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Synthesis and sintering of biomimetic hydroxyapatite nanoparticles for biomedical applications

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Abstract Synthesis of monodisperse nanoparticles with uniform morphology and narrow size distribution as achieved by nature is a challenge to materials scientists. Mimicking the process of biomineralization has led to the development of biomolecules mediated synthesis of nanoparticles that overcomes many of the problems associated with nanoparticle synthesis. Termed as biomimetics this paradigm shift in the philosophy of synthesis of materials is very advantageous for the design-based synthesis of nanoparticles. The effect of concentration of a protein named bovine serum albumin on particle size, morphology and degree of crystallinity of biomimetically synthesized hydroxyapatite particles, has been studied. Results establish 0.5% protein as the required concentration to produce 30–40 nm sized hydroxyapatite particles with an optimum degree of crystallinity as required for biomedical applications. These particles synthesized under certain stringent conditions are found to have stoichiometric calcium:phosphorus ratio of 1.67 and exhibit restricted grain growth during sintering.

1 Introduction

Calcium hydroxyapatite (HAp, $Ca_{10}(PO_4)_6(OH)_2$) the main inorganic constituent of the bones and teeth has been intensively researched in the last decade in order to produce third generation bone grafts and implants to replace the prevalent allograft and metallic supports such as dental filling in the form of powder, in bone in the form of sintered blocks etc. HAp is being used in several forms like powders, granules, sintered porous blocks and even as coatings over metallic implants. Granules and blocks of HAp are derived from powder and have major roles as space fillers, scaffolds and low load bearing implants in orthopedics. Nanosized HAp powder (size <100 nm) possessing uniform size and morphology has many applications in different fields of medicine ranging from targeted drug delivery to designed load-bearing implants [1–5]. Need for such intensive work on HAp is because, nowadays, requirements of third generation bioceramics are not just limited to biocompatibility; they also demand bioactivity and resorbability. Such functionality of HAp can however be achieved only by exerting a control over its morphological features during synthesis. In biomedical industries, the absence of effective micro-structural control in a co-precipitation process is the main limitation in producing functional HAp nano-particles at a commercial level. Synthesis of nanosized HAp powders from natural sources, though attractive, can never be a solution to industrial requirement [6]. The recent emergence of biomimetic technologies is definitely showing signs of providing a feasible solution to such problems [7–10]. However, a biomimetic route that uses only simulated body fluid (SBF) for synthesis of HAp powder lacks microstructural control because of the absence of biological macromolecules in SBF. In nature, biological macromolecules provide pre-organized matrices for controlled nucleation and oriented growth of HAp nanoparticles [11, 12]. These nanoparticles in a constrained environment then undergo self-assembly forming bioactive three-dimensional bones and teeth. Based on the concept of biomineralization, Sinha and co-workers have demonstrated the synthesis of nanosized HAp particles (30 nm–40 nm) using both synthetic and biopolymers as supramolecular matrices [13]. The present manuscript

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reports the detailed systematic studies carried out with nanosized HAp particles produced biomimetically using the globular protein bovine serum albumin (BSA). Among the different globular proteins, BSA is most commonly available and normally stable up to 78°C in the presence of a high ionic concentration [14]. The effect of protein concentration on both morphological features and crystallinity of HAp nanoparticles and also their structural, thermal, chemical characterization and sintering studies has been analyzed.

