ray (EDX) to figure out the impact of the SBF states on the trademark properties from claiming. That vicinity of functional group of the specimen before and after immersion will be indicated by FTIR. SEM equipped with an energy dispersive X-ray (EDX) is applied to spot the morphologies of the specimen. FTIR spectra show the functional group of HAp specimen ranging from 3000 to 1087 cm^{-1}. The morphological of HAp specimen were compared from commercial HAp and synthesized HAp after SBF soaking. From the morphologies shown in SEM, new apatite layer formation was foreseen from their SBF behavior pattern. The formation of major peak of Ca, P, and O show in EDX analysis is confirm the formation of apatite layer.**

Keywords - Hydroxyapatite; In-vitro; Simulated Body Fluid (SBF); Tissue Engineering

I. INTRODUCTION

In the recent years, due to ageing UK population, increased dynamism of people's lives and growing life expectancy, the clinical demand for bone replacement and repair had growing well. Hydroxyapatite (HAp) with chemical formula $Ca_{10}(PO_4)_6(OH)_2$ is an important inorganic biomaterial. Because of its chemical and structural similarity with the mineral phase of bone and teeth, it is suitable to be used for hard tissue repair [3]. To serve the demand on bone replacement, Hap is identified as most suitable bone substitution materials.

HAP may be generally utilized within musculoskeletal methods because it's chemical and crystallographic comparability to the carbonated apatite in mankind's bones and teeth. Great mechanical properties with predominant biocompatibility of sintered HA make it well favored bone and tooth implant material. For two decades, HAp has been utilized in bone tissue engineering because of its fantastic candidates for bone repair and recovery. In spite of HA is bioactive and osteoconductive, its mechanical properties would inadequate, making it unable to be used as a load bearing implant [6].

A revised SBF solution was proposed in 2003 to take into account the fact that a large proportion of calcium and magnesium species present in serum is bound to proteins and hence unavailable for apatite precipitation [1]. The revised SBF solution had a 40% lower calcium concentration and a 33% lower magnesium concentration [5].

The use of SBF for bioactivity testing has widely used since 1987. After 20 years of research in this field, the formation of apatite on a material dipped in SBF is a proof of its bioactivity and can be used to anticipate its bone bonding ability in vivo [5]. Besides, SBF also has been used widely for in vitro assessment of the bioactivity of artificial materials by examining their apatite-forming ability in the fluid. From the other side, SBF also has been used to prepare bioactive composites by forming bone-like apatite on various types of substrates [2].

The simulated body fluid is prepared in laboratory with the ionic concentration nearly similar to human blood plasma according to procedure developed by Kokubo. The appropriate quantities of reagents like NaCl, $NaHCO_3$, KCl, $K_2HPO_4.3H_2O$, $MgCl_2.6H_2O$, $CaCl_2$, Na_2SO_4 and tris buffer are dissolve in 1L of double distilled water so as to have ionic concentration of various inorganic similar to those of the human blood plasma. Table 1 provides the ion concentration of SBF and its comparison with human blood plasma according to Kokubo. It shows that the ion concentration of SBF is similar to the concentration of human blood body.

Table 1: Ion Concentration of SBF and Human Blood Plasma

Ionic concentration ($mmol/dm^3$)	Simulated Body Fluid	Blood Plasma
Na^+	142	142
K^+	5.0	5.0
Mg^+	1.5	1.5
Ca^+	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

Therefore, the main emphasis of this research work is to stimulate the in vitro behavior of HAp specimen over the simulated body fluid. The performance of in-vitro characterization of HAp is analyzed by Fourier Transform Infrared (FTIR), Scanning Electron Microscope (SEM) and Energy Dispersive X-ray (EDX).

