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Influence of sonication on the physicochemical and biological characteristics of selenium-substituted hydroxyapatites

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Although the material hydroxyapatite (HAP) has excellent porous, biocompatible, and biodegradable properties, its mechanical strength and microbial inhibition rate are not adequate for its direct use in bone tissue engineering or in constructing artificial teeth. To overcome some of its limitations, in the present study, we have formed an organic–inorganic composite with an altered internal structure *via* doping selenium (Se) cations into the lattice of HAP. We have synthesized Se-substituted HAP (Se-HAP) composites with different Se/P ratios (0.01, 0.05, and 0.1 M) *via* a wet chemical route in which two different sets of samples were collected (1) after only precipitation (referred to as the precipitation method) and (2) after precipitation followed by sonication (referred to as the sonochemical method). FTIR and Raman spectroscopic analyses confirmed the successful doping of Se into the HAP matrices, while powder XRD studies indicated their highly crystalline nature, which was significantly influenced by Se doping. The XRD data also showed that the Se-HAP particles formed by the precipitation method have a size of 56 nm and those formed by the sonochemical method have a size of 29 nm. Morphological analysis by means of SEM and TEM indicated that the sonochemical method produces well-defined rod-shaped particles, while the precipitation method produces particles with agglomerated structures. Hemolytic studies confirmed that the Se-HAP particles are biocompatible, and that the hemolytic ratio increases with the Se content. In addition, antibacterial studies indicated that Se-HAP responds quite well against a Gram-positive strain (*S. aureus*), on a par with the response to a Gram-negative strain (*P. aeruginosa*). Finally, *in vitro* cell viability and proliferation studies indicated an increase in the proliferation capacity of non-cancer cells (NIH-3T3 fibroblasts) and a considerable reduction in the viability of cancer cells (MG-63 osteosarcoma). Based on the overall analysis, the Se-HAP samples formed by the sonochemical approach could have potential for biomedical applications in bone cell repair, growth, and regeneration.

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1. Introduction

In recent years, there has been increasing demand for the research and development of novel orthopedic biomaterial implants with structure and properties, *viz.*, porosity, osseointegration, mechanical strength, thermal stability, and antibacterial ability, as similar as possible to those of natural bone. From this point of view, hydroxyapatite (HAP) has been found to be one of the most suitable naturally occurring biomaterials, as bone contains widely distributed apatite crystals (about 65–70% mass interspersed in the collagen) and HAP is biocompatible and biodegradable.^{1,2} Hence, taking the properties of HAP into consideration, this material has been employed in many different applications. However, it suffers from the limitation of poor mechanical strength (particularly the fatigue properties) and thus is not suitable for use in direct bulk form for high load-bearing

applications such as orthopedics and dental materials.^{3,4} Many different approaches are being followed to improve the mechanical resistance, thermal stability, and biocompatibility of HAP by replacing some of the Ca^{2+} ions in its crystal lattice with metal ions such as Ag, Ti, Mg, Zn, Mn, Se, Sr, *etc.*, in which the [metal]/[Ca] molar ratio has a significant influence on the fundamental properties.^{5–16} In addition, the synthesis of HAP *via* various routes, including precipitation, sonochemical, hydro-/solvothermal, solid-state, sol-gel, self-propagating combustion synthesis, and emulsion/micro-emulsion synthesis, can generate particles with different properties, such as size, shape, surface charge and functionality, thermal and mechanical strength, biocompatibility, biodegradability, *etc.*^{4–12} This tailoring of HAP's properties could make it a suitable implant material for the regeneration of bone tissues *via* increased osteogenesis or as a surface coating material to improve the bioactivity of implants.

To explore the behavior of HAP-based nanocomposites for bone imaging and regeneration purposes, various composites have been formed by the substitution of the HAP lattices with many different inorganic metals/metal oxides; for example, in a recent study, a HAP-MgO composite fabricated by the microwave irradiation technique was found to have greatly improved mechanical properties (compressive strength of 111.20 ± 5 MPa and fractural toughness of 136.98 ± 5 MJ m⁻³), hydrophilicity, and biocompatibility in terms of cell viability.⁶ In a similar study, a mixed composite of nano-HAP (99.5 wt%) and graphene nanoparticles (0.5 wt%) in three-dimensional form resulted in an exceptionally strong material (with a fracture toughness of ~ 116 MJ m⁻³, Young's modulus of ~ 98 GPa, and compressive strength of 96.04 MPa) with porosity, an interconnected morphology, and biocompatibility.⁷ Other metal-doped HAP complexes being developed include Ag-Ti coated HAP for enhanced stability and corrosion resistance,⁸ Zn-HAP for biocompatibility,⁹ TiO₂-HAP for increased cellular activity,¹⁰ Mg-HAP for improved scaffolding and porosity,^{6,11} Pt-Se-HAP for potent anticancer activity,¹² Sr-HAP for bioactivity and biocompatibility,¹³ Mn-HAP for improved stability and strength,¹⁴ and Se-HAP for biocompatibility, cell growth, and anticancer activity of bone marrow cells.^{15,16}

Among the many different metal-doped HAPs, we have been interested in Se (selenium)-doped HAPs, as this composite can help to treat osteosarcoma, a common type of bone cancer, in a sustainable way.^{15,16} In one study, toxicity testing of Se-HAP loaded with the antioxidant catechin in human osteosarcoma cells (MNNG/HOS cell line) found that it exhibited antitumor activity provided by the generation of reactive oxygen species (ROS) *via* a caspase-3-dependent pathway.¹⁷ This investigation seems to indicate potential for the treatment of bone cancers through the development of antitumor immunity and with minimal side effects to the stem cells, thereby reducing the probability of recurrence