2 Materials and methods

In situ synthesis of nanosized and microporous HAp particles was carried out using BSA. Freshly prepared calcium nitrate tetrahydrate solution of strength 0.4 M was made alkaline using ammonia: water in the ratio 1:2. The pH of the solution was maintained between 9–11. Four different aqueous solutions of BSA (i.e., Fraction V powder, SRL, India), 0.05%, 0.1%, 0.25% and 0.5% was made in double distilled water and added to the calcium solution. Ca $(NO_3)_2$ and BSA of each of the above concentrations respectively was gently stirred to avoid denaturation of the protein, incubated at 75°C in a hot air oven for 24 h 0.156 M-diammonium hydrogen phosphate was made alkaline using ammonia: water in the ratio 1:1, pH between 9–11, and this was added gradually to the above-incubated mixture. Milky white coloration was observed almost instantaneously, which was allowed to age for 48 h at a temperature of 30 ± 2°C, after which it was decanted and washed thoroughly with de-ionized water. The slurry was then spray dried using an indigenous Spray Drier that was fabricated by S. M Scientech, Kolkata, India with a capacity of 1000–1200 ml slurry. The HAp powder was compacted uni-directionally at a load of 5 Ton and sintered at temperatures of 1000°C, 1200°C and 1300°C respectively. The HAp samples were structurally characterized using scanning electron microscopy (SEM, JSM-840 A, JEOL with an accelerating voltage of 15 KV, no coating was required as HAp is a piezoelectric material), transmission electron microscopy (TEM, CM 200, Philips with an accelerating voltage of 200 KV, the powder was dispersed in water, sonicated and a drop taken on a carbon coated copper grid and dried), X-ray diffractometry (XRD, PTS 3003, Seifert, targets Co Kα $\lambda = 1.7889$ Å, with 2θ in the range of 10–50°@ 0.5°/min and Cu Kα target, $\lambda = 1.5418$ Å, 2θ in the range of 10–70°@ 0.5°/min respectively) Differential Thermal Analyser (DTA, Seiko A320, heating rate 10°C/min with a temperature range from 30–1300°C) and Fourier transform infrared spectroscopy (FT-IR-410 JASCO, with wavenumbers in the range of 3700–500 cm^{-1}) techniques.

3 Results and discussion

Composite materials of nanosized HAp particles and organic polymers need to be studied because the bioactive apatite content in existing composites is not adequate. It is normally lower than 45% due to the large size of HAp particles, in comparison to 60% nanosized HAp content in natural bone and this restricts the bioactivity of the composite. In this paper, nano-HAp crystals are *in situ* synthesized in BSA matrices; very similar to the way Nature synthesizes inorganic crystals at normal temperature and pressure without any environmental hazards. The control achieved in size, shape and morphology of these particles is noteworthy. TEM studies carried out on all the four HAp samples synthesized using different protein concentrations revealed a systematic variation in the size of the particles (Fig. 1). 0.05% BSA concentration yielded highly anisotropic HAp particles of 1–1.5 μ in length and 20–25 nm width (Fig. 1a). Doubling the protein concentration to 0.1% reduced the aspect ratio to half the earlier one (Fig. 1b). Further increase of protein concentration to 0.25% reduced the aspect ratio by limiting the length of anisotropic HAp particles in the range of 250 nm–300 nm (Fig. 1c) and finally at 0.5% BSA concentration we obtained monodisperse HAp powder of 30 nm–40 nm with acicular morphology (Fig. 1d). The observed variation in the aspect ratio of the synthesized HAp particles as a function of protein concentration may be attributed to the modifying effects of BSA on crystallization of HAp [15–17]. The apatite crystals in natural hard tissues are formed as thin needles, with a size of 5–20 nm by 60 nm and over 100 nm long in enamel. The shape and size of the nano-HAp crystals synthesized by us are similar to the apatite crystals in natural bone and this similarity is the first step towards making a biomimetic composite. It is interesting to note that XRD-patterns of HAp particles synthesized in four different protein concentrations revealed an otherwise contradictory feature with respect to morphological studies (Fig. 2a). One may note that an increase in protein concentration from 0.05% to 0.5% has led to a systematic sharpening in the diffraction pattern with the appearance of discrete diffracting planes. HAp nano-particles synthesized with 0.05% BSA reveals only two planes (211) and (002), whereas the HAp particles synthesized with 0.5% BSA revealed the presence of all major peaks namely (211), (002), (112), and (202). It has been established that the presence of BSA modifies the kinetics of HAp precipitation and its crystallinity through a complex process that brings systematic changes in the XRD pattern [18] The comparative XRD, as reported by us also shows a reduction in particle size of HAp with increasing protein concentration. A better crystallinity of the precipitated HAp particles with increasing BSA concentration may be a result of more and more secondary bond forming possibility of the protein.

