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

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A B S T R A C T

These research was conducted to study synthesize of the hydroxyapatite (HAP) and characterize the nanoparticles structures and to synthesize the nanofibrous hybrid structure formed through self- assembled peptide (FKFSFEFEFKFK) with HAP as inorganic material. At the same time, these research was conducted to characterize and analysis the morphology of nanofibrous hybrid structure formed through self-assembled peptide (FKFSFEFEFKFK) with water and sodium perchlorate. Furthermore, the method of these research divided into two section; first, the preparation of HAP and concentrated HAP with peptides and second section is characteristic of HAP and HAP with peptide (FKFSFEFEFKFK). Simple precipitation method is used to prepare HAP and the preparation of HAP with peptide (FKFSFEFEFKFK) prepared by using sodium perchlorate salt and water. By using several equipment such as Scanning geology microscope, Fourier Transform Infrared Spectroscopy (FT-IR), Inductive Coupling Plasma (ICP) and Powder X-ray Diffraction (XRD); the nanoparticle can be characterized. By conducting the research, we can better understand the effects of surface charge on cellular uptake and biocompatibility of HAP nanoparticles. Moreover, the mechanical properties of HAP and the surface area of HAP by combination with self-assemble peptide (FKFSFEFEFKFK) can be improved by further study about the HAP nanoparticles

Keywords:

Nanofibrous, Hydroxyapatide, peptides, FKFSFEFEFKFK, self-assembled, Mineralization.

1. INTRODUCTION

Hydroxyapatite (HAp, Ca5 (PO4)3(OH) 2) is the main inorganic constituent with calcium phosphate ceramic mineral present in vertebrate bones and teeth.Through research on HAP nanofibrous in detail can help to improve the mechanical properties of HAP and the surface area of HAP by combination with self-assemble peptide. The morphology of the nano-fibrous formed through self- assembled peptide in water and sodium perchlorate salt. Molecular self-assembly is process molecules are spontaneous organization of peptides into nanostructures such as vesicles, nanotubes, nanofibers, β-sheets and helical ribbons by using processes driven by free-energy that include, hydrogen bonding, electrostatic, Van der Waals, and π-π stacking interactions. Next, this research paper concentrating on characterization of HAP and HAP with peptides by using equipment of Scanning Electron Microscope (SEM), Fourier Transform Infrared Spectroscopy (FT-IR), X-ray diffraction (XRD), and Inductive Coupling Plasma (ICP) to study the behaviour of the nanoparticles formed.

The control over the bio functionality and structure of self-assembled peptide must be increased which can improve their application on vaccine and drug delivery. These peptides are basic building blocks for targeted drug-delivery carriers. One of the important structure as building block peptide is helical peptide. The hydrogen bonding between backbone amides which form right-handed-helices will result in helical structure with a periodicity of 3.6 residue spectrum (Perez 2014). The side chains of the amino acids involved need to interact with other helices, as they protrude outwards from the helix. Beta-sheet is another structure consist of alternating sequence of hydrophilic and hydrophobic amino acids, providing the peptide backbone an amphiphilic property that directs formation of beta-sheets(Habibi, Kamaly et al. 2016). Moreover, the formation of beta-hairpins in proteins will form when the orientation of two beta-sheets in anti-parallel directions. A beta–hairbin structure proposed by Shneideretal, where it design with sequence of VKVKVKVKVDPPTKVKVKV. Another structure of self-assembled peptides is using an amino acid pairing peptide (AAPP) strategy. This model used combinations of amino acid pairings that self-assembled including hydrogen bonds, ionic bonds, weak interactions and hydrophobic interactions. Cyclic peptides is result from self-assemble nanotubes which adopt flat conformations or stack via hydrogen bonding. The side chains amino acid of the peptide ring are oriented outward.

