RESEARCH ARTICLE

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Novel Green Chemistry Synthesis of Nano-Hydroxyapatite using Soya Milk as a Natural Stabiliser

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Present work describes a novel green chemistry synthesis of Nano – Hydroxyapatite (HAP) using Soya Milk (SM) as a Natural Stabiliser. The HAP is a biocompatible material and widely used in orthopaedic and dental applications. Nano-HAP powders are characterized using XRD, SEM, EDS and FTIR. These results show that the nano- HAP powders obtained by Green Chemistry Synthesis using Soya Milk (SM) as a Natural Stabiliser (NS) appear to be quite promising due to optimized pH and Ca/P ratio values that can be adapted for future research and development.

Introduction

Biomaterials belong to a multidisciplinary science. Rapid growth in development of nano technology has led to greater development in its synthesis. Hydroxyapatite (HAP) in general is the inorganic and hardest material of human body with a chemical formula of $\mathbf{Ca_{10}(PO_4)_6(OH)_2}$. Recent studies report that it has been widely used in biomedical applications like bone tissue repairs and bone replacement materials [**1**]. HAP is the main inorganic component used in orthopaedic, dental implant coatings on hard tissues and as filters for bone defects due to its similar attractive properties. It has excellent biocompatibility that mimics the behaviour of natural bones or teeth. Due to its bioactivity and bio – affinity, the HAP offers a strong interaction and attachment between the bone formation at the interfaces, by providing a local source of Ca^{2+} and $(PO_4)^{3-}$ions. These ions are very useful for mineralisation of bony tissues [**2**].

Nano particles of HAP exhibit much higher bioactivity than the micro size due to its unique properties. It provides much larger interface giving rise to higher bioactivity, biocompatibility and Osseo conductivity. Nano phase of the HAP represents promising class of orthopaedic and dental implants with improved Osseo integrative properties [**3**].

According to literature nano-HAP, compared to conventional ceramic formulations, has unique properties such as surface grain size, pore size, wet ability etc., that can control protein interactions for example adsorption, configuration and bioactivity by modulating subsequent osteoblast adhesion and long-term functionality [**4**].

Nano-HAP is currently used for several applications due to its functional response. It is used in marrow cavity in the process of implant re-sorption and bone substitution, as it can help for bone remodelling. It has a greater ability to tissue response in quicker implant surface turnover studied by Du *et. al.,* [**5**]. It is also important in many industrial applications, such as catalyst support, liquid-chromatographic columns, lighting materials powder carriers, chemical sensors, ion conductors, retardant of cancer cells and drug delivery agent etc. [**6-8**]. Nano-HAP promotes faster bone regeneration. It has been shown that it possesses desirable biocompatibility and bioactivity and has an ability to form direct bonding to regenerate bone without intermediate connective tissue [**9**].

In addition to this, it can be used to control physical properties in the bone implantation bed. Muller Mai *et. al.* [**9**] have tested these nano particles of the HAP and nano apatite or organic implant in Vivo. Bone itself is a composite consisting of nano-HAP with an average length ~ 40-60nm and a width of ~ 25nm embedded in a collagen matrix [**10**]. It has been observed that all the materials mentioned above are suitable for bone replacement and for drug release such as antibiotics, growth factors or other substances [**9,10**].

Tumours, trauma, congenital developmental deformities and severe inflammatory diseases often vary in degree of bone detects. Traumatic pain during the bone transplantation using nano-HAP has become one of the important methods of bone repair and replacement [**11,12**].

Recently, synthesis and characterization of nano - HAP powders have been carried out through green chemistry route using rice water (RcW) as natural stabiliser as a precursor [**13**]. It has been shown that the synthesised

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powders are poly dispersive and in nano range. It is known that the Soya Milk (SM) is a good source of both the calcium and phosphorous that have been reported for improved bone health. In addition, the selection of a particular natural stabiliser depends on its dispersive ability [**14**]. The effect of SM on the synthesis of nano - HAP is not reported. Therefore, it has been decided to use SM as a natural stabiliser to produce nano - HAP. Present work is thus related to the synthesis of nano -HAP through green chemistry synthesis. The synthesised powders are subsequently characterised using different techniques.