II. METHODOLOGY

A. Preparation of Simulated Body Fluid (SBF)

Simulated body fluid was prepared in laboratory with the ionic concentration nearly similar to human blood plasma. The SBF is prepared based on procedure develop by Kokubu (Kokubo method) [11]. There are four steps to prepare SBF solution which is cleaning, dissolution of chemicals, adjustment of pH and storage.

i. Cleaning

It is important to do cleaning process in order to sterilize all the apparatus used. Firstly, all the apparatus were cleaned by using dilute hydrochloric acid solution, sterilizing agent and ultra-pure water in this order. Next, all the bottles etc. were immersed in diluted hydrochloric acid solution for several hours. Then, the bottles were removed from the solution and washed with tap water well. The bottles etc. were immersed in sterilizing liquid for overnight. After that, the bottles etc. were removed from the liquid and washed with ultra-pure water well. The bottles were washed with ion-exchanged water for several times and their mouth were covered with wrapping film.

ii. Dissolution of Chemicals

In this step, 750mL of ultra-pure water was put into a 1000mL beaker. Polyethylene beaker was used. The water was stirred with magnetic stir with heater and temperature was maintained at 36.5˚C. Next, the beaker was placed in clean bench to avoid dusts. Each chemical as in Table 1 was added one by one until from order 1 to order 8 into the water in the order given, after each reagent was completely dissolved. The chemical was weighed with the bottle. The water was added. The remaining chemical on the weighing bottle was washed with ultra-pure water and the solution was added in the water. Reagent 9 is added little by little with less than 1g, in order to avoid local increase in pH of the solution.

Table 2: Reagents for Preparation SBF (pH 7.25, 1L) [8]

Order	Reagent	Amount (g/l)
1	NaCl	7.996 g
2	$NaHCO_3$	0.350 g
3	KCl	0.224 g
4	$K_2HPO_4.3H_2O$	0.228 g
5	$MgCl_2.6H_2O$	0.305 g
6	1 kmol/m^3 HCl	40 cm^3
7	$CaCl_2$	0.278 g
8	Na_2SO_4	0.071 g
9	$(CH_2OH)_3CNH_2$	6.057 g
10	1 kmol/m^3 HCl	Appropriate amount for adjusting pH

iii. Adjustment of pH

The pH must be adjusted to obtain standard pH. The pH meter was calibrated with fresh standard buffer solution. The temperature of the solution in the beaker was checked after reagent 9 was added and the electrode of pH meter was immersed into the solution. The pH was measured while the temperature was at 36.5˚C. At this point, the pH is approximately 7.5. The solution was titrated with 1 kmol/m3 of HCl solution to adjust the pH to 7.25(or 7.40). The solution was transferred from the beaker to a 1000 mL of glass volumetric flasks. The remaining solution inside the beaker was washed with ultra-pure water several times and was added to the flasks. Ultra-pure water was added to the solution and the total volume of the solution was adjusted to 1000 mL. The flask was shaken well. The flask was kept at room temperature of approximately 20˚C. After cooling, ultra-pure water was added again to the solution to 1000 mL, and the flask was shaken well throughout the process.

iv. Storage

The polyethylene (polystyrene) bottle of 1000 mL was rinsed with a bit of the prepared solution (SBF) three times. The solution was transferred from the flask to the polyethylene bottle and stored in a refrigerator at 5-10˚C.

B. In-Vitro Test

To observe the apatite layer into the composite surface of HAp specimen, in vitro test was carried out. The HAp powder that has been converted into the pallet was immersed into the SBF solution at body temperature and was incubated for 2 to 4 weeks without refreshing or adding SBF solution [11]. The samples were filtered, washed with water three times and dried at room temperature. To determine the effects of the SBF conditions and soaking period on the characteristic properties of the SBF, the samples were observed under FTIR, SEM and EDX measurements.

III. RESULTS AND DISCUSSION

A. Fourier Transform Infrared Spectroscopy (FTIR) Analysis

FTIR spectroscopy was employed to characterize the presence of characteristic bonds and group of difference functional groups present in HAp $Ca_{10}(PO_4)_6(OH)_2$. The representative FTIR spectrum shows all characteristic absorption peaks of HAp.

The FTIR analysis was used to access the bioactivity of HAp specimen post-immersion in SBF for 25 days. FTIR spectra of 20S HAp is shown in Figure 2 before and after soaking in SBF 25 days.