Bottom-up is method used to produce nano-sized self-assembled peptides which derivatives according to their building blocks.(Habibi, Kamaly et al. 2016) Molecular self-assembly is process molecules are spontaneous organization of peptides into nanostructures such as vesicles, nanotubes, nanofibers, β-sheets and helical ribbons. Peptide amphiphiles (PAs) another self-assemble method which have ability as novel biomaterials for regenerative medicine become most noticeable peptides over past decade. There are four domain of a class of Pas has been developed by Stupp

laboratory. Domain I usually from an unbranched alkyl group, domain II consist of peptide sequence forming b-sheet, domain III consist of one to three charged amino acids to impart aqueous solubility and domain IV using a bioactive signaling epitope that can be recognize by nanofiber structure. **(**Matson, Zha et al. 2011). Another type of self-assembling method was designed by using molecule without a designated alkyl tail domain (Matson, Zha et al. 2011). A series of self-complementary with 16-residue peptides have uncharged and charge residue alternatively develop by Zhang and the idea basically from the yeast protein zuotin. Self-assembled hydrogel through solid-phase methodology, the material properties can be change by small amino-acid sequence. In the preparation of biological scaffolds, individual molecules used for self- assembly makes it possible to determine the properties of the larger material (Habibi, Kamaly et al. 2016)

Gene delivery is one of the HAP nanofibrous application where antibiotic drugs were entrapped into the apatite hybrid materials made by template regulated precipitation which is often porous and the drug can deliver in control manner. The HAP nanoparticles can combine with several genetic materials and protein which used in loading gene and protein. Next application is delivering drugs to the central nervous system. The main challenge of peptides delivered to the CNS can be overcome by modification made and increasing the stability of the drug in the plasma and promoting endocytosis transportation across the blood–brain barrier. Intra-Ocular drug delivery is restricted by efflux pumps and many static (different layers of sclera, a, and retina, including blood-retinal barriers and blood-aqueous) and dynamic (lymphatic clearance, conjunctival blood flow, choroidal, and dilution by tears) barrier. In cardiovascular drug delivery, growth factors (GFs), other proteins and hormones can enhance the survival and promote myocardial regeneration. Moreover, by using bone drug delivery, transferring drug to bone tissue for the disease like, osteosarcoma or osteoporosis is very challenging because of the complex mineralized nano and micro structure of bone. The function of nanoparticles used is for delivery of anticancer drugs as they can reduce undesirable side effects and can increase the efficacy of treatment.

2. METHODOLOGY

The method used to synthesis HAP and hydroxyapatite with peptide (FKFSFEFEFKFK) were described. The research on method was separated into two sections. Firstly, preparation of HAP and HAP with peptides. Second section is characteristic of HAP and HAP with peptide.

2.1. Materials

The material used in this research are $Ca(NO_3)_2.4H_2O$, $NaH_2PO_4.2H_2O$, deionized water, hydrochloric acid, ammonium Hydroxide solution (28%-30% NH_3H_2O), calcium, sodium perchlorate, peptide, phosphorus and potassium hydroxide.

2.2 Preparation of HAP

The synthesis of HAP nanoparticles will prepare 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 will be stirred vigorously and heated to about 85°C. Next, the concentrated ammonium hydroxide solution with concentration about 28% - 30% need to be added to the solution and this will induce the nanoparticles precipitation. This experiment conducted at 85°C for 24 hour as to ensure that the complete conversion of the starting material to hydroxyapatite. Then, this mixture will cooled to the room temperature and the material was settled in the container. The excess liquid was decanted off as the fresh deionized water will added and the mixture will stir concisely before allowing the solid to settle and decanting once again. Lastly, the dilution and decanting process will repeated until the pH of the mixture was below 9.

2.3 Preparation of supersaturated HAP

The preparation of supersaturated hydroxyapatite (HAP) solution started from step dissolving HAP powder with ratio of (calcium and phosphate of 1.67) , in solution containing 100 mM of hydrochloric acid and had final concentration of 50 Mm of calcium. Then, 40 mL from stock solution will pipetted into the clean polythene container and by adding the distilled water, the volume of solution increase to 450mL. The pH was adjusted to 7.01 by adding 0.5 M potassium hydroxide. Next, the sodium perchlorate need to be added until reach the final concentration which is 200 mM and the final volume is adjusted to 500 mL with distilled water.