Experimental section

Preparation

Preparation of nano-HAP went through several developmental procedures. From past four decades a number of techniques have been developed to prepare hydroxyapatite. Some of these techniques are solid state, sol-gel processing, precipitation, hydrothermal reaction, microwave sintering, spray pyrolysis and emulsion processing [**15**]. However, the most convenient method of synthesis of nano-HAP has been found to be the wet-chemical precipitation method. The synthesis of nano - HAP has been carried out under normal temperature and pressure conditions. The HAP material in present study is synthesised using Yagai and Aoki approach as indicated by Buyer *et. al.*, [**14**].

$$10\ Ca(OH)_2 + 6\ H_3(PO_4) \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 18\ H_2O \quad (1)$$

Ammonium Hydroxide is used as a leaving agent or acid regulator in the above equation. It is a safe reagent that is used as an acid regulator in food and drug administration. Its pH controlling abilities make it an effective antimicrobial agent and aqueous ammonia is an excellent acid neutraliser.

$$10\ Ca(OH)_2 + 6\ H_3PO_4 + 20\ NH_4OH \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 20\ NH_3 + 38\ H_2O \quad (2)$$

Modified synthesis

In this paper, the synthesis of nano-HAP powders using SM as a Natural Stabiliser (NS) is reported at three different concentrations that are drawn at three different temperatures. As mentioned above, Soya or Soy is a rich source of protein and it contains all essential amino acids. It is used as a milk substitute in infant feeding, as an alternative to infant lactose intolerance. Drinking Soya Milk or Soy Milk boosts the Calcium and Iron levels in the human body [**16**]. It accelerates the consumption of B-complex, and is a rich source of vitamin B2, vitamin B12, that makes our bones stronger and builds sturdier teeth and gum [**17**]. Because of these abilities, it is used as a NS in the preparation of nano-HAP. Experimental results show that it affects enamel hardness in teeth [**16,17**].

Using the bio mimetic coating method, biologically active agents can be added to the supersaturated solutions and gradually be co-precipitated with calcium phosphate crystals forming a layer on the metal implants. This creates the possibility of uniformly distributed antibiotic layer or bio mimetic coating and releases it at a controlled rate by preventing local post-surgical infection [**17**]. SM is used as a NS as it is economically within the reach and is readily available.

It is known that the Soya Milk (SM) is a great source of calcium, the most important mineral of human body that is beneficial for bones and teeth. Humans need calcium to build and maintain strong bones and teeth as they constitute 99% of the body's calcium. Calcium promotes better bone health keeping them stronger and maintaining their structure intact hence reducing the risk of fractures. Consuming enough calcium can help to strengthen bones and reduce the risk of osteoporosis. Soya milk can also support our cardiovascular system as it is an excellent source of potassium which is deeply connected to lower blood pressure and irregular pulse. It can also help for healthy brain due to rich availability of omega -3 fatty acids [**12,18,19**]. Therefore, the SM has been used as a precursor.

Fresh soya is cleaned and soaked for 24 hours. Subsequently, the soaked grains are grounded and filtered to get SM. Homogeneous Precipitation technique plays a key role in modified synthesis. Addition of SM decreased the initial temperature and increased the reaction time. In other words, reaction temperature is inversely proportional to the concentration of NS and directly proportional to the rate of reaction. There is no specific rule for the concentration of NS that has to be added. So, in the present synthesis, 5mL, 10mL and 15mL of SM is used as an NS.

$$10\ Ca(OH)_2 + 6\ H_3PO_4 + 20\ NH_4OH + \times(SM) \rightarrow Ca_{10}(PO_4)_6(OH)_2 .\times(SM) + 20\ NH_3 + 38H_2O \quad (3)$$

Eq. (3) represents modified reaction of preparation of nano-HAP, where 'x' is the number of moles of SM used as NS. The schematic representation of synthesis of nano - HAP powders is shown in **Fig. 1**.

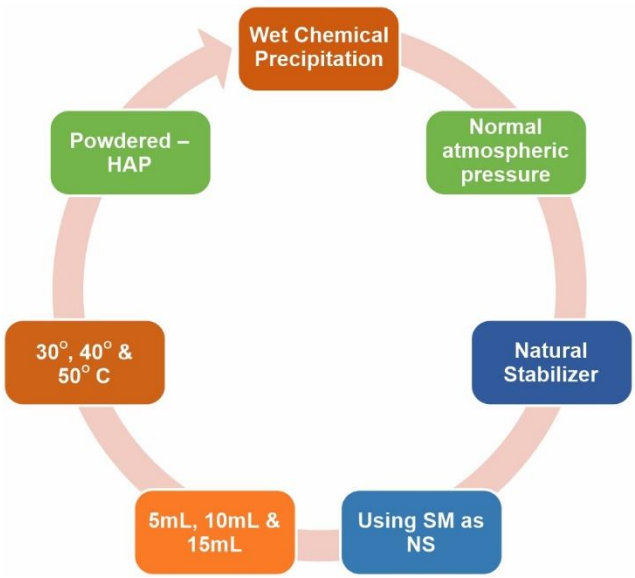

Fig. 1. Schematic representation of preparation of nano-HAP.

