NANOFIBROUS BIO-INORGANIC HYBRID STRUCTURE FORMED THROUGH SELF-ASSEMBLED PEPTIDE (FKFSFEFEFKFK)

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***Abstract*— The purpose of this study is to synthesis and characterized Hydroxyapatite (HAP), to investigate and study the hybrid structure that will form through self-assembled between HAP and (FKFSFEFEFKFK) peptide and to characterize the hybrid structure between HAP and (FKFSFEFEFKFK) peptide in different medium of distilled water and potassium chloride. The technique of synthesizing HAP was done by wet chemical method where Calcium Nitrate Tetra Hydrate was allowed to mix with Sodium Di-hydrogen Phosphate in deionized water producing calcium/phosphorus (Ca/P) with ratio 0f ~1.67. The pH was adjusted until reaching pH7 where HAP is best work at pH7 condition. Then the HAP synthesis was used to mineralize with peptide (FKFSFEFEFKFK). The assembly nanoparticles form through the mineralization was allowed to characterize by using Fourier transform infrared spectroscopy (FTIR), Powder X-ray diffraction (XRD), Inductive coupling plasma (ICP) and under microscope. Fourier transforms infrared spectroscopy (FTIR) was used to characterize the functional groups of synthesis HAP nanoparticles on molecular level. Powder X-ray diffraction (XRD) was used to see the selected reactions on the surface of HAP such as its intrinsic properties and crystalline structure while Inductive coupling plasma (ICP) monitor the concentration [Ca2+] of the solution phase during the mineralization. Observation under microscope was done to observe sample surface structure and the size distribution. In summary, it shows that the synthesis of HAP is maintained based on its molecular level, intrinsic properties and crystallinity. Mineralization under medium distilled water can form a better and clear size distribution compared to under medium potassium chloride.**

Keywords*— Hydroxyapatite, Mineralization, Peptide, Self-assembled, Wet chemical method

I. INTRODUCTION

Several attempts had been performed during last year in order to fabricate scaffolds that will mimic the natural tissues. This multidisciplinary field is growing fast in contribution of biology, medicine and engineering. Million bone injuries occur in the US every year according to statistic that in turns will need nearly million bone grafting procedures. Therefore other alternatives treatment of bone injuries is crucial to be exploring for bone tissue engineering to replace autografts and allograft technique that have disadvantaged outcome and to overcome the potential challenges while doing surgery for this technique. The scaffold can reduce the need for multiple surgeries associated with the removal of metallic implants (Zahra Rezvani et al, 2016).

Other reasons that contribute to the research of Nano-hybrid scaffolds are due to the huge number of teeth lost because of dental caries, trauma or periodontal diseases. The application of Nano fibrous hybrid structure and tissue engineering will overcome this problem by allowing a few natural or synthetic biomaterials that have similar morphology, porosity, size, shape and biocompatibility with extracellular matrix to combine with growth cells and stem cells. This combination might allow the regeneration of tissue possible [7].

The challenge in engineering is to construct a bio- mimicking extracellular matrix (ECM) with effective mineralization. Thus, many on-going researches are focusing on the methodology to synthesis the biomaterials and to find the best method to fabricate the biomaterials to form excellent scaffolds that is comparable to the native structure of tissue [3].

In tooth engineering field, the biomaterials that will be scaffolds can be anything whether it is natural or synthetic, rigid or non-rigid as long as the combination of biomaterials meet the requirement such as the rate of degradation match the rate at which new tissue is formed without leaving a toxic environment to the host tissue. Some biomaterial like collagen is too soft to replace the native tissue of tooth although it match a few requirement such as bioactivity and biocompatibility, in order to overcome this weakness, inorganic material such as hydroxyapatite (HAP) is use because it have better mechanical strength than collagen, biocompatible and most importantly have similar structure with extracellular matrix. Thus the study of biomaterial itself is crucial so that the bad side of biomaterial can be overcome by scaffold with any other combination of biomaterials [7].

