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RESEARCH ARTICLE

MICROWAVE ASSISTED STUDY ON BIO MATERIAL NANO HYDROXYAPATITE CRYSTAL (*HELIX POMATIA*) IN SIMULATED BODY FLUID

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ABSTRACT

A inexpensively low-cost Nano hydroxyapatite (Ca_{10} $(PO_4)_6$ $(OH)_2$ was synthesized from the biomaterial such as land snail-shell *(Helix Pomatia)* using di-ammonium hydrogen phosphate $(NH_4)_2HPO_4$ under microwave irradiation. The grained powder of land–snail shell was dissolved in diluted hydrochloric acid and a solution di-ammonium hydrogen phosphate was slowly added to the mixture while maintaining the pH at 9-10 using ammonium hydroxide(NH_4OH), followed by microwave irradiation for 30 min .The Nano hydroxyaptite (nHAP) after microwave irradiation was collected and calcined at 900° C for 2h, The synthesized HAp as nano crystal of 20-30 nm scale was soaked in simulated body fluid (SBF) for various period of time likely 7, 14 and 30 days respectively. After the particular period of soaking; the residue was collected, washed and dried at hot air oven. The powdered mass was characterized by XRD, FTIR, FE-SEM, and FE-TEM analysis. The prepared powder sample using land snail shell under microwave irradiation is hopeful source of carbonated hydroxyapatite (CHAP) with tremendous properties and can be considered for a better bone substitute material and the prepared HAP powder due to the formation of apatite on its surface was observed.

INTRODUCTION

The hydroxyapatite is one such material which is extremely used as bone substitute for the damaged bones; teeth and etc in the field of bio-medical applications. Chemically, they are made up of carbonate, small amount of magnesium and trace of other elements. As per the literatures review, the general methods which incorporate to prepare hydroxyapatites are precipitation, sol-gel, combustion synthesis and plasma etc (Brown P W *et al*., 1991, Young R.A *et al*., 1982, Arita I.H *et al*., 1995, Brendel T *et al*., 1992, Bartenfelder U.M.A *et al*., 1991, Roy D.M *et al*., 1974**).** However, conventionally they can be synthesized through hydrothermal, micro emulsion and mechano chemical process (Bezzi G *et al*., 2003, Anee Kuriakose T *et al*., 2004, Hattori H *et al*., 1990, Remant Bahadur KC *et al*., 2008, Muray M G *et al*., 1995, Lim G K *et al*., 1996, Kim W *et al*., 2000). Very recently the microwave coupled hydrothermal protocol offers an worth full route for synthesizing HAP with different morphologies have been reported (Yeong B *et al*., 2001). Interestingly, the synthesis of HAP employing biological waste is another valuable method because of its commerciality and environmental benignity. In addition, according to kokubo and takadama's experimental results, a bioactivity of HAP could be proved by immersing in simulated body fluid for allowing to access apatite formation. Aspired by the above work, we attempted and checked the biocompatibility of as synthesized HAP to be attributed to bone bonding species and to form *in-vivo* apatite arrangements (Sadat-Shojai M *et al*., 2010, Marc Bohner, Jacques Lemaitre *et al*., 2009, Kokubo T *et al*., 2006, Amir Abbas *et al*., 2014, Ivone Regina de oliveira *et al*., 2015).

MATERIALS AND METHODS

Synthesis of Hap

The skeletons of land-snails shells were collected and washed with tap water, followed by distilled water to get relieve of surplus deposits and muds. They were air and vacuum dried for 24h and crushed by pistol mortar to obtain powdered mass of 200 mesh size particles. The stoichiometric amount of land snail-shell powder was dissolved in dilute hydrochloric acid and the mixture was added to a solution of di-ammonium hydrogen phosphate at 9-10 P^H using aqueous ammonia. The resulting mixture was stirred for 30 min and immediately transferred to a domestic microwave oven and irradiated at 800W energy of frequency employing 2.45 GHZ for 45 min continuously. After the irradiation, the residue was washed thrice with de-ionized water and then dried in a air oven at 60°

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C for 2h (Murugan R *et al*., 2003, Nedunchezhian G *et al*., 2015). The pictorial representation of HAP synthesis method is shown in fig.(1)