Material characterization

The powders obtained using equation (3) were subjected to X-ray diffraction (XRD) studies in INEL (France) equinox 3000 equipment attached with a copper source (Kα radiation) and position-sensitive detector (PSD). The XRD equipment was operated at 40 kV and 30 mA. The XRD patterns were used to measure the crystallite sizes of the powders [**13**].

Fourier transform infrared spectroscopy (FTIR) was studied in order to obtain the spectrum in an FTIR instrument (MODEL: IRPrestige21, cat. no. 206–73600-36, serial no. A21005002961LP, Shimadzu Corporation, Kyoto Japan) that can be operated at 220V/230/240V~ 50/60Hz [**13**].

The shape and sizes of the particles were examined in scanning electron microscope (SEM). SEM studies were carried out in ZEISS equipment, EVO 18 special edition [**13**].

Small angle X-ray scattering (SAXS) was used to measure the size, shape and distribution of the particles of powdered nano-HAP. The equipment model and make are Xeuss 1.0, and Xenocs, France. It is operated with duel energy Mo and Cr micro source, with camera length 2400 mm and q range: 0.024 to 14 per nm where q is the scattering wave vector [**13**].

Results and discussion

Effect of SM on pH

The pH is the main reason for which a biomaterial is identified to be the best material that can be used at its best. The HAP nano particles change the pH due to its solubility of different concentrations of NS as its Capping Agent (CA). It is mainly due to the strength of the bonding between the surfaces of NPs and one end of the Capping Agent. An addition of SM as a NS has increased the pH of the synthesised powder at different concentrations. During the process, the steric repulsion has reduced the extent of agglomeration. This in turn also varies pH values. It is observed that at 5 mL, 10mL, 15mL concentrations, the pH values are 9.9, 10.6 and 11.3, respectively (**Table 1**).

Table 1. Effect of NS on pH, Time and Temperature of nano-HAP synthesised at different concentrations.

Concentration of SM used as NS (mL)	pH	Time (minutes)	Temperature (ºC)
0	10.8	18	83
5	9.9	35	72
10	10.6	28	63
15	11.3	21	54

Present results (Table 2) reflect that there is a 0.7 increase for every 5mL increase of SM. Interestingly, the 5mL addition of SM decreases the pH value in comparison to that of the pure nano -HAP. The variation of pH values at different concentrations of NS is shown in **Fig. 2**. The Wet-Chemical precipitation method offers a molecular level of mixing the reactants such as Calcium and Phosphorous based NS. This influences its pH as well as Ca/P ratio. Apparently, it is known that the pH value of nano-HAP should be greater than 10.0 is optimum for biomedical applications [**15**].

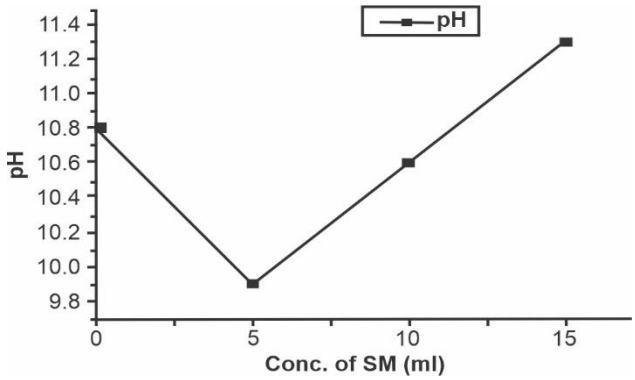

Fig. 2. pH of nano- HAP with 0 mL, 5 mL, 10 mL and 15 mL concentrations of SM as NS.