HAP had been widely used in many fields especially in biomedical applications such as bone tissue engineering materials and drug release control. The reasons why HAP is so attractive because it have similarity in terms of chemical with inorganic component of bone matrix, biocompatibility with soft tissue such as muscle and gums, slow biodegrade, osteoductive and osteoinductive good, strong affinity, and the chemical bonding of HAP with the host cells is advantages compared to other bone substitutes for instance metallic implants and allorgrafts [3].

The methodology to synthesis HAP is crucial to improve the morphology and characteristic of HAP so that it can compete with the synthetic HAP available and the existing and natural HAP because HAP is then will be used as scaffold in important industry such as medical and bone tissue engineering. There are a lot of methodologies to synthesis HAP chemically and biologically. Every method perform usually will undergoes several analysis to

make sure that the HAP form is good enough, bioactive and non-toxic to human using Fourier transform infrared spectroscopy (FTIR), Zeta potential measurement, Powder X-ray diffraction (XRD), Scanning electron microscope (SEM) and transmission electron microscopy TEM. HAP was synthesis by various techniques in a few research back then which are Solid state reactions, sol-gel synthesis, Chemical precipitation, Micro emulsion, Hydrothermal reactions, Co-precipitation [3], Wet chemical methods, mechano-chemical [1], High-gravity precipitation [2], green synthesis [6] and spray drying method [9], homogeneous precipitation, RF plasma spray, combustion synthesis, ultrasonic spray freeze-drying, sonochemical synthesis [5].

A growing interest in the investigation of self-assembling nanostructures has contributed to the attraction on self-assembling between inorganic material (eg: Hydroxyapatite) with polypeptide based copolymers. This polypeptide based copolymers shows a good promise in terms of good building blocks allowing greater control on intra and intermolecular interactions. This building blocks offer a stable structure for scaffolds by interaction based on bonding such as hydrogen bonding, electrostatic interaction, π-π stacking and van der Waals.

In a case of drug, to develop a drug for a medical purpose, a large molecule such as protein can be used as a therapeutic option. The using of protein in the scaffold can be very challenging thus a good understanding on nature of protein and conformational structure characteristic is necessary.

II. METHODOLOGY

2.1 Methodology

2.1.1 Synthesis of HAP nanoparticles

The synthesis Of HAP was performed by mixing of 60 mmol (14.17 g, 0.2 mol l−1) of Calcium Nitrate Tetra Hydrate and 36 mmol (5.62 g, 0.12 mol l−1) of Sodium Di-hydrogen Phosphate in 300 ml of deionized water. The solution is basically producing 300ml calcium/phosphorus (Ca/P) with ratio 0f ~1.67.The solution was stirred vigorously and heat at the same time to 85˚C before adding 300 ml of concentrated ammonium hydroxide solution (28–30%). Nanoparticle precipitation was going to be induced after the addition of ammonium hydroxide solution. The mixture was allowed to heat for 24 hours at 85˚C to ensure the hydroxyapatite completely produce by utilizing the starting material, then the material was cooled down to room temperature to allow the solid to settle at the bottom of the container and the excess liquid was going to decanted off. Fresh de-ionized water was added and the solution was stirred again. The settle down and decanted off process was repeated once again until pH value of mixture below 9 [4].

2.1.2 Preparation of supersaturated HAP

Preparation of stock solution was done by dissolving HAP 1.67 ratio of Ca/P powder in a solution containing 100 mM of hydrochloric acid until the final concentration calcium reach 50 mM. Then stock solution of 40mL was pipette into a clean polythene container, and the distilled water was added to achieve final volume of 450mL. 0.05 M of potassium hydroxide was added gradually so that the pH reached 7.01. Final concentration of 200 mM was expected to reach by adding sodium chloride, and then distilled water was added to meet final volume of 500 mL. The concentration of the resultant HAP-supersaturated solution was [Ca2+] = 4 mM.