2.3 Preparation of Mineralization of supersaturated HAP with FKFSFEFEFKFK peptides.

The sodium perchlorate will be added until reach the final concentration which is 200mM and the final volume increase by adding distilled water to 500mL. The mineralization process of supersaturated HAP and peptide done by mixing both peptide and supersaturated HAP with volume of 20 µL and 200 µL respectively in glass tube. Next, this solution will be incubated in the incubator at 36.7°C in order to vaporize the solvent water and the mixture will centrifuged, (Liang Chen 2011). The solid materials obtain will be re-suspended in water and to remove the soluble salts, the particles need to be rinsed. Finally, the nanoparticle characterized by using SEM.

2.4 Characterization of HAP

2.4.1 Fourier Transform Infrared Spectroscopy (FT-IR)

FT-IR is to identify the existence of surface group and it record all sample of HAP. FT-IR was used to characterise the functional groups of untreated and surface modified HAp nanoparticles. For each spectrum, 16 scans between the wavenumbers of 4000 and 400 cm^{-1} were recorded in the transmission mode by a potassium bromide method.

2.4.2 Powder X-ray Diffraction (XRD)

XRD was performed within a 2θ with range of 20° till 60° on as Scintag Pad V x-ray diffractometer with Cu Kα radiation about 1.54 Å and Ni filter. Besides, the scans of bulk powders were run at 40 kV and 35 mA.

2.4.3 Fourier Transform Infrared Spectroscopy (FT-IR).

HAP- peptide was freeze dried after 72 hour incubation and then was mixed with KBr as to do the characterization by FT-IR.

2.4.4 Inductive Coupling Plasma (ICP)

ICP was used to detecting mom-metal and metals at concentrations as low as one part in 10^{15}.

2.4.5 Geology Microscope

A geology microscope uses a system of multiple lenses to provide magnification. A typical compound microscope will include a viewing lens that magnifies an object 10 times, and four secondary lenses that magnify an object 4, 10,20 and 40 times.

3. RESULTS AND DISCUSSION

The result obtain by analysis of HAP, supersaturated HAP and mineralization where self-assembled peptide (FKFSFEFEFKFK) combine with supersaturated HAP.

3.1 Analysis on FT-IR

FT-IR spectroscopy is an effective tool to identify the absorption bands related to the functional groups of PO_4 and OH, as well as the characteristics of HAp and its precursors employed during synthesis.

Table 1: FT-IR absorption bands of synthesized HAP functional groups

Chemical groups	Absorption bands (cm^{-1})	Description
CO_3^{2-}	870-1650	Substitutes phosphate ion, B-type HAp is formed
OH^-	630-3570	OH^- ions prove presence of HAp
Adsorbed water	2600-3600	Under influence of thermal treatment, absorption band becomes narrower
HPO_4^{2-}	875-880	Characterizes HAp with deficient of calcium.
PO_4^{3-}	960	V1: Phosphate decomposed
	460	V2: Phosphate decomposed.
	1000-1040	V3: Indicates that under influence of temperature, phosphate decompose
	555-602	V4: Phosphate decomposed.
NO_3^-	820 and 1380	Synthesis residue that disappears during the calcifying process

By referring to FT-IR absorption bands table, chemical group of absorption band from graph can be obtain and the most common chemical groups in FT-IR spectrum of HAP are PO_4^{3-}, CO_3^{2-} and OH.

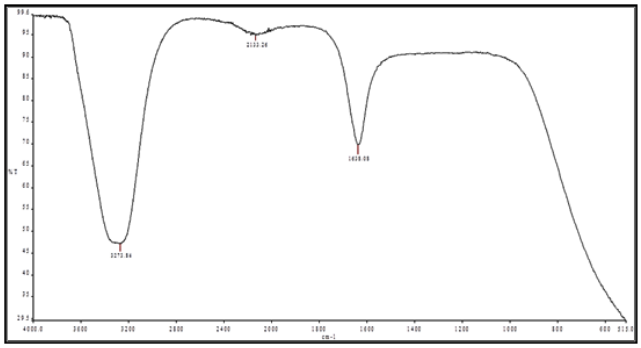

Figure 1: FTIR absorption band liquid concentrated HAP from Sample 1

Table 1: liquid concentrated HAP from sample 1

Wavenumber cm^{-1}	Functional group
3273.84	Adsorbed water
2133.26	OH^-
1638.08	CO_3^{2-}