X-ray diffraction

The nano-HAP using the above discussed modification is characterised using powdered X-ray diffraction to know the structural analysis of the material. The XRD patterns are shown in **Fig. 3**. The XRD patterns of nano-HAP exhibit sharper peaks which indicate better crystallinity. The peak positions are in good agreement with the nano-HAP peaks of JCPDS, ASTM No. 09-0432 (2003). The XRD diffraction peaks obtained with relative d-spacing values at crystal planes (002), (211), (301), (222), (213), (004) of hexagonal system of HAP and other peaks are also matching with synthesised nano-HAP. The XRD results obtained here are in good agreement with the reported results of Buyer *et. al.*, 2000 [**21**].

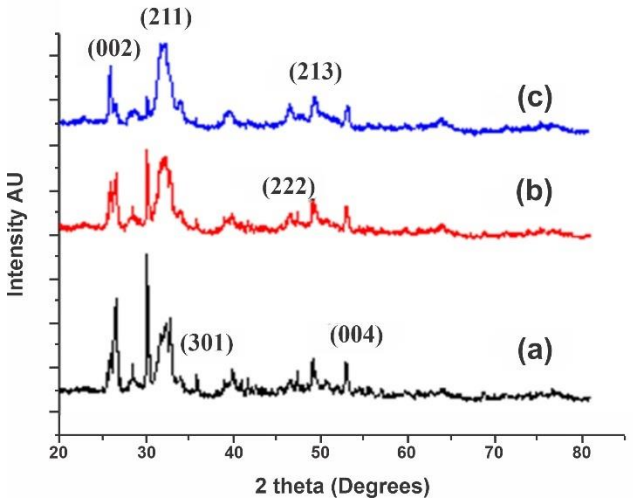

Fig. 3. XRD patterns of nano HAP synthesized at SM concentration: (a) 5 mL, (b) 10 mL and (c) 15 mL.

Nano-HAP synthesised with different concentrations of NS gives crystallites of different sizes. Natural Stabiliser is responsible for preventing uncountable growth of

particles, particle size and particle agglomeration and its solubility. The crystallite size of the synthesised powder can be calculated using Scherer's formula [**22**].

$$Dp = 0.94\lambda / \beta \cos\Theta \quad (4)$$

where, Dp = average crystallite size, λ = X-ray wavelength, β = FWHM of peak (line broadening in radians) and Θ = Bragg angle.

It is observed that the average crystallite size is 17.45 nm at 15mL SM as NS and its smallest crystallite size is 4.86 nm. The results of crystallite sizes are given in **Table 2**.

Table 2. Average crystallite size of nano-Hap at 5 mL, 10 mL, 15 mL concentrations studied at 30 ºC, 40 ºC and 50 ºC.

NS (SM)	Crystallite size (nm)						Ave. Crystallite size (nm)
	(002)	(211)	(301)	(222)	(213)	(004)	
0 mL	35.02	16.33	20.85	20.57	22.17	27.86	23.883
5 mL/30°C	19.41	39.58	30.06	38.55	32.58	30.00	31.696
10 mL/30°C	37.33	40.33	29.75	42.94	53.98	24.14	38.078
15 mL/30°C	31.76	---	29.16	40.10	17.96	23.48	28.492
5 mL/40°C	42.77	21.99	31.71	55.25	17.65	23.72	32.182
10 mL/40°C	20.34	---	36.18	13.86	17.87	24.74	22.598
15 mL/40°C	30.73	---	---	16.34	14.90	19.22	20.298
5 mL/50°C	43.87	18.54	32.53	15.44	20.69	24.53	25.933
10 mL/50°C	33.64	---	46.62	---	13.85	21.03	28.785
15 mL/50°C	28.95	4.86	19.99	14.88	15.45	20.57	17.450

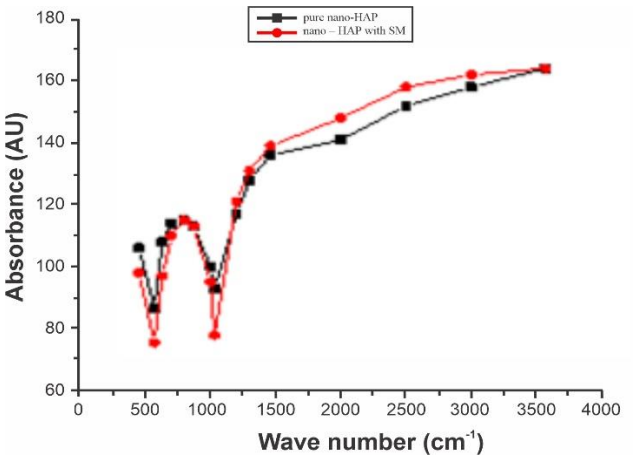

Fig. 4. FTIR Spectra: (a) 0 mL SM as NS and (b) 15 mL Conc. of SM as NS of nano – HAP.