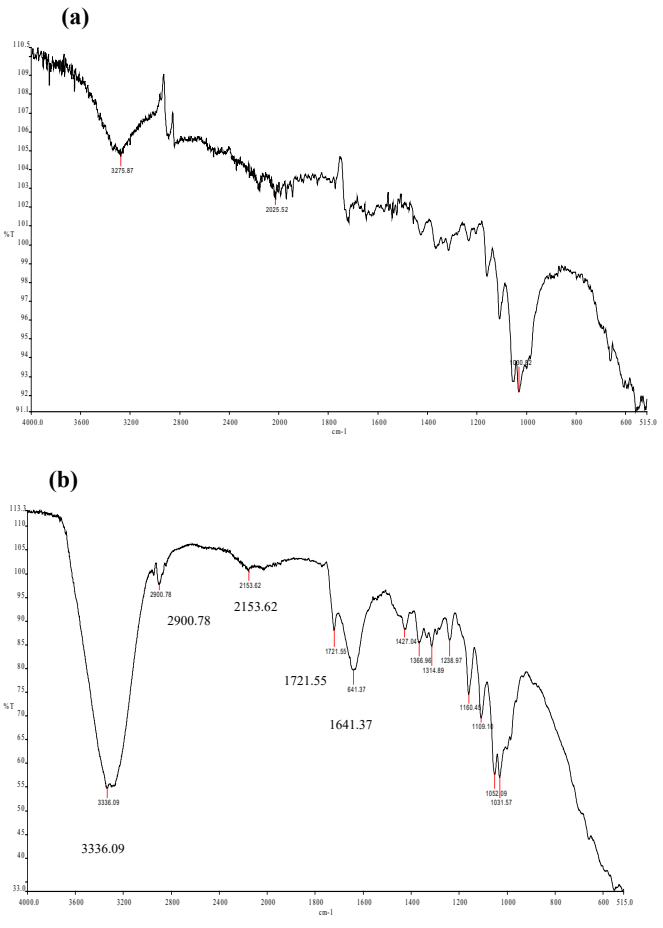
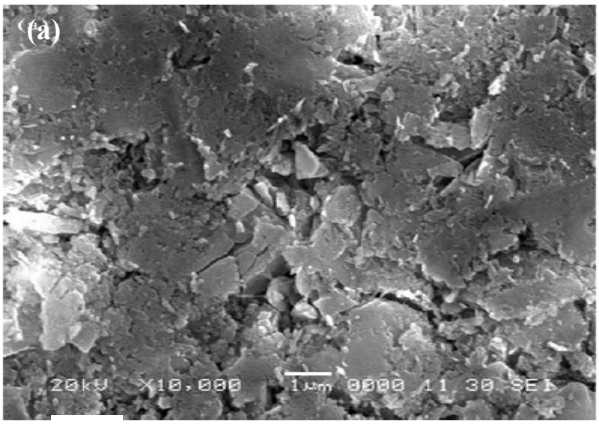


Figure 1: FTIR Spectrum of HAp specimen; (a) before soaking in SBF, (b) after soaking in SBF for 25 days

FTIR of HAp shown the intensity of HAp bands such as amine, alkyne in HAp sample decreased post-immersion for 25 days compared to before immersion. The band associated to the stretching modes of C-H bond was observed at 2900.78 cm^{-1}. The bend at 1721.55 cm^{-1} and 1641.37 cm^{-1} shown the C=O and C=C respectively. The bends at 1427.04 cm^{-1}were attributed to the stretching modes of the hydroxyl groups, respectively. The strong stretching FTIR was observed at 1160.43 cm-1 and 1109.10 cm^{-1} due to vibration of alcohol group. Hence, from result, it shows that the bioactivity of the HAp specimen is occurred after immersion in SBF solution.

B. Scanning Electron Microscope (SEM)/ Energy Dispersive X-ray (EDX) Analysis

Scanning Electron Microscope (SEM) was performed using a voltage of 10,000 kV in order to observe the surface morphology of HAp specimen after 25 days of SBF immersion. The comparison between pure HAp specimen and after immersion into SBF is shown in figure 2 and 3.