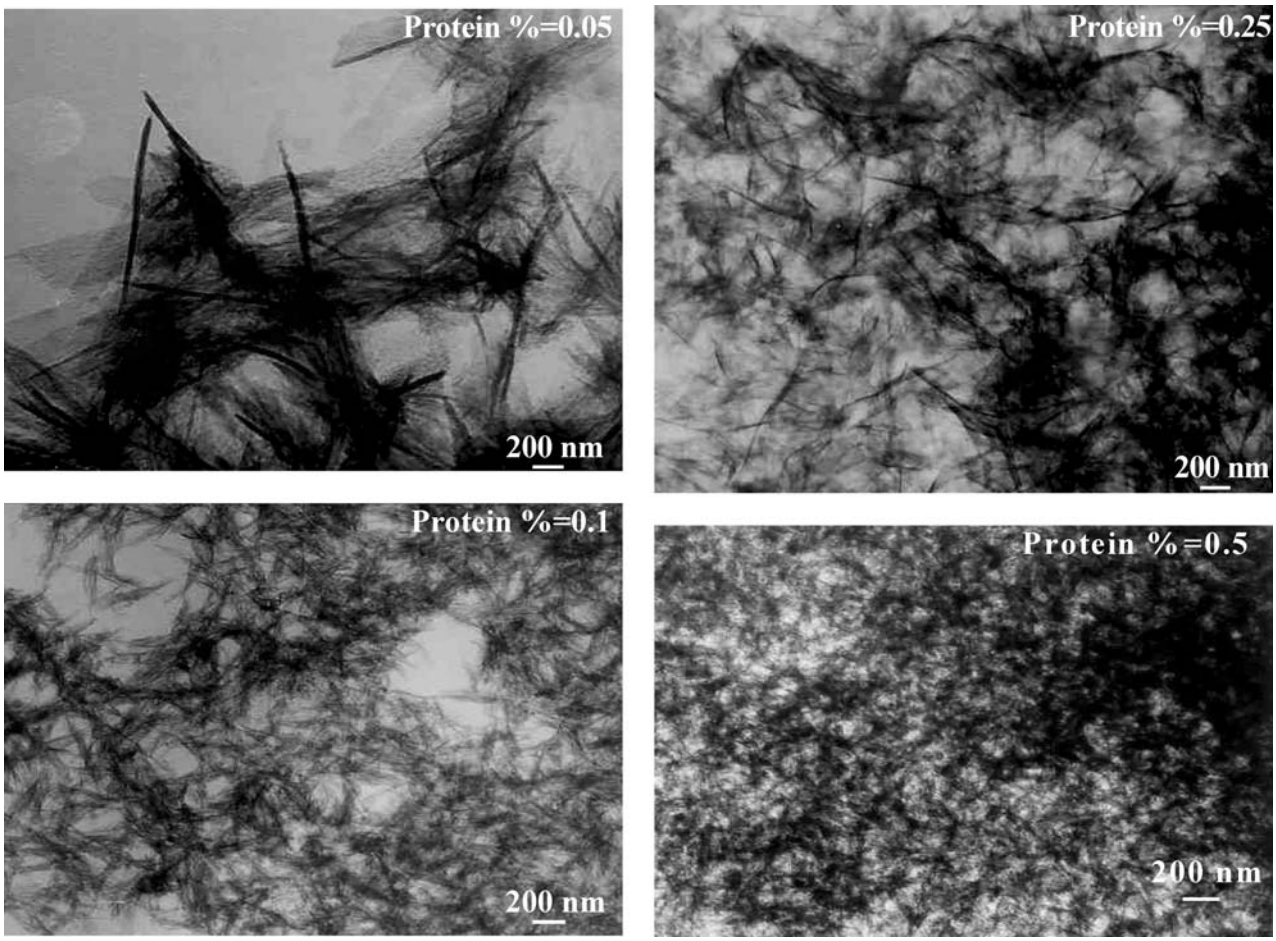

Fig. 1 TEM Bright field images of nanosized HAp particles synthesized in different protein concentrations; HAp particles in (a) 0.05% BSA, (b) 0.1% BSA, (c) 0.25% BSA and (d) 0.5% BSA.