2.1.3 Mineralization of HAP and peptide

200 µL solution of peptide (with a concentration of 3.0 × 1012 PFU/mL, pH ~7.5) and 200 µL supersaturated HAP solution (pH ~7.01, [Ca2+] = 4 mM) were mixed together in a glass tube to form a clear solution. To vaporize the solvent water slowly the solution was allowed to age in an incubator at 36.7 °C. The mixture was centrifuged. The solid materials forms were re-suspended in water, transferring onto a TEM grid, soluble salt was removed by rinse [8].

2.2 Characterization of nanoparticles

2.2.1 Fourier transforms infrared spectroscopy (FTIR)

Fourier transforms infrared spectroscopy model Shimadzu was used to characterize the functional groups of synthesis HAP nanoparticles and to determine its functional group on molecular level [4].

2.2.2 Powder X-ray diffraction (XRD)

Powder X-ray diffractometer model Rigaku was used to see the selected reactions on the surface of HAP such as its intrinsic properties and crystalline structure. Perform within 1θ/min range of 20˚C to 60˚C. The bulk scan will be run at 40kV and 35Ma [4].

2.2.3 Inductive coupling plasma (ICP)

Inductive coupling plasma was to monitor the [Ca2+] of the solution phase during the mineralization [8].

2.2.4 Microscope

Microscope was used to observe sample surface structure on the range of micrometer.

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III. RESULTS AND DISCUSSION

A. Characterization of HAP powder synthesis.

In the present study, the synthesis of bio-inorganic material is crucial to reach maximum outcome for the self-assembly of hybrid structure. Many on-going researches are done on how to synthesis inorganic materials specifically HAP (Hydroxyapatite). Focusing on the surface charge of the hydroxyapatite is one of the ways to maximize the process of assembly between organic and inorganic material. Although which kind of surface charge of HAP to be synthesis is still not clear we speculate that the normal or untreated way of synthesis HAP could biocompatible with (FKFSFEFEFKFK) peptide. FTIR analysis was done to provide better understanding on what had happen on the molecular level of these HAP syntheses. Consisting of three different HAP with the same method of synthesis namely sample A, B and C, Figure 1, 2 and 3 indicate that the most characterized chemical groups in the FTIR spectroscopy are carbonate ion, phosphate ion, nitrate ion and hydroxide ion.

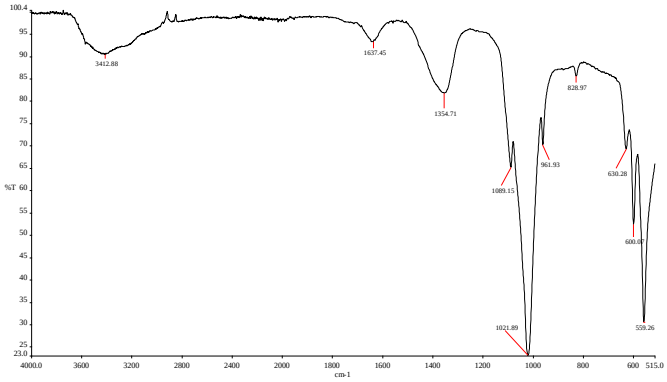

Figure 1: FTIR analysis sample A of HAP powder

Table 1: Data interpretation for FTIR sample A powder

Functional groups	Absorption bands (cm-1)
Adsorbed water	3412.88
Carbonate ion	1637.45
Nitrate ion	1354.71
Nitrate ion	1089.15
Phosphate ion	1021.89
Phosphate ion	961.93
Nitrate ion	828.97
Hydroxide ion	630.28
Phosphate ion	600.07
Phosphate ion	559.26