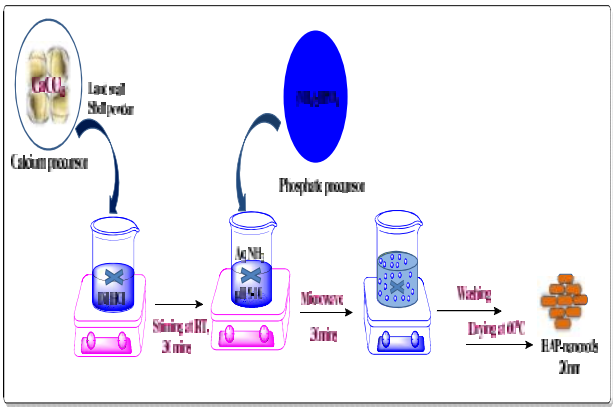

Figure 1 Synthesis Method of HAP

Table 1 Ion Concentration (mm) of SBF and Human Blood Plasma

ION	SIMULATED BODY FLUID	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^-	148.8	103.0
HCO^{3-}	4.2	27.0
HPO_4^{2-}	1.0	1.0
SO_4^{2-}	0.5	0.5

Table 2 chemicals were used in the preparation of SBF solution with De-ionized water

ORDER	REAGENT	AMOUNT (IN GM PER LITER).
1	NaCl	7.996
2	$NaHCO_3$	0.350
3	KCl	0.224
4	$Na_2HPO_42H_2O$	0.228
5	$MgCl_26H_2O$	0.305
6	1M-HCl (About 90% of HCl to be added)	40 Ml
7	$CaCl_22H_2O$	0.278
8	Na_2SO_4	0.071
9	$(CH_2OH)_3CNH_2$	6.057

Evaluation and Measurement

The equipped sample was studied for their phase purity with X-ray powder diffraction (XRD-877) using Cu-kα radiation. The FTIR spectra were obtained to identify the functional groups using a spectrum RXI Perkin Elmer. The morphological studies were supported using by FE-SEM (JEOL JSM 6701-F, USA) and FE-TEM (JEOL 2100 F, Japan).

Synthesis of Simulated Body Fluid (Sbf)

A metastable buffer solution is known as SBF, it contain calcium and phosphate ions formerly supersaturated with respect to the apatite. Therefore SBF is prepared as follows, and even a little, undesired variance in both of the preparation steps and storage temperature may significantly involve the phase purity and high temperature stability of the equipped HAP Powder, as well as the preparation of kinetics of the precipitation method.

Merck-grade NaCl (99.5%), $NaHCO_3$ (99.5%), KCl (99.0%), $Na_2HPO_42H_2O$ (99.5%), $MgCl_26H_2O$ (99.0%), $CaCl_22H_2O$ (99.0%), Na_2SO_4 (99.5%), $(CH_2OH)_3CNH_2$ (99.5%) and HCl were used in the preparation of SBF for this study. Put 750 ml of de-ionized water in to a 1000 ml of beaker (polyethylene beaker).Stir the water and keep its temperature at 37°C with magnetic stirrer with heater. Add each chemical given in the table 2 in to water until # 8, one by one in the order given in the table 2, after each reagent was entirely dissolved. Add #9 should be little by little with less than about 1g, in order to avoid local increase in P^H of the solution. put the electrode of the P^H meter in the solution measure its p^H value while the temperature at 37°C. If we add 1M-HCl solution with pipette to adjust the p^H 7.40.Then add and adjust the total volume of the solution to 1000 ml and stored the solution at 5-10°C in the refrigerator (Kokubo T *et al*., 1990).

X-Ray Diffraction Analysis

The X-Ray diffraction (XRD) analysis of the sample was done by X-Ray diffractometer using Cu-kα radiation. The XRD spectra of calcinated HAP and the sample which immersed in SBF are shown in fig (2). From the spectra, the strongest intensity peaks observed at 25.84°, 31.87°, 32.95° and 34.02°.representing [(002), (211), (300),(202)] plane. The prepared HAP shows the characteristic peaks with poor crystalline. In order to improve the crystallinity, the HAP is heated to 900°C for 2 hours in a furnace then the furnace cooled. After the heat treatment these HAP were soaked in SBF for various periods like 7, 14 and 30 days respectively, the result shows well crystallized pattern with sharp peaks. The originated peak position is in good agreement with standard JCPDS card No. 09-0432, and JCPDS Card No-89-6437, considered to calculate the grain size is estimated including HAP, using the Scherer formula

$D=k\lambda/\beta\cos\theta$. Where
$\beta=FWHM\times\pi/180$
$K=0.94$
$\lambda=1.5406$ Å