A heat-treatment for three hours of the HAp powder produced using 0.5% BSA at temperatures 800, 1000 and 1250°C respectively enhanced the degree of crystallinity of the powder from 39.28 to 97.78% without indicating any signature of phase-decomposition (Fig. 2b). Average particle sizes of 25.5 nm, 45.75 nm and 54.25 nm were obtained by using Scherer formula: peak width of major peaks at half maxima. This confirms the inhibition of diffusion of atoms of as-precipitated HAp nanoparticles, hence the very narrow size distribution. It is interesting to note that the as-synthesized powder structurally matches with the HAp present in younger bones while the one heat-treated at 1250°C revealed the diffraction pattern akin to the mature bone mineral. The EDX analysis of the as-precipitated HAp powder with 0.5% BSA revealed Ca/P molar ratio ∼1.67, matching with that of bone mineral where as heat treated powder confirmed the deficiency of phosphate ions (Ca/P ∼2.124). This variation indicates a possibility of transformation of HAp into tri-calcium phosphate and calcium oxide. Thermal analysis of the precipitated powder on the other hand manifested a weight loss of 20% by a three-step process in the temperature zone below 600°C and above that no significant weight loss could be observed (Fig. 3). This behaviour may be attributed to the loss of adsorbed water on the surface of nano-particles as well as combustion of the protein. The third one is constituted from two stages, which may be attributed to the condensation of the HPO4 ions to pyro-phosphate ions.

The FTIR spectra of as-precipitated HAp particles displayed characteristic bands due to hydroxyl and phosphate groups (Fig. 4). The band at 3573 cm^{-1} is due to an OH stretching vibration confirming the presence of hydroxyl ion in the apatite lattice. Adsorbed water also displays a broad band in the range of 3700–3500 cm^{-1} and a relatively sharper band at 1630 cm^{-1}. These bands are always present in biological apatites thus establishing a close resemblance of the powder being studied here with the biological one. The band at 1385 cm^{-1} signifies the presence of amide group from the protein. The bands present at 1092 cm^{-1}, 1046 cm^{-1} and in the range of 631–560 cm^{-1} highlight the presence of orthophosphate ions in the apatite crystal lattice [19]. The density of the as precipitated and heat-treated powder was measured by typical Archimedes method; a density of 2.61 g/cc