Fourier Transform Infrared Radiation (FTIR)

FTIR spectra were obtained using an FTIR instrument that can be operated at 220V/230V/240V~ 50/60Hz. The FTIR spectroscopy analysis is used to identify the presence of hydroxyl group and phosphate groups. The results of nano-HAP synthesised using 15 mL concentration of SM as NS are displayed in **Fig. 4**. The observed functional groups are given in Table 3. The peaks at 3569 cm^{-1} and 630 cm^{-1} are characteristic of vibrations of Hydroxyl group confirming the diffraction evidence [**23-27**]. Peaks observed at 1030 cm^{-1} and 570 cm^{-1} are the characteristic vibrations of PO_4^{3-} [**24-25**]. The CO_3^{2-} bands are observed at 870 cm^{-1} and also at 1460 cm^{-1} [24-26]. It might be due to the adsorption of atmospheric carbon dioxide during the sample preparation. However, from XRD, it is observed that the presence of CO_3 groups does not influence the purity of nano-HAP [**25-27**]. There are anti-symmetric modes in the peaks because of variation in the absorbance factor of 0 mL nano-HAP and nano-HAP with 15 mL SM. This may be due to the influence of NS.

Table 3. Wave numbers and relative functional groups

Wave Number cm^{-1}	Functional Group
3569	OH^-
630	OH^-
870	CO_3^{2-}
1460	CO_3^{2-}
1030	PO_4^{3-}
570	PO_4^{3-}

Scanning Electron Microscope (SEM) Analysis

Morphological study of nano particles of synthesised Hydroxyapatite using SM as NS is studied using Scanning Electron Microscope (SEM). Crystal morphology and the sizes were analysed by scanning electron microscopic study. Micrographs of SEM of different nano-HAP samples prepared at different conditions are studied. **Fig. 5** displays the SEM images of the nano-HAP with 15mL concentration of SM solution used as a NS. SEM images of nano-HAP at 20 µm, 10 µm, 2 µm, 1µm and 200 nm magnifications are showing that the synthesised nano-HAP particles have different dimensions and are poly dispersive in nature. The image exhibits the rod shape structures of particles with dimensions of 215-240 nm and the smallest particle size of the nano-HAP is observed as 215.5 nm. The observed size of particle - 1, 2, 3 and 4 are 215.5nm, 222.6 nm, 222.6nm and 240.7 nm, respectively.

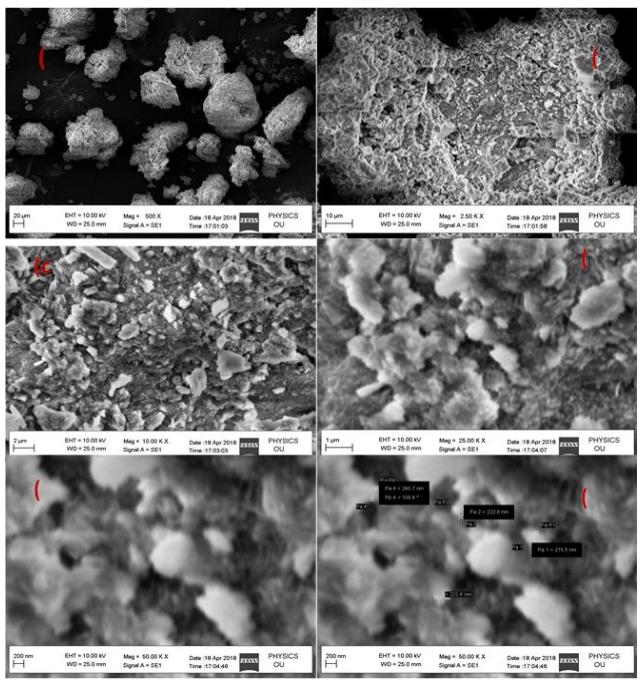

Fig. 5. Scanning Electron Micrographs of nano-HAP synthesised with 15mL SM concentration.

EDS

The EDS elemental analysis has been performed in order to define the elemental composition of synthesised nano-HAP samples. A representative SEM image along with EDS Spectrum of 15mL SM nano-HAP is displayed in **Fig. 6**.