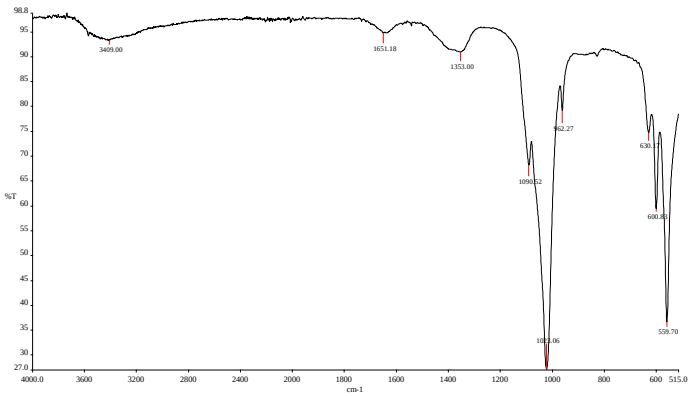

Figure 2: FTIR analysis sample B of HAP powder

Table 2: Data interpretation for FTIR sample B powder

Functional groups	Absorption bands (cm-1)
Hydroxide ion	3409.00
Hydroxide ion	1651.18
Carbonate ion	1353.00
Nitrate ion	1090.52
Phosphate ion	1023.06
Phosphate ion	962.27
Hydroxide ion	630.17
Phosphate ion	600.83
Phosphate ion	559.70

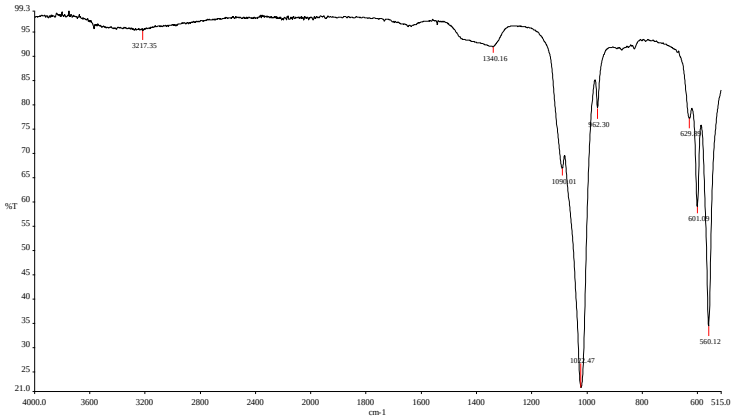

Figure 3: FTIR analysis sample C of HAP powder

Table 3: Data interpretation for FTIR sample C powder

Functional groups	Absorption bands (cm-1)
Hydroxide ion	3217.35
Carbonate ion	1340.16
Phosphate ion	1090.01
Phosphate ion	1022.47
Phosphate ion	962.30
Hydroxide ion	629.89
Phosphate ion	601.09
Phosphate ion	560.12

The intrinsic properties and crystalline structure are other important properties of Hydroxyapatite to be observe instead of its molecular level. If there is any chemical reaction occur during the experiment, the intrinsic properties and the crystalline structure could change thus powder X-ray diffraction was used because it provide platform to observe the crystalline structure of HAP. Hydroxyapatite synthesis was observed under graph of Powder X-ray diffraction (XRD).There were basically three samples that had been observed namely sample A, B, and C. The results for each sample were recorded in Figure 4, 5 and 6. Based on XRD analysis obtained from Figure 4 for HAP powder sample A, the nanoparticles had the characteristic peaks of crystallinity at 22-23˚C, 26˚C, 28-30˚C, 32-34˚C, 40˚C, and 47-54˚C. While for XRD analysis obtained from Figure 5 for HAP powder sample B, the nanoparticles had the characteristic peaks of crystallinity at 22-23˚C, 26˚C, 28-30˚C, 32-34˚C, 40˚C, and 47-54˚C. XRD analysis obtained from figure 6 for HAP powder sample C indicates that the nanoparticles had the characteristic peaks of crystallinity at 22-23˚C, 26˚C, 28-30˚C, 32-34˚C, 40˚C, and 47-54˚C.