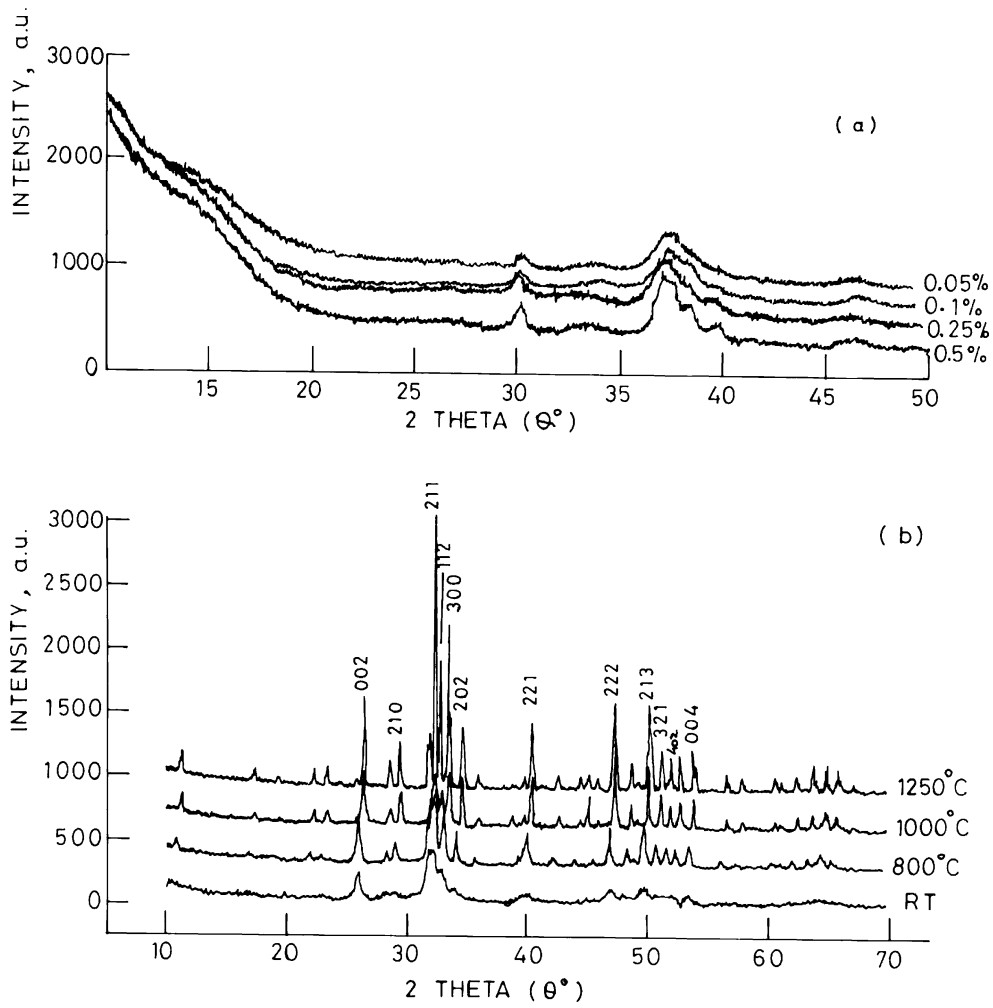

Fig. 2 X-ray diffraction pattern of (a) as-precipitated HAp nanopowder with varying protein concentration (b) enhancement of degree of crystallinity of nanosized Hap powder synthesized with 0.5% protein and subsequent heat-treatment at different temperatures.

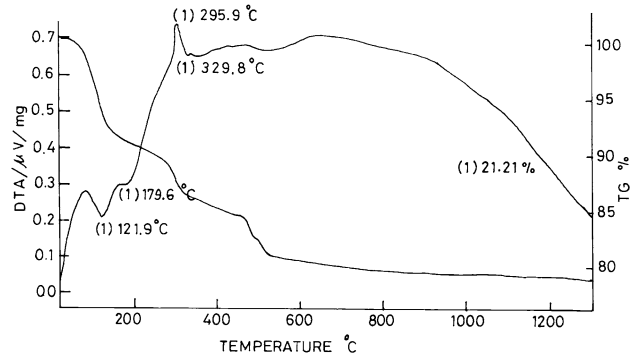

Fig. 3 Differential thermal and gravimetric analysis of nanosized HAp powder synthesized with 0.5% protein.

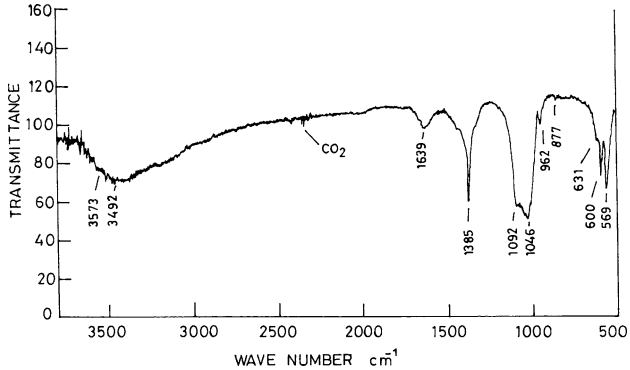

Fig. 4 FTIR spectra of synthesized HAp powder exhibiting characteristic bands of phosphate and hydroxyl groups.