Table 3 Grain size of HAP Before and after soaked in SBF

S.no	Samples	Crystallite Size (nm)
1	HAP before soaked in SBF	16.76
2	HAP soaked in SBF 7 days	17.01
3	HAP soaked in SBF 14 days	25.95
4	HAP soaked in SBF 30 days	31.19

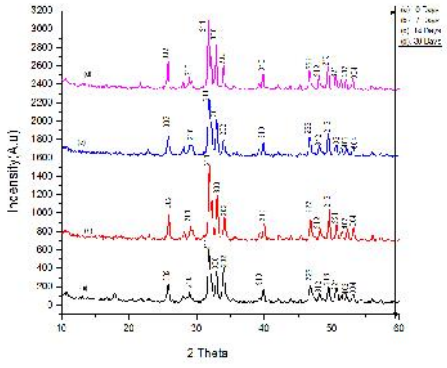

Figure 2 XRD –Analysis of hydroxyapatite powder after soaked in SBF (a). 0 Days (b). 7 Days (c). 14 Days (d). 30 Days.

The grain sizes were calculated 16-31 nm for the HAP and sample after immersed in SBF solution. These particles increased with increasing soaking times. The apatite on the surface of a material in SBF is useful for the *in –vivo* bone bio activity of the material (Bose S *et al*., 2010, Bianco A *et al*., 2009).

FTIR Studies

The FTIR spectrum of equipped and calcinated HAP after immersion of SBF solution shows the distinguishing peaks matching to stretching vibration of PO_4^{3-} ions at around 1041.69 cm^{-1} and the peaks at 570.06 cm^{-1} - 604.10 cm^{-1} are assigned to the deformation of PO_4^{3-} ions (Zyman Z *et al*., 2011). The broad OH- stretching band around 3643.05 cm^{-1} - 3402.31 cm^{-1} suggest the adsorption of H_2O molecules. A peak at 1406.26 cm^{-1} – 1575.28 cm^{-1} represents the existence of carbonate at trace level. The observed doublet at around 604.10 cm^{-1} – 570.06 cm^{-1} are established the development of apatite. these peaks are due to bending mode of P-O bonds in phosphate group , These peaks at 1406.26 cm^{-1} –1575.28 cm^{-1} and 760.45 cm^{-1} - 872.70 cm^{-1} are the stretching mode of CO_3^{2-} (Parhi P *et al*., 2004, Park Y *et al*., 2008). This may be weak bonding between carbon and oxygen in the current study.

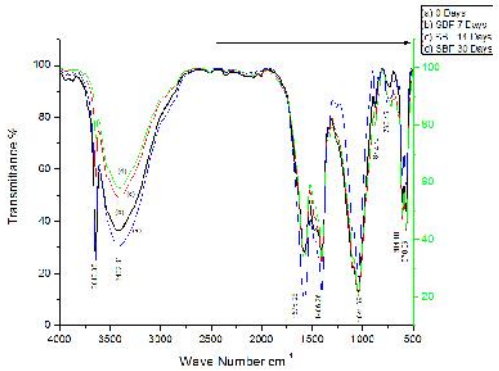

Figure 3 FTIR – Characteristics of hydroxyapatite powder soaked in SBF (a). 0 – Days (b). 7- Days (c). 14 – Days (d). 30 - Days

Sem Analysis

The morphologies of synthesized, calcinations of HAP powder after immersion of SBF are shown in the fig. (4). the synthesized calcinations powder have almost regular and sphere-shaped in structure with little agglomerated. The calcination process was a delaying effect on sintering at low temperature. At The temperature range of 900^oC, the grains are more rounded and the arrangement is reduced which agrees with values higher in respect to Hydroxyapatite and the occasion to recognize a former mechanism of Particle rearrangement. But at higher temperatures due to the decreasing of the probability of grain growth, the densification can be improved (Yoon S *et al*., 2005). After incubation of 7 days of the sample in to the SBF solution the surface morphology should be covered with little amount of calcium phosphate and have almost regular and cauliflower shaped structure. After incubation of 14 days the morphology of the surface strongly packed with increased calcium phosphate coating with regular and agglomerated. The immersion of 30 days in SBF solution the HAP surface should be increased with coating of calcium phosphate to form an appetite layer more closely packed. The EDS analysis of HAP immersed in SBF after 30 days shows the presents of Ca, P, and O .These major peaks were prove the escalation of calcium phosphate layer on the HAP surface (Ji-Ho Park *et al*., 2006, Zhao Xuhui *et al*., 2009, Pradnya N.Chavan *et al*., 2010, Farzadi A *et al*., 2011, Anjuvan singh *et al*., 2012, Megha P.Mahabole *et al*., 2012, Marcele Florencio Neres *et al*., 2013).

