Nanofibrous Bio-inorganic Hybrid Structure Formed through Self-Assembled Peptide (FEFEFEFEFKFK) in Distilled Water and Calcium Acetate

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***Abstract*— This research is conducted in order to understand the synthesis of the hydroxyapatite (HAP) and to characterize the nanoparticles structure formed through self-assembled peptide, to study the synthesis of the nanofibrous hybrid structure formed through self-assembled peptide by using HAP, and to investigate and characterize the morphology of the nanofibrous formed through self-assembled peptide (FEFEFEFEFKFK) in water and salt (calcium acetate). This research is categorized into two parts: the synthesis and mineralization of HAP and the characterization of nanoparticles. Wet precipitation reaction was used in synthesizing Hydroxyapatite (HAP) sample at temperature of 80 ℃. Fourier Transform Infrared Spectroscopy (FT-IR) found that the HAP powder assigned with functional groups of PO_4^{3-}, OH^-, and CO_3^{2-}. Inductive Coupling Plasma (ICP) analysis found that Ca^{2+} concentration of supersaturated HAP measured is 1.009mM compared to the standard HAP with 4mM Ca^{2+}. Powder X-ray Diffraction (XRD) analysis revealed that no secondary crystalline phase as found from the HAP powder and the distance between the atomic layers in the crystal is 0.3096nm at peaks 25.878^0 correspond to reflection (002). The synthesized HAP The formation of biomaterials forms through biomimetic self-assembly by generating apatite hybrid from the mineralization of HAP with self-assembly peptide able to create biodegradable and biocompatible characteristics to be used in the biomedical field especially in tissue engineering is expected. In addition, this makes it more favourable to be used in the biomedical field as the mechanical strength and surfactant of HAP nanocrystals can be improve.**

***Keywords*— nanofibrous, bio-inorganic hybrid, peptide, calcium acetate**

I. INTRODUCTION

The organic or inorganic hybrid nanomaterial became extremely widespread and the combination of these materials at the level of nanoscopic is not peculiar anymore, [1]. Nanoparticle assembly shows an energetic and functional role in almost every related field. It is critical to control and manipulate the assembly of nanoparticles. Other than that, the building blocks unexpectedly organize into ordered structures through thermodynamic processes and other constraints. Nanoparticle assembly is an operational approach for preserving the chemical properties of nanoparticles and also can be applied to improve nanoparticle performance [2].

According to N. Habibi et al, 2016, peptides and peptide derivatives are depending on their building blocks were viewed as self- assembled peptides. Recently, many applications are highlighted with relevant examples when these structures become basic building blocks. The building blocks of peptides and proteins commonly come from amino acids. This is because; they are able to induce a high molecular complexity starting from relatively simple molecules which being at the molecular basis of the living world, [3]. Synthetic polypeptides derived from large and natural amino acids are very beneficial building blocks for fabricating self-assembling structures due to their high physicochemical stability, diversity in sequence and shape, suitability for large scale synthesis, biodegradability and biocompatibility, [4].

Synthetic HAP, $Ca_5 (PO4)_3(OH)_2$) is one of the favourable material to use in dental and medical applications. In fact, the HAP nanoparticles have numerous benefits over conventional-sized HAP bulk ceramics such as for their large surface-to-volume ratio, reactivity and biomimetic morphology of the HAP nanoparticles.

According to M. Okada, T. Matsumoto, 2015, these properties showed that HAP nanoparticles are very useful in the fillers for composites, carriers for drugs and reparative materials for damaged enamel, [5]. According to M. Swetha et al 2010, hydroxyapatite HAP, $Ca_{10} (PO4)_6(OH)_2$ nanocrystallites and collagen fibrils is one of the complex inorganic–organic nanocomposite materials that well controlled in overall numerous length scales including nanoscale. In fact, they can be altered by changing the components for monomers with dissimilar ratios, acquaint with several functional groups to them or adjusting the polymerization conditions. The mechanical properties can be enlightened by the addition of nanoparticles into the biopolymer matrix. Hence, producing nanocrystallites of calcium phosphate (CaP) salts with natural polymer is the core technique in accomplishment the artificial biomaterials as bone substitutes in biominesis inspired, [6].