The three results that had been obtained from XRD analysis show that it is constant with the results from previous study and constant with the HAP phase of (ICDD 09-432). It is suggested that the reactions on HAP surface and the intrinsic properties of HAP and the crystalline structure, are maintained

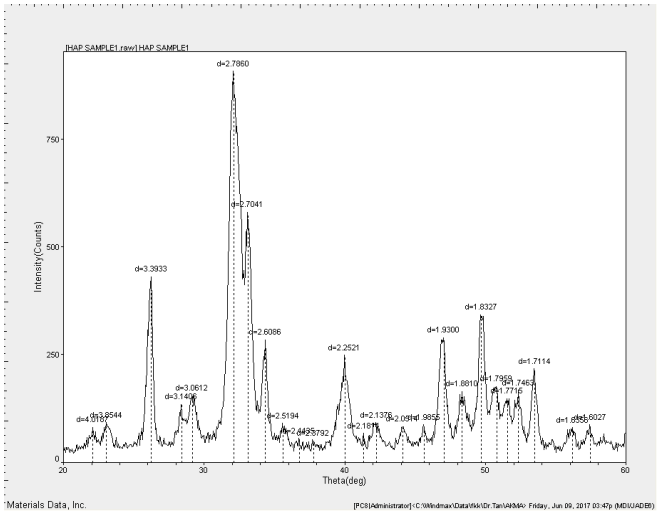

Figure 4: XRD analysis for HAP powder sample A

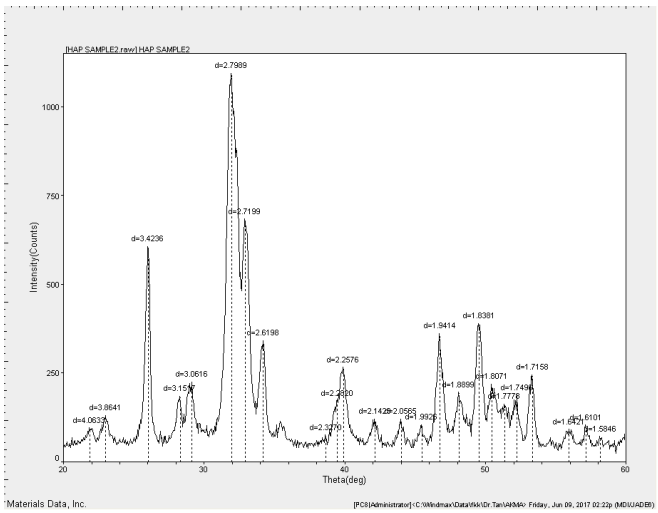

Figure 5: XRD analysis for HAP powder sample B.

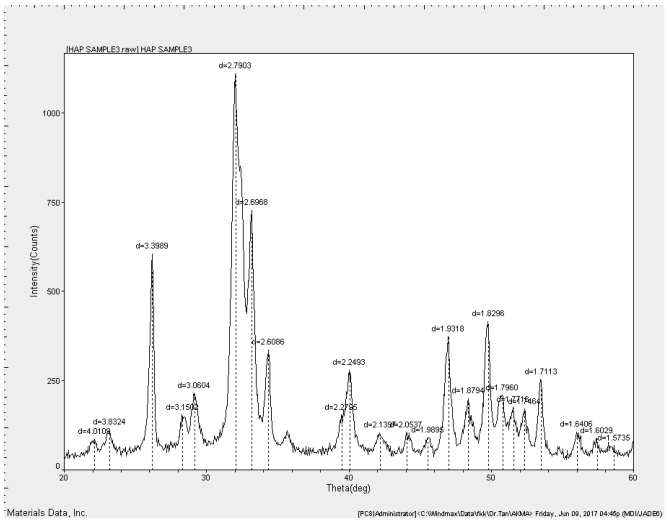

Figure 6: XRD analysis for HAP powder sample C

B. Characterization of saturated HAP

Recent research had been done that shows the important of free cationic precursors on the mineralization of HAP with other organic material such as phage. One of important free cationic precursors is calcium ions. This calcium ion help initiates the formation of bundle-like in the present of cationic precursors usually phosphate ion between HAP and organic material by electrostatic interaction. The reaction between anionic and cationic precursors causing accumulation within the bundle and contribute to the supersaturated of the local environment. The standard HAP ratio is 1.67 with pH 7 making the material more stable and less soluble. We then tested the idea of the research on the synthesized of supersaturated HAP following calcium/phosphorus (Ca/P) with ratio 0f ∼1.67 and the final concentration of calcium expected to be [Ca2+] = 4mM