(b)

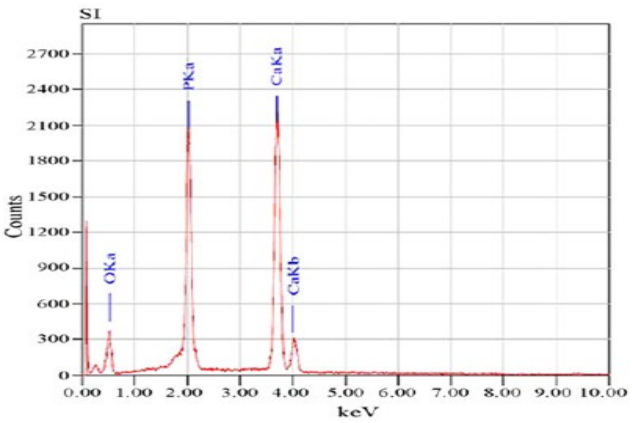
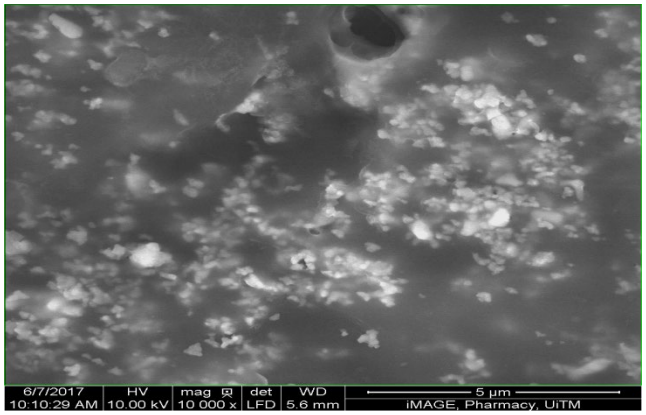
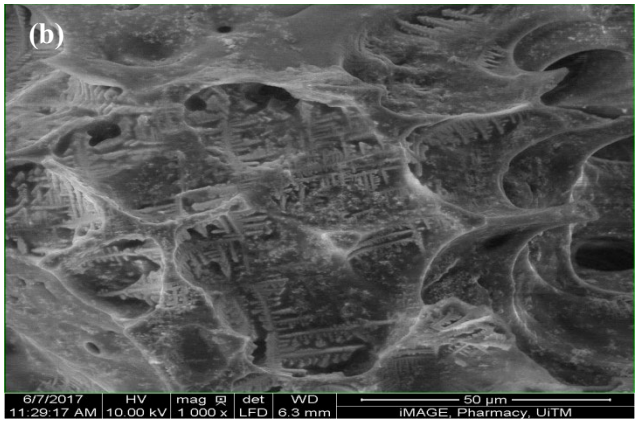
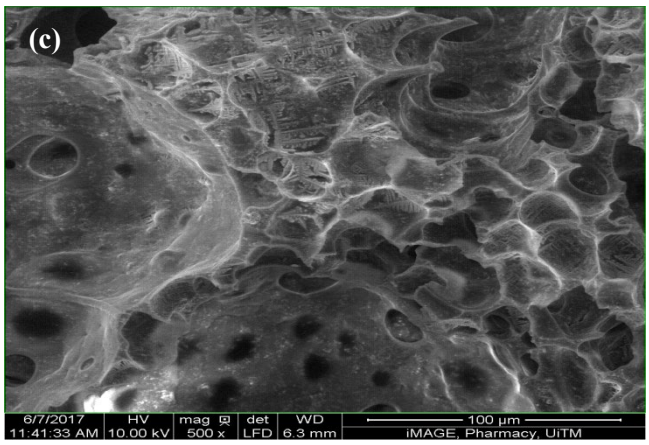


Figure 2: (a) SEM image of pure HAp specimen before immersion in SBF, (b) EDX Spectrum of HAp specimen before immersion in SBF [11]







(d)