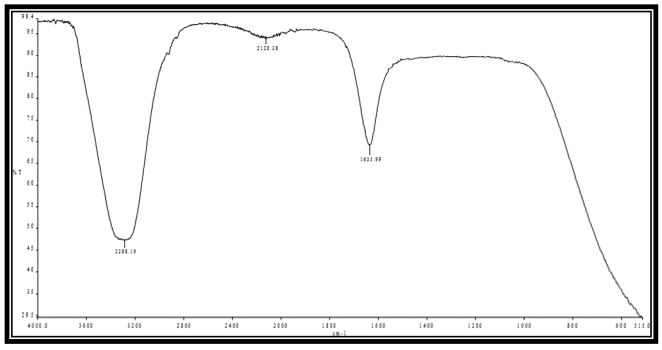

Figure 2: FTIR absorption band liquid concentrated HAP from sample 2

Table 2: liquid concentrated HAP from sample 2

Wavenumber cm^{-1}	Functional group
3288.19	Adsorbed water
2120.38	OH^-
1635.99	CO_3^{2-}

Analysis performed by FTIR spectroscopy allowed to determine the functional group present in the supersaturated HAP solution. The absorption band from Table 1 at about 3273.84 cm^{-1} and absorption band from table 2 at 3288.19 cm^{-1} represents the adsorbed water from sample 1 and sample 2 respectively. The band at 2133.26 cm^- from sample 1 and band at 2120.38 cm^{-1} from sample 2 is characterized as the bending mode of OH^-. The broad absorption band from 1638.08 cm^{-1} and 1635.99 cm^{-1} indicates the existence of the bending mode of carbonate group in the supersaturated HAP structure.

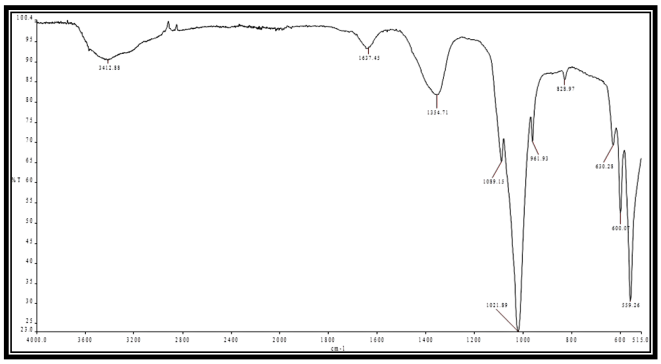

Figure 3: FTIR absorption band powder HAP from sample 1

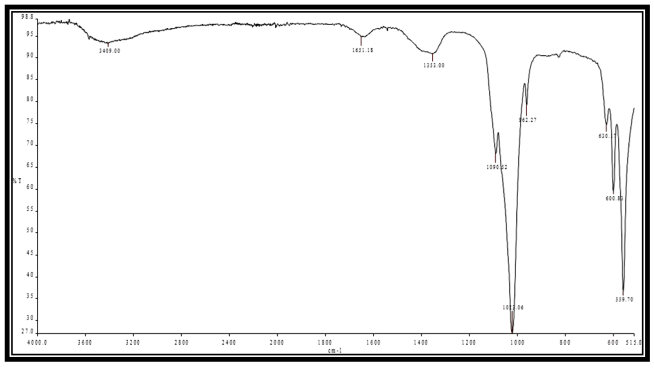

Figure 4: FTIR absorption band powder HAP from sample 2

Table 3: HAP powder from sample 1

Wavenumber cm^{-1}	Functional group
3412.88	Adsorbed water
1637.45	CO_3^{2-}
1354.71	CO_3^{2-}
1089.15	PO_4^{3-}
1021.89	PO_4^{3-}
961.93	PO_4^{3-}
828.97	NO_3^-
630.28	OH^-
600.07	PO_4^{3-}
559.26	PO_4^{3-}

The analysis of HAP powder was conducted to investigate the present of functional group on molecular level. The infrared bands of untreated HAP are shown in figure 3 and 4 with sample 1 and 2 respectively. From table 3, the bands at 1089.5, 1021.89, 961.93, 600.07 and 559.26 cm^{-1} are characterized as the bending mode of phosphate. The band at 1637.45 and 1354.71 shows the carbonate group in the HAP structure. The adsorbed water present in the band of 3412.88. From table 3 nitrate group also present with band of 828.97.