Inductive coupling plasma was performed to monitor the [Ca2+] of the solution phase during the mineralization (Tao He et al). From the ICP analysis obtained, it is indicate that the sample 2 have the nearest calcium concentration with the expected result which are (4.17mM), follow by sample 4 (5.25Mm), sample 1 (3.4mM) and the least to be choose for mineralization is sample 3 (2.9Mm). Although the reasons why the value of calcium ion's concentration is differ from one sample to another is still not clear, we speculate that during the experiment, chemical reactions occur while adding hydrochloric acid and potassium hydroxide to stabilize the pH. The amount of potassium hydroxide added was differing.

Table 11: Analysis of inductive coupling plasma for supersaturated HAP liquid

	Name of sample	Mg/L	mM
UNK-002	Supersaturated HAP liquid 1	136.00	3.4
UNK-003	Supersaturated HAP liquid 2	167.28	4.17
UNK-004	Supersaturated HAP liquid 3	115.63	2.9
UNK-005	Supersaturated HAP liquid 4	210.53	5.25

To get a better understanding on the molecular level of supersaturated HAP, FTIR analysis was done on each sample. Figure 7, 8, 9 and 10 indicate the functional groups that present in the supersaturated HAP of sample 1, 2, 3 and 4. The chemical group present was constant which are adsorbed water, hydroxide ion and carbonate ion. It was mention earlier from previous research that in order for the HAP to form mineralization with other organic material, the present of anionic precursors is important such as phosphate ion but none of the sample shows the present of phosphate ion. The mineralization of HAP with peptide (FKFSFEFEFKFK) was expected to be failed. These situations occur probably because of the chemical reactions that happen between hydrochloric acid, water or potassium hydroxide during the supersaturated step of HAP. The molarity of hydrochloric performed was 50 while the advice molarity of hydrochloric acid is 10. The sample might also vaporize because it had been left for a few days before undergoes supersaturated step. The supersaturated HAP produce was in the form of liquid, the phosphate ion might accumulate at the bottom of the tube causing small amount of phosphate ion was taking out for FTIR analysis.

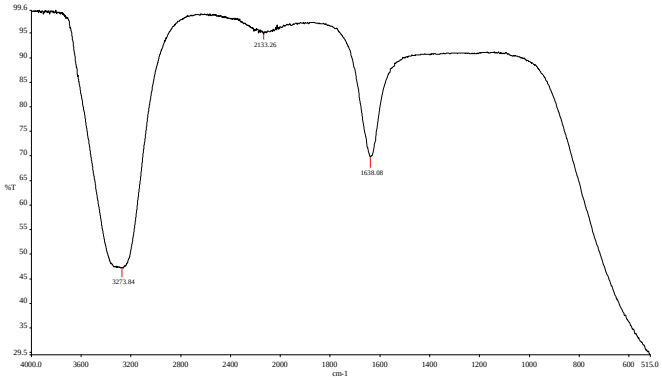

Figure 7: FTIR analysis for supersaturated HAP liquid sample 1

Table 7: FTIR analysis for supersaturated HAP liquid sample 1

Functional groups	Absorption bands (cm-1)	Description
Adsorbed water	3273.84	Under influences of thermal treatment (narrowed the absorption band)
Hydroxide ion	2133.26	Proves presence of hydroxide ion in the HAP
Carbonate ion	1638.08	Substitution phosphate ion