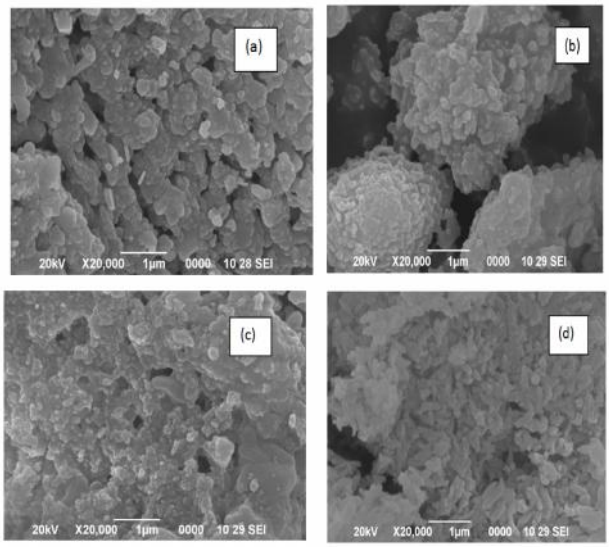

Figure 4 SEM Images of Hydroxyapatite Powders.
(a) HAP before soaked in SBF (b). HAP 7 - days soaked in SBF
(c). HAP 14 - days soaked in SBF (d).HAP 30 - days soaked in SBF.

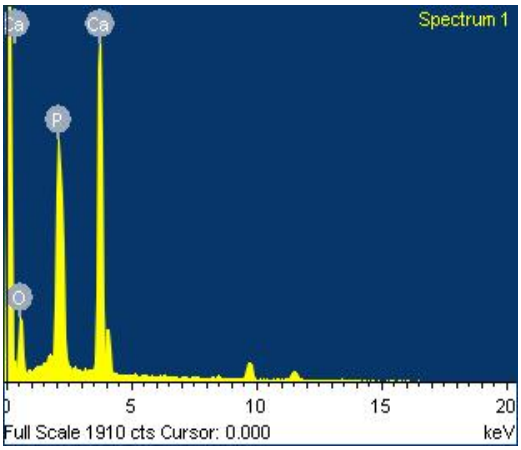

Figure 5 EDAX Spectrum of HAP Soaked in SBF after 30 days.

TEM Analysis

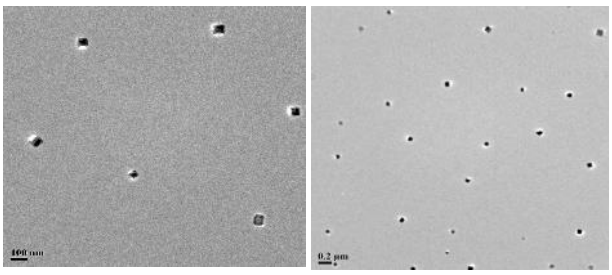

Figure 6 TEM Images of HAP Soaked in SBF after 30 days.

To further confirm the shape and size of the nano-HAP crystallite, they were investigated by TEM analysis. Fig.(6) demonstrates the micrograph of the as-synthesized calcinated HAP powder after incubated 30 days in SBF solution shows highly agglomerated and crystalite like structure to confirm the

surface coated with the formation of apatite layer and nano-HAP powder with average size of 100 nm; displays poor agglomeration of the individual particles with 20nm size of nano-HAP powder. From the micrograph, it has been observed that the particles were highly agglomerated as crystallite like shape and the size of particles were found to be 30 nm (Chandrasekar A *et al*., 2013, Enas Ismail., 2015). This result was more hopeful with the grain size calculated by Scherer formula.

CONCLUSIONS

Stoichiometric pure and thermally stable HAP powder was synthesized effectively using land snail shell and di-ammonium hydrogen phosphate under microwave irradiation, then the HAP powder was calcined to 900^{o}C and immersed for different period of time. The purity of the prepared HAP powder after soaked in simulated body fluid was verified by various methodical techniques having a brilliant Physico Chemical and *in-vitro* physiological properties. The HAP can be used as a bone substitution for filling bone deficiencies and as coating substance an orthopedic. And also the land snail shell initiate HAP is a possible Nano bio-ceramics which could be important for bio-medical applications.

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