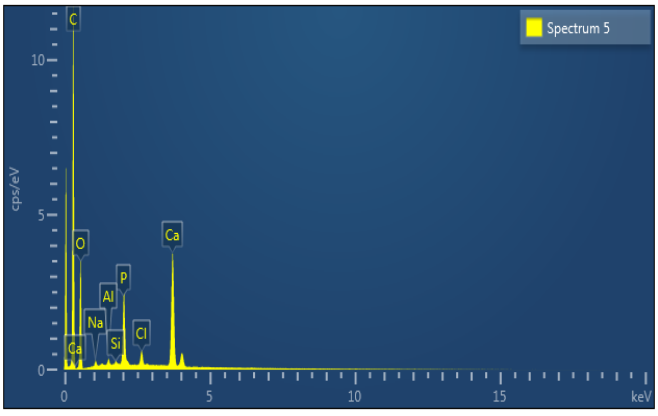


Figure 3: SEM images of the HAp specimen after immersion in SBF for 25 days; (a) HAp with the size of 5µm, (b) HAp with the size of 50µm, (c) HAp with the size 100µm, (d) EDX spectrum of HAp specimen after immersion in SBF for 25 days for the size of 100 µm

From figure 2(a and b), the surface of pure HAp is found to be plain and dense surface. The presence of compositional elements of Hap such as Ca, P, and O were detected by EDX. Figure 3(a, b, c, and d) shown the morphology of SEM and 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. Generally, the formation of a dense apatite layer was observed on porous bioactive materials from SBF solution, but the formation of porous interlinked apatite layer from SBF was rare as reported.

The osteointegration and osteoconduction properties are enhance from these apatite layers. The osteointegration and osteoconduction properties are enhance from these apatite layers. Osteoconduction is the process by which osteogenesis is induced. Osteoconduction implies the recruitment of immature cells and the stimulation of these cells to develop into osteoblasts. In a bone healing situation such as a fracture, the majority of bone healing is dependent on osteoinduction. Which means that, osteoconduction is bone grows on a surface (Albrektsson et al., 2001). Osseointegration is the stable anchorage of an implant achieved by direct bone-to-implant contact. Osseointegration is possible in other parts of the body, but its importance for the anchorage of major arthroplasties is under debate. Ingrowth of bone in a porous-coated prosthesis may or may not represent osseointegration.

This sort of action indicates that after incubation in SBF, HAp specimen accelerate the process of mineralization in the region for genuine bone material on useful investigations.. Energy Dispersive X-ray analysis of the HAp specimen in SBF after 25 days shown the presence of elements Ca, P, O as well as C, Na and Cl. The major peaks due to Ca, P, and O confirmed the growth of calcium phosphate layer on the HAp surface, as describe in Figure 3(d).

When a material is incubated in SBF solution, the formation of apatite layer on the surface of pellet goes through a sequence of chemical reactions like spontaneous precipitation, nucleation and growth of calcium phosphate [11]. It has been recommended that surface chemistry plays a crucial role in this process and even the functional groups of materials have a large effect on the bone bonding property. As mention earlier, the HAp structure consists of Ca, PO_4, and OH groups closely packed together.

The negativity of HAp surface is highly depend on the OH^- and PO_4^{3-} groups while the positivity of HAp is depend on Ca^{2+} ions. The process of apatite formation mainly depends on negative group, which in turn depends on the large number of negative ions (i.e. OH^- and PO_4^{3-}) on the surface. During incubation period, the positive Ca^{2+} ions from SBF are attracted by the OH^- and PO_4^{3-} ions present on HAp surface. Therefore, the surface gains positive charge with respective to the surrounding SBF and further attracts the negatively charged OH^- and PO_4^{3-} ions from the SBF. This leads to formation of the apatite layer [11].

IV. Conclusion

In conclusion, the formation of apatite layer on HAp surface and the development of functional groups are identified by SEM equipped with EDX and FTIR. The growth of apatite layer on the HAp surface is identified as highly porous. The formation on major peak on surface on EDX due to Ca, P, and O confirm the growth of calcium phosphate layer on the HAp surface. However, it is difficult to observe the bioactivity of the HAp due to some limitations such as lack of sample which difficult to make comparison of HAp surface along a time. Hence, it is recommended to increase the amount of HAp specimen and observe the formation the apatite layer of HAp surface along the soaking period in SBF solution.

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