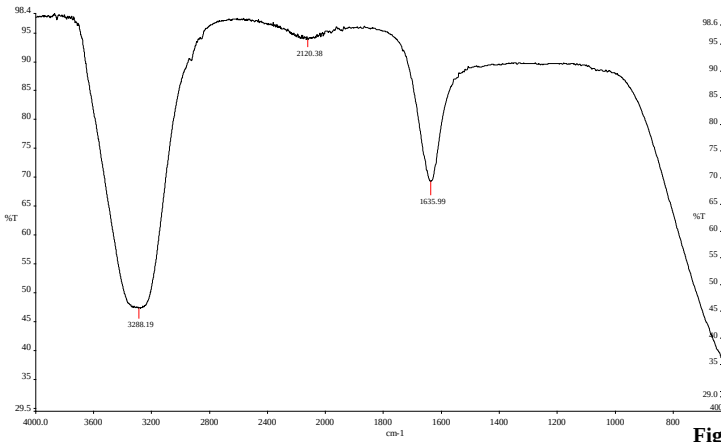

Figure 8: FTIR analysis for supersaturated HAP liquid sample 2

Table 8: FTIR analysis for supersaturated HAP liquid sample 2

Functional groups	Absorption bands (cm-1)	Description
Adsorbed water	3288.19	Under influences of thermal treatment (narrowed the absorption band)
Hydroxide ion	2120.38	Proves the presence of hydroxide ion in HAP
Carbonate ion	1635.99	Substitution of phosphate ion

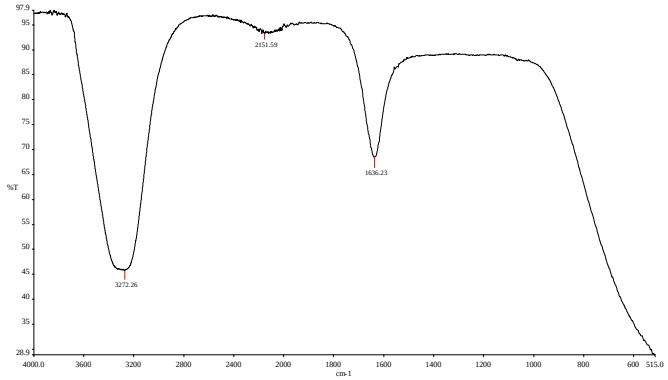

Figure 9: FTIR analysis for supersaturated HAP liquid sample 3

Table 9: FTIR analysis for supersaturated HAP liquid sample 3

Functional groups	Absorption bands (cm-1)	Description
Adsorbed water	3272.26	Under influences of thermal treatment (narrowed the absorption band)
Hydroxide ion	2151.59	Proves the presence of hydroxide ion in HAP
Carbonate ion	1636.23	Substitution of phosphate ion

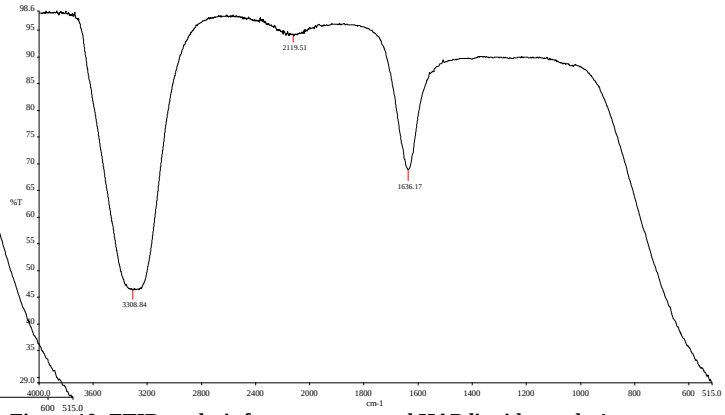

Figure 10: FTIR analysis for supersaturated HAP liquid sample 4

Table 10: FTIR analysis for supersaturated HAP liquid sample 4

Functional groups	Absorption bands (cm-1)	Description
Adsorbed water	3273.84	Under influences of thermal treatment (narrowed the absorption band)
Hydroxide ion	2133.26	Proves the presence of hydroxide ion in HAP
Carbonate ion	1638.08	Substitution of phosphate ion