Previous studies, most researchers have showed the similarity of HAP nanoparticles with other nanomaterial as they are normally non- toxic and become sources for cell damage *in vitro*. These nanoparticles showed more positive biocompatibility compared to rod-like nanoparticles. All earlier studies indicate the biocompatibility of HAP nanoparticles are very supportive in designing of HAP nanoparticles and further understanding for effects of surface charge on cellular uptake should be done, [7]. Therefore, our studies investigated the formation of new nanofibrous structures and its application to replace the existing materials with biocompatibility materials. The mechanical properties of HAP and the surface area will be improvising combination with self-assemble peptide, (FEFEFEFEFKFK) and the morphology of the nanofibrous will be formed through self-assembled peptide in water and calcium acetate.

II. METHODOLOGY

2.0 Materials and Method

The technique required to accomplish the goals of this study was focused into two significant sections which were (i) preparation and (ii) characterization of HAP

2.1 Preparation of powder hydroxyapatite (HAP)

The synthesized of HAP powder were prepared by mixing of 300 ml solution with calcium/ phosphorus (Ca/P) ratio of 1.67 with 14.17 g of Ca $(NO_{3)2}.4H_2O$ and 5.62 g of $NaH_2PO_4.2H_2O$ in 300 ml of deionized water. After the mixing, the solution was stirred vigorously and heated to about 85°C. Ammonium hydroxide solution with concentration at 28% - 30% was added and the nanoparticles precipitation was induced. This solution was hold at 85°C for 24 hour as ensure that the complete conversion to hydroxyapatite. The mixture was cooled to room temperature and let settled in the container. The excess liquid decanted off after addition of fresh deionized water and the mixture was stirred concisely before allowing the solid to settle and re-decanted again. These procedures were repeated until the pH of the mixture is below 9.

2.2 Preparation of Hydroxyapatite with peptide (FEFEFEFEFKFK)

The HAP and peptide treatment was prepared with two significant section which were (i) preparation of supersaturated hydroxyapatite (HAP) solution and (ii) mineralization.

2.2.1 Preparation of supersaturated hydroxyapatite (HAP)

The stock solution was prepared by dissolving 5.1839g of HAP powder in 100mL solution containing 100 mM of hydrochloric acid and had final concentration of 50 Mm of calcium. Then, 40 mL from stock solution was pipetted into the clean polythene container and the increased the volume to 450 mL by adding the distilled water. The pH was adjusted to 7.01 with 0.5 M potassium hydroxide. The calcium acetate was added until reach the final concentration which was 200 mM and the final volume was adjusted to 500 mL with distilled water. At the end, the concentration needed for the resultant HAP-supersaturated solution is $[Ca2^+]$.

2.2.2 Mineralization of HAP with peptide

Then mineralization process was done by mixing together the solution of peptide (FEFEFEFEFKFK) which was about 200 µL and 200 µL of supersaturated HAP solution in the glass tube as to form a clear solution. Then, this solution was incubated in the incubator at 36.7°C in order to vaporize the solvent water and the mixture is going to centrifuge. The solid materials were re-suspended in water and then rinsed the particles as to remove the soluble salts. Then, the solutions were transferred into characterization process,

2.3 Characterization of hydroxyapatite (HAP) and peptide

2.3.1 ***Fourier Transform Infrared Spectroscopy (FT-IR).***

FT-IR was used to characterize the functional groups of untreated and surface modified HAP nanoparticles. For each spectrum, 16 scanned within 4000 - 400 cm^{-1} wavenumbers and were recorded in the transmission mode by a potassium bromide method.

2.3.2 ***Inductive Coupling Plasma (ICP)***

ICP was used to determine the concentrations of metals which are Ca and P in the dried precipitates. The powders were dissolved in 1 ml nitric acid and subsequently diluted with deionized water to a total volume of 10 ml. The quantitative measurement was carried out against standard solutions that contain defined concentrations of all ions of interest.