Table 6: HAP powder from sample 2

Wavenumber cm^{-1}	Functional group
3409.00	Adsorbed water
1651.18	CO_3^{2-}
1353.00	CO_3^{2-}
1090.52	PO_4^{3-}
1023.06	PO_4^{3-}
962.27	PO_4^{3-}
630.17	OH^-
600.83	PO_4^{3-}
559.70	PO_4^{3-}

From figure 4, the present of phosphate functional group identified at bands 1090.52, 1023.06, 962.27, 600.83 and 559.70 cm^{-1}. The infrared bands at 1651.18 and 1353.00cm^{-1} represent the carbonate group and band at 630.17 cm^{-1} represent the hydroxyl group. 3409 cm^{-1} represent the bending mode of adsorbed water.

3.2 Analysis on ICP

UNK-001	std 2ppm	2.0731	---	0.0549	0.0030
UNK-002	sample 1	136.00	---	0.0730	0.0032
UNK-003	sample 2	167.28	---	0.0905	0.0040
UNK-004	sample 3	115.63	---	0.0616	0.0022
UNK-005	sample 4	210.53	---	0.1147	0.0051

Figure 5: calcium ion concentration in supersaturated HAP

ICP is one of the equipment used to analyse the present of calcium ion concentration. The required concentration of calcium ion which is 4mM; controlled by ICP. In detail, ICP analysis is conducted to determine the amounts of Ca and P extracted by acid from HAP powder. The concentration of the resultant HAP-supersaturated solution is important in the preparation of supersaturated HAP. To obtain better result, 4 samples of supersaturated HAP are analyse to identify the concentration of the calcium ion. During mineralization, Inductive coupling plasma was performed to observe the [Ca2+] of the solution phase. From the ICP result obtain, it is shown that the sample 2 have the nearest calcium concentration with the expected result which are (4.17mM), next nearest value from sample 4 which is 5.25Mm, followed by sample 1 with 3.4mM, and at last from sample 3 with 2.9Mm concentration of calcium ion. The reason for different value of calcium concentration obtain was not clear. It may be because of the amount of potassium hydroxide added was different. Thus reaction occur between hydrochloric acid and potassium hydroxide to stabilize the pH produce different concentration of calcium ion. The suitable temperature was 70℃ and pressure favoured was 9.5atm for synthesis HAP powder with Ca/P ratio close to 1.67.

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3.3 Analysis on XRD

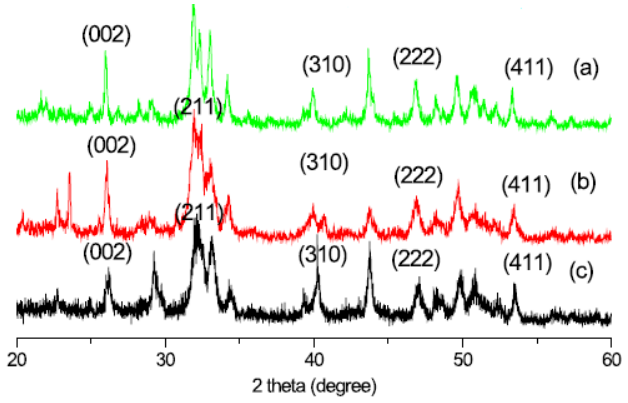

Figure 6: XRD pattern of (a) is main diffraction peaks for sample A, (b) is main diffraction peaks for sample B and (c) is main diffraction peaks for sample A.

One of the important properties observe in hydroxyapatite is the crystalline structure of HAP. Thus XRD is used to analyse the crystalline nature of HAP nanostructure and phase purity at different temperature. There were three samples that had been observed namely sample A, B, and C and the result can be seen in figure 6.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). The main diffraction peaks for sample A,B and C present at 2Θ regions of 26˚, 29 ˚, 32 ˚-34 ˚, 40 ˚, 47 ˚-54˚, which are consistent with the HAP phase(ICDD 09-432). It is suggested that the reactions on HAP surface and the intrinsic properties of HAP and the crystalline structure, are maintained. The result obtain from XRD analysis is the expected result and it is constant with the results from previous study.