C. Characterization of HAP with peptide (FKFSFEFEFKFK) under medium of distilled water and potassium chloride.

The supersaturated HAP from sample 2 was chosen to be mineralized with peptide (FKFSFEFEFKFK) because the concentration of calcium ion present in the supersaturated HAP was advice to be use which was 4mM. The mineralization of HAP with peptide was observed under microscope to see the detail morphology and the size distribution of itself and with different medium which were distilled water and potassium chloride. Comparing the mineralization, under potassium chloride, and under medium distilled water, it can be seen that the morphology and the size of bundle like form under distilled water is more clearly to be seen forming a bundle-like structure than the others. The mineralization of HAP with peptide without medium was seen to be self-assembly too forming accumulation but the bundle-like is not clear. Adding salt which is potassium chloride might destroy the peptide protein because of its acidic phase causing a not clear image of mineralization. Thus adding distilled water as a medium can be a very promising way to develop new hybrid structure perhaps for bio-engineering field as well as tissue engineering.

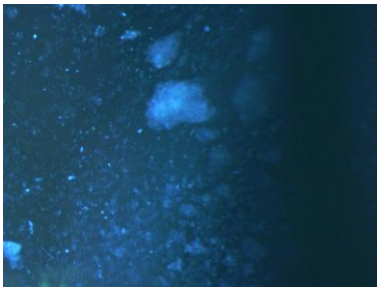

Figure 11: Mineralization of HAP with peptide (FKFSFEFEFKFK)

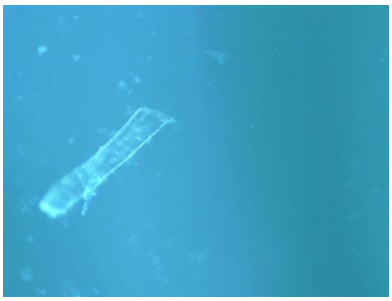

Figure 12: Mineralization of HAP with peptide (FKFSFEFEFKFK) on medium Distilled water

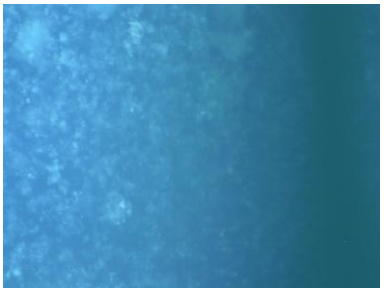

Figure 12: Mineralization of HAP with peptide (FKFSFEFEFKFK) on medium potassium chloride

IV. CONCLUSION

In summary, HAP nanoparticles and supersaturated HAP was synthesized. The effect on molecular level, the effect of chemical reaction and the concentration of calcium obtained in the HAP was study, analyzed and characterized. The mineralization of HAP with peptide (FKFSFEFEFKFK) was investigate and characterized on its biocompatibility and structure in different medium which are distilled water and potassium chloride. The HAP synthesis contain the most characterized chemical groups in the FTIR spectroscopy which are carbonate ion, phosphate ion, nitrate ion and hydroxide ion. HAP also shows constant with the HAP phase of (ICDD 09-432). The reactions on HAP surface, the intrinsic properties of HAP and the crystalline structure are maintained. The three samples from HAP powder synthesize can be used for supersaturated HAP. The result for molecular level of supersaturated HAP shows that the only chemical groups present were absorbed water, hydroxide ion and carbonate ion. The concentration of calcium ion was indicate by using inductive coupling plasma showing that sample 2 is the best to be chosen for mineralization. The mineralization under distilled water has better biocompatibility with HAP and peptide than in potassium chloride. These results gives a deeper understanding on the synthesis of HAP, the suitable ratio and concentration of calcium ion to be used and the best biocompatible medium to be used for various applications especially in bio-engineering and bio-medical field.

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