2.3.3 ***Powder X-ray Diffraction (XRD)***

XRD was involved in characterization of these particles and performed within a 2θ with range of 20° till 60°mwith Cu Kα radiation about 1.54 Å with scanning speed 3°. Besides, the scans of HAP powders were run at 40 kV and 35 mA.

2.3.4 Geology microscope

BX41-P polarizing microscope was used to examine the morphology of the acquired HAP- peptide composite specimen. This sample was prepared by place the samples onto the glass slide. Then morphology of the sample will examine by using 4X, 10X, 20 X and 40 X magnifications.

III. RESULTS AND DISCUSSION

A. FT-IR Absorption bands of the synthesized HAP

Hydroxyapatite Ca_{10} $(PO_4)_6(OH)$ $_2$ is dominated and the most major mineral phase in the solid tissues of the vertebrates. It consisted of the same ions that form mineral part of teeth and bones. A biological HAP usually has lacking of calcium and it is always substituted with a carbonate. Probably, two types of carbonate substitution are:

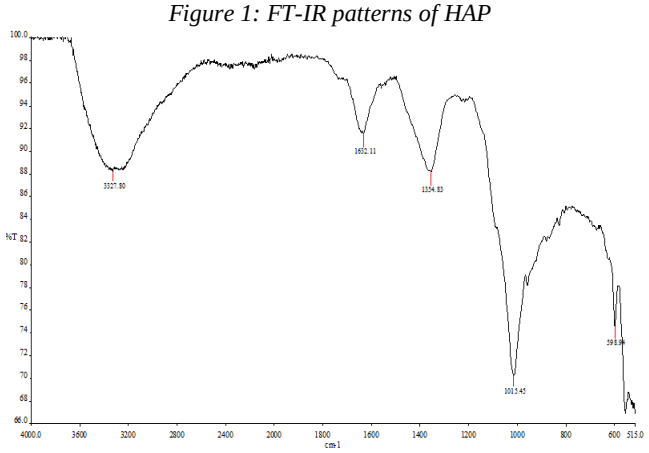
1. Direct substitution of OH- with CO_3^{2-} (A-type substitution $(CO_3)^{2-} \leftrightarrow 2OH-$).
2. Necessity after charge compensation, PO_4^{3-} substituting tetrahedral group with CO_3^{2-} (B-type substitution).

Table 1: FT-IR absorption bands of synthesized HAP chemical groups, [8]

Functional groups	Absorption bands (cm^{-1})	Description
OH^-	630-3570	OH^- ions prove presence of HAP
CO_3^{2-}	870-1650	Substitute phosphate ion
HPO_4^{2-}	875-880	-Characterizes HAP with deficient of calcium -Refers to non-stoichiometric HAP
NO_3^-	820-1380	Synthesis residue that disappears during the calcifying process
PO_4^{3-}	460	v2
	560-600	v4
	960	v1
	1000-1120	v3
Adsorbed water	2600-3600	Under influence of thermal treatment, absorption band becomes narrower

Figure 1 shows the FT-IR spectra of HAP powders. The characteristic bands in Table 1 showed in the sample spectra allocated here: (a) two bands were observed at 1632.1 cm-1and 1354.83 cm-1 due to the stretching mode of carbonate group $(CO_3)^{2-}$, (b) the band at 3327.80 cm^{-1} arises from OH-, the bands at 1015.45 cm-1, 598.94 cm^{-1} and 558.26 cm^{-1} arise from PO_4.

Figure 1: FT-IR patterns of HAP



The FT-IR analysis showed all typical absorption characteristics of hydroxyapatite. In addition, some carbonate contents were seen $(CO_3)^{-2}$ peak around 1600 cm^{-1}, which an indication of the presence of carbonate apatite. This might have invented through the absorption of carbon dioxide from the atmosphere. Therefore, the functional group of the HAP powder predicted from this analysis are compared with the result of H. Eslami et al 2008, for confirmation. Thus, it is observable that the synthesized powder is certainly hydroxyapatite, [9].