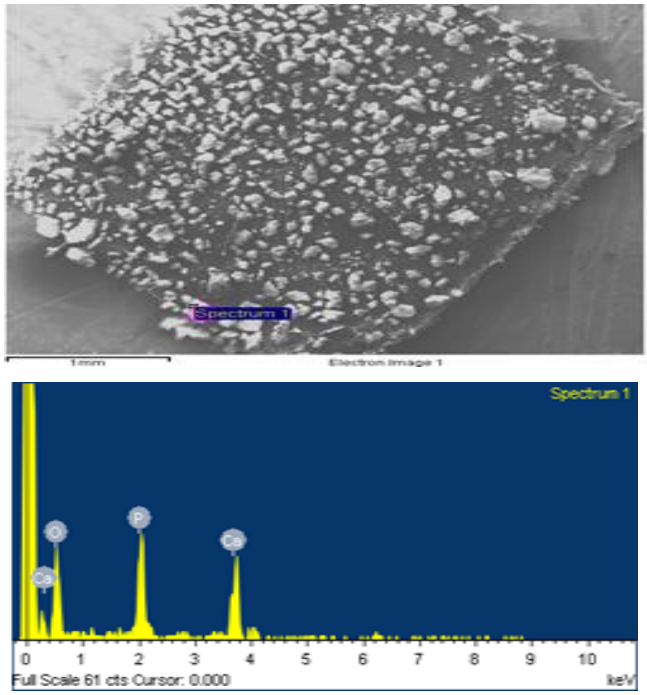

Fig. 6. SEM image and EDS Spectrum of 15mL SM nano-HAP.

The Ca/P stoichiometry of calcium phosphate nano particles strongly influence their performance under biological conditions that have not been totally examined or described till date. For this reason, the relation between Ca/P of nano to microparticulate of calcium phosphate substrates and their biological properties such as osteoblasts (bone-forming cells), viability and collagen production are still under the process of vigorous research. The nano-HAP requires correct calcium to phosphorous molar ratio in the final product. A number of Ca and P NS combinations are employed for wet-chemical precipitation synthesis. Thae Ca/P ratio of calcium phosphate synthesized with 15mL SM concentration as NS are given in **Table 4**.

Table 4. Ca/P of nano-HAP with SM 15mL Concentration.

Element	wt. %	atom%
Oxygen (O)	41.53	61.65
Phosphorous (P)	21.23	16.28
Calcium (Ca)	37.24	22.07
Ca/P wt.% = 1.75		
Ca/P atom% = 1.36		

The Ca/P of synthesised sample of 15mL SM taken at 50 °C is 1.75 by its wt.% whereas for the stoichiometric natural HAP, the Ca/P is 1.67. Morphological study using SEM images of the same sample shows the particle size is 215 nm, and its Ca/P is 1.75 by its wt.%. It is confirming the calcium concentration of synthesised nano-HAP has to be in between the ranges of 1.65-1.85. The Ca/P of synthesised nano-HAP of the same sample by atom % is 1.356. From literature, it is known that the Ca/P of calcium phosphate is very important that should be considered when selecting various dental and orthopaedic applications [**26-27**].

Small Angle X-ray Scattering (SAXS)

Small Angle X-Ray Scattering (SAXS) is an analytical technique giving access to information about the structure of materials at a Nano- and meso-scale. Measurements can be made on almost any sample but most often it is used for soft matter and nano-structural materials. SAXS is a primary characterisation tool for polymers, surfactants, colloids, proteins, porous media, Nano-particles and nano-composites. SAXS can be performed on samples in a dynamic or controlled environment. SAXS provides unequivocal determination of nano scale structural and morphological information and size determination of micro and nanoparticles with mean diameter ranging between a few nano meters and ~ 300 nano meters [**28**].

SAXS can provide mean size of a crystalline material. In this paper, the SAXS technique of the synthesized pure nano-HAP, nano-HAP synthesized using 5mL, 10mL, 15mL SM as Natural Stabiliser are studied. The particle size of pure nano-HAP for the volumes 5mL, 10mL, and 15mL SM used as NS and synthesised at 50°C are given in **Table 5**. The minimum particle size of 15mL NS is ~ 6 nm. The intensity vs. scattering wave vector is same in all the cases and corresponding volume distribution reveal that the particles are poly disperse in nature (**Fig. 7** and **Fig. 8**).