3.4 Analysis on Geology Microscope

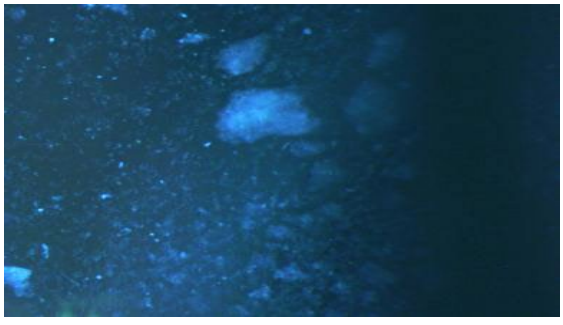

Figure 7: Mineralization of HAP with peptide (FKFSFEFEFKFK)

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 in sample 2 is closed to 4mM. The mineralization of HAP with peptide was observed under geology microscope to see the detail morphology and the size distribution. The bundle-like structure was observed from mineralization of HAP with peptide (FKFSFEFEFKFK).

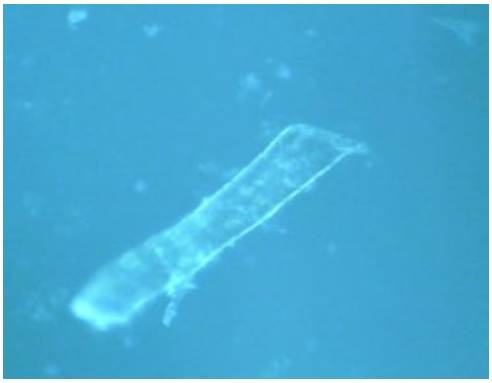

Figure 8: HAP nanoparticles in distilled water medium.

The mineralization of supersaturated HAP with FKFSFEFEFKFK peptide which form nanoparticles, analysed by using geology microscope. The distilled water medium was used during mineralization of HAP nanoparticles and the result shown in figure 8. The morphology of nanoparticle were observed and figure 8 clearly show a rod shape of particles and smooth surface morphology.

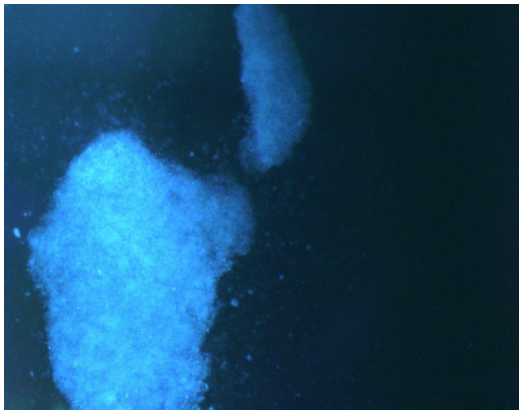

Figure 9: HAP nanoparticle in sodium perchlorate salt medium.

The result from figure 9 shows the mineralization of supersaturated HAP with FKFSFEFEFKFK peptide which form nanoparticles in sodium perchlorate medium. The result obtain was not in good agreement with the expected shape. These may be because of the alkaline medium which induced the nucleation of the calcium phosphate phase over the hydroxyapatite phase. Due to the existence of a number of phosphate compounds, this calcium phosphate system indicate as the most complex material.

Conclusion

The method of the research which divided into three section successfully conducted where first method is the preparation of HAP and second method to produce supersaturated HAP and third method is mineralization of supersaturated HAP with peptide (FKFSFEFEFKFK). By using several equipment such as Scanning geology microscope, Fourier Transform Infrared Spectroscopy (FT-IR), Inductive Coupling Plasma (ICP) and Powder X-ray Diffraction (XRD); the nanoparticle can be characterized. By conducting these research, the effect of surface charge on cellular uptake and biocompatibility of HAP nanoparticles can understand better. 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.

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