Table 1: Functional groups presence in HAP

Wavenumber (cm^{-1})	Stretching mode	Functional group
3327.80	Ion stretching	OH^-
1632.11	Asymmetric stretching	CO_3^{2-}
1354.83	Asymmetric stretching	CO_3^{2-}
1015.45	Asymmetric stretching	PO_4^{3-}
598.94	Asymmetric bending vibration	PO_4^{3-}
558.26	Asymmetric stretching	PO_4^{3-}

B. Concentration of calcium ions [$Ca2^+$] in HAP solution

ICP is used to analyze the molar ratio of Ca/P by determine the concentration of calcium ion. Concentration of calcium ion is important to mineralize the peptide with supersaturated of HAP solution.

The first analysis of HAP powder was done by dissolved into 0.1M HCL solution and the favourable concentration, [$Ca2^+$] needed is 50mM.

Table 2: Concentration of Calcium, [$Ca2^+$] in HAP before supersaturated.

Sample 1	Conc. of HCL	Conc. of Ca^{2+} (ppm)	Conc. of Ca^{2+} (mM)	Temp while dissolving (°C)	pH
1st analysis	0.1	1982.8	49.5	80	3.87
2nd analysis	0.1	2170.7	54.2	80	3.87
3rd analysis	0.1	1832.0	45.7	80	3.87
Average: 49.8mM					

Then, the second analysis was conducted as to analysis the targeted concentration for supersaturated HAP. This was done by dissolved in 0.05M of KOH solution and the standard concentration for this HAP is 4Mm.

. Table 2: Calcium concentration, [Ca2+] in supersaturated HAP

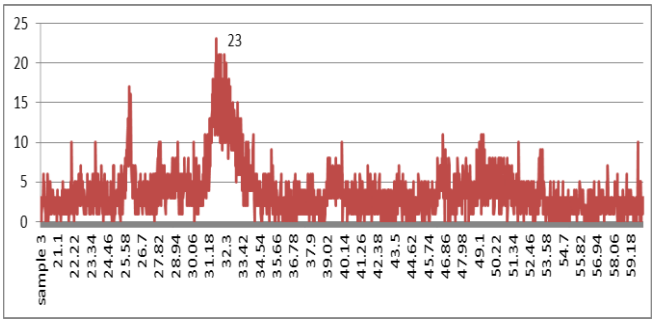
Sample 1	Conc. of KOH (M)	Conc. of Ca^{2+} (ppm)	Conc. of Ca^{2+} (mM)	Temp while dissolving (°C)	Final pH
1st analysis	0.05	82.830	2.067	80	7.01
2nd analysis	0.05	28.650	0.715	80	7.01
3rd analysis	0.05	9.8650	0.246	80	7.01
Average: 1.009 mM					

Chemical precipitation is a method that is used to synthesize the hydroxyapatite, HAP powders with molar ratio of Ca/P =1.67 which are suitable for tissue engineering application. The mechanism of acidic interaction with HAP involves with wo phases. In the first phase, an acid bonds to surface calcium of

HAP with formation of calcium-acid complexes. While in the second phase, the acid the either remain attached to the HAP surface with only limited decalcification involved or the calcium-acid complexes will detach, resulting in a substantial decalcification effect, [10].

C Size, crystalline structure and phase of Hydroxyapatite (HAP) powder

Figure 2: XRD's pattern of HAP



A typical powder XRD pattern of a synthesized nanometer sized HAP powder is presented in Figure 2. Inspection of Figure 2 reveals the presence of crystalline nanometer sized HAP phases, which were found to be consistent with the phases listed in the ICDD database. The main indices for nanometre sized HAP: (002), (211), (300), (202), (130), (002), (222) and (213), [11]. The distance between atomic layers of the synthesized nano-HAP powder was calculated from the XRD pattern using Bragg's Law;

$$2d \sin\theta = n\lambda$$

d: distance between atomic layers in a crystal
λ: wavelength of incident X-ray
n: integer (0.9)
θ: diffraction angle

Table 3: Result XRD analysis for HAP powder

Peak Search Report (1 Peaks, Max P/N = 1.6)							
[sample 1.raw] sample 1							
PEAK: 21-pts/Parabolic Filter, Threshold=0.0, Cutoff=0.0%, BG=7/0.5, Peak-Top=Summit							
2-Theta	d(Å)	BG	Height	I%	Area	I%	FWHM
25.878	3.44	4	13	100	144	100	0.188

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By using formula mentioned above, the distance between atomic layers in HAP crystal was calculated and the comparison was done with standard HAP 0.3096.