of the precipitated HAp powder whereas a maximum density of 2.98 g/cc was obtained after heat-treating the powder at 600°C for 3 h. On the other hand, HAp powder during compaction and sintering exhibited a large variation in the density. The green compact revealed a density of 1.8 g/cc that reduced to as low as 1.5 g/cc at 285°C. However further increase in sintering temperature led to an increase in the bulk density and we could record the maximum density (~2.98 g/cc, 94% of theoretical density) at 1300°C. Initial drop in the density could be attributed to the removal of adsorbed water and the protein matrix. Sintering study of the synthesized powder showed the typical sintering behavior of ceramic powders.

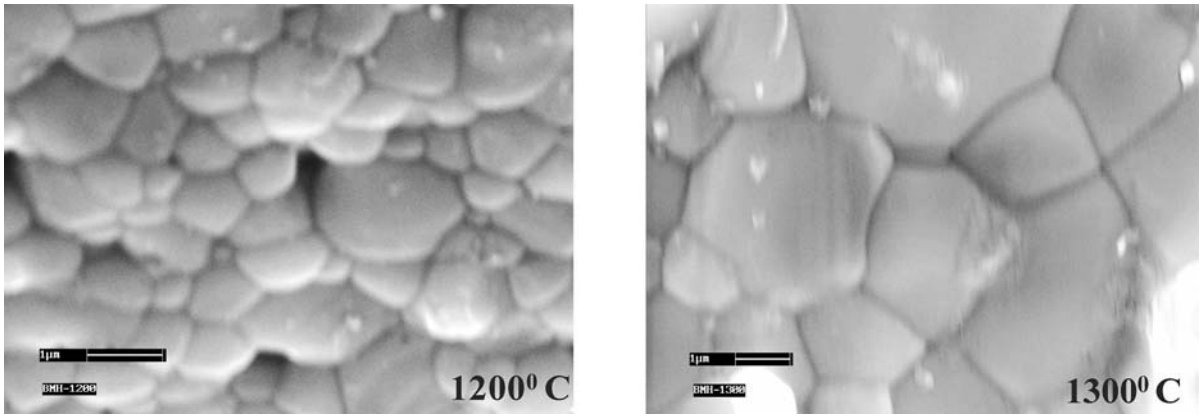

Fig. 5 SEM studies on sintered HAp (a) HAp nanopowder sintered at 1200°C and (b) sintered at 1300°C.

SEM studies of the sample sintered at 1200°C revealed average grain size in the range of 200–300 nm, one may even notice the presence of the particles less than 100 nm in size (Fig. 4a). Even 1200°C sintered product revealed porosity in the structure but at 1300°C there was no porosity, but abundance of HAp particles in the sub-micron size (Fig. 4b). The difficulties encountered in the sintering process may directly be correlated with a very narrow size distribution of the as-precipitated particles under going compaction and thus establishes the control of proteins on the morphological features of the synthesized nano-particles. An ideal ceramic powder should have maximum number of uniform sized particles and its sintered compacts should have well-defined pore size distribution and our result is very close to that from microstructural observation.

4 Conclusion

In order to make improved materials from natural polymers, much more knowledge of polymer structure/property relationships and control of morphology will be needed. However, biomimetics-using biomolecules sure seems the way ahead where biomedical applications of HAp are concerned. A single phase of HAp cannot meet the functional needs of bone implants because of brittleness and insufficient toughness. Since natural vertebrate bone is a nanocomposite material in which an assembly of Hap nanoparticles effectively reinforces collagen fibres, we have used a hybrid organic-inorganic approach, close to natural processes in developing bioaffinitive composites comprising BSA and HAp.

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