Table 5. Particle size of pure nano-HAP, SM 5 mL, 10 mL, and 15 mL as NS synthesised at 50 °C.

Type	S1	S2	S3	S4
0 mL NS	7.3	16.1	23.3	33.0
5 mL NS	4.5	10.6	14.5	22.0
10 mL NS	4.1	9.6	13.2	20.8
15 mL NS	5.8	12.0	18.1	29.6

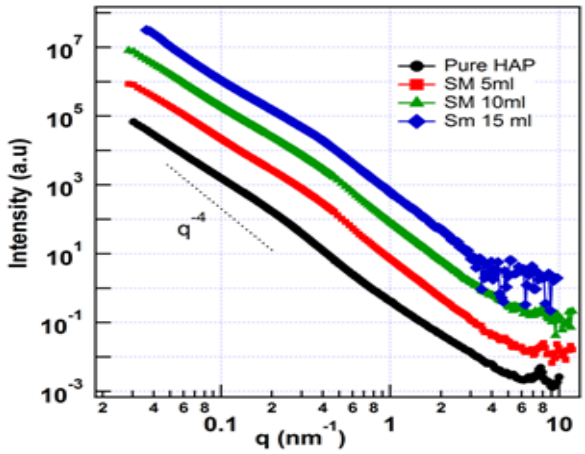

Fig. 7. Intensity vs. Scattering wave vector of pure-HAP, nano-HAP of 5 mL, 10 mL of 15 mL SM as NS.

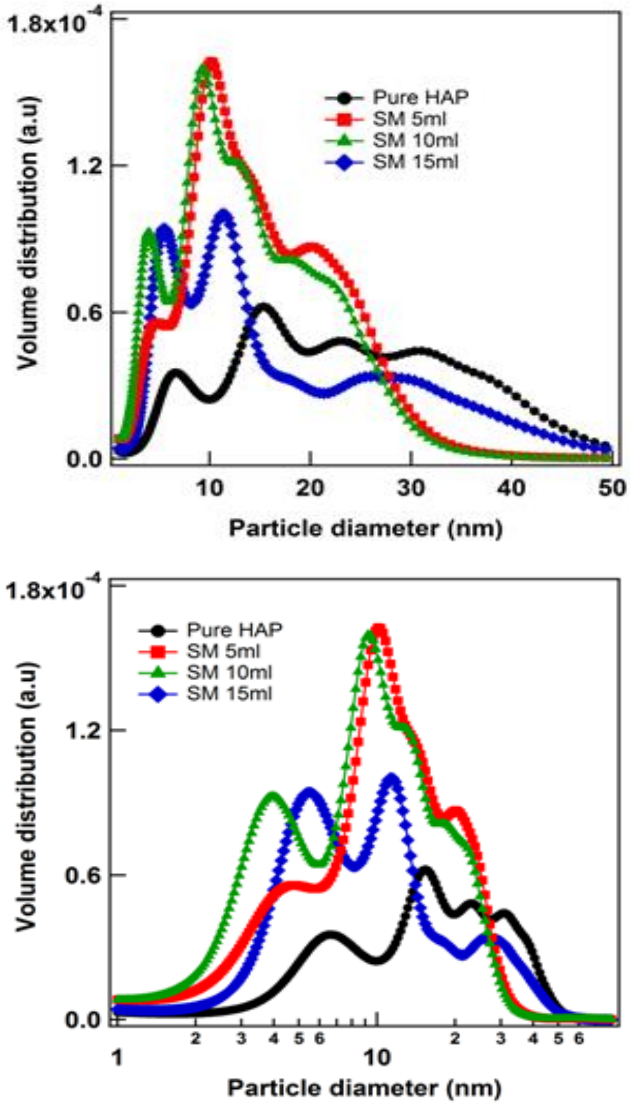

Fig. 8. SAXS image of particle diameter (nm) and volume distribution of pure-HAP, nano-HAP of 5 mL, 10 mL of 15 mL SM as NS on linear and log-log plots.

Conclusion

The synthesised nano HAP powder obtained by Green Chemistry Synthesis using Soya Milk as a Natural Stabiliser appears to be quite promising due to optimized pH and Ca/P ratios.

Acknowledgement

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Keywords

Novel green chemistry synthesis, nano-HAP, Natural Stabiliser (NS), biocompatible material, Soya Milk (SM).

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