Table 4: Standard of distance of atomic layers for HAP's crystals,[12].

Crystallographic Plane (h k l)	Angle (θ)	d-Spacing (nm)	Intensity (%)
0 0 2	25.879	0.3440	40
2 1 1	31.774	0.2814	100
1 1 2	32.197	0.2778	60
3 0 0	32.902	0.2720	60
2 0 2	34.049	0.2631	25
3 1 0	39.819	0.2261	20
2 2 2	46.713	0.1943	30
2 1 3	49.469	0.1841	40
3 2 1	50.494	0.1806	20
0 0 4	53.145	0.1722	20

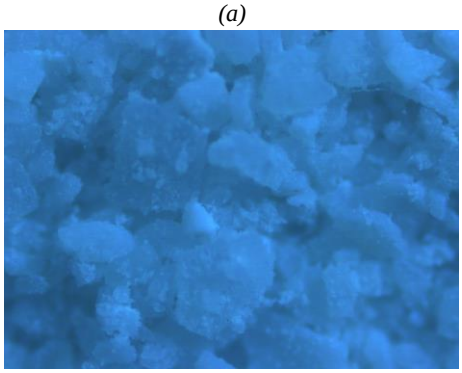
Table 6: Comparison between standard HAP and Sample 1

Parameter	Standard HAP			Sample 1		
	Peak 1	Peak 2	Peak 3	Peak 1	Peak 2	Peak 3
2θ (º)	25.879	31.774	32.908	25.878		
I (%)	40	100	60	100		
d (nm)	0.3440	0.2814	0.2778	0.3096		
Crystallinity	Highest			Low		

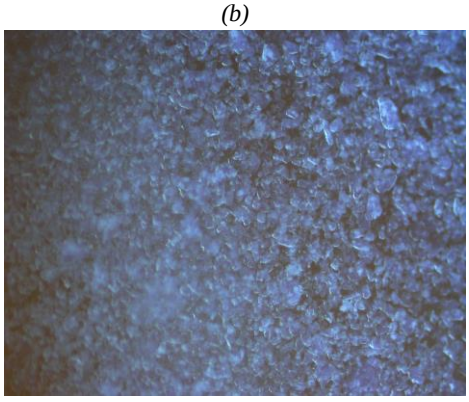
The synthesized HAP powder of has lower crystallinity and crystallite size. This is identified by comparing the diffraction peaks pattern, where the diffraction peaks pattern of standard HAP is narrower and well separated as compare to diffraction peaks as show in Figure 2. From Table 6, its shows that only peak at 25.878^0 show the presence phase of HAP corresponds to reflection (002).

D Morphology of the acquired HAP- peptide

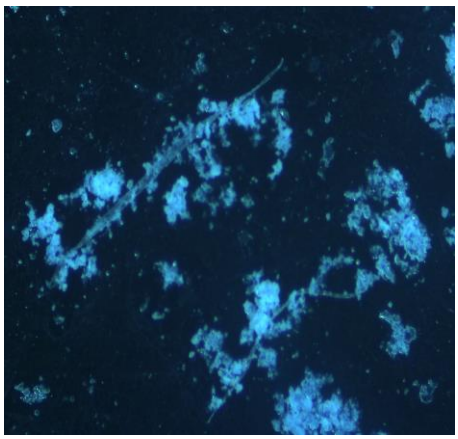
(a)



(b)



(c)



(d)

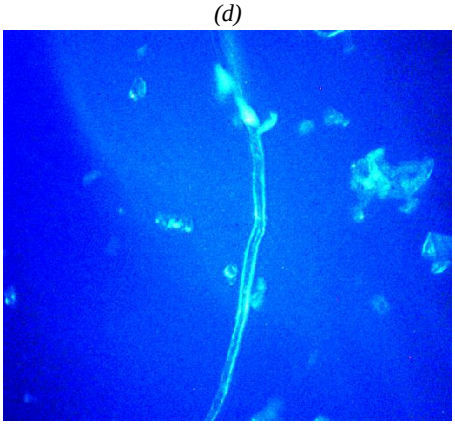


Figure 3: Images from geological microscope (a)HAP powder (b) Supersaturated HAP with 10X magnification (c) Mineralized HAP in distilled water and (e) Mineralized HAP in calcium acetate solution

From the figure above, (a) indicate the porous structure of HAP powder. This porous property driven the HAP to become compatible as it is provided excellent mechanical fixation and allowed chemical bonding between bioceramics and bones. For medical applications, it is significant to consider this properties as the surrounded biomaterial will chemically react with their environment and they should not create undesired effects on their adjacent or distant tissues, [13].

(c) and (d) show the image of mineralized HAP with peptide dissolved in distilled water and calcium acetate solution. This was done as to investigate the effect of OH- ion and Ca2+ ion towards the mineralized HAP's surface. Therefore, a rod shape structure was found and the structure was observed not only in distilled water but in calcium acetate solution too. However, the structure was found in calcium acetate is more clear as compare with (c). In this study, 130Mm of calcium acetate is used as the molarity of human body fluid is in the range of 130mM until 140Mm. This will ensure that the formation of new nanofibrous structure is compatible with human bone.

HAP formation does not occur directly but proceeds through amorphous calcium phosphate precursors in mineralizing environments. It is believed that negatively charged amino acids (E) contained carboxylate and phosphorylated residues which play a key role in controlling HAP mineralization. However, positively charged (K) and polar amino acids can also interact with calcium phosphate groups and can influence HAP particle size and crystallinity, [14].

Surface charge of HAP nanoparticles can be modified by immobilization of amino acids. Acidic amino acids like glutamic acids, (E) lead to negatively charged surface meanwhile zwitterionic amino acids can prevent HAP surface from nonspecific protein adsorption and even provide clear antifouling properties as they contained two counter-charged groups and maintained overall charge neutrality. Growth of HAP crystals is often inhibited by the adsorption of organic compounds such as proteins and amino acids. This feature is highly interesting since the decreasing on the degree of crystallinity should affect the mineralization of HAP and even could make possible accomplishment of controlled solubility amino acids. The surface of HAP crystal is negatively charged possibly due to better surface migration of $PO4^{3-}$ groups, which is considered to have an incredible impact on organic adsorption. The interaction of basic residues and $PO4^{3-}$ ion of HAP mineral surface has been considered as a contributing factor for the binding affinity of positively charged synthetic peptide to HAP surface, [15]

IV. Conclusion

The objectives for this study is divided into three sections and successfully conducted. The first goal is to synthesis HAP. Yet, the synthesis of HAP by wet method is not achieved the standard due to result of the concentration of calcium {Ca^{2+}]. The standard concentration of calcium should be 4Mm and this value is favourable for combination with peptide. However, the synthesis HAP is used and proceeds to the next experiment, combination with peptide. Then, the morphology of mineralized HAP is clearly shown in calcium acetate solution and distilled water. Nonetheless, the formation of fibril structure in calcium acetate long and clear than in distilled water but, the existing of the rod shape is more in distilled water. All of the characterizations are done by using SRD, FT-IR, ICP and Optical Microscope and all results are discussed. Overall, the new formation of the nanofibrous structure by combination of HAP and peptide (FEFEFEFEFKFK) are compatible to human body composition. This is accepted as fibril structure form in 130Mm calcium acetate is represented the fluid body composition.

Acknowledgment

This research was supported by Faculty of Chemical Engineering UiTM Shah Alam. The author wishes to acknowledge Dr Tan Huey Ling and Dr Harumi Veny for their generous assistance and meaningful discussion to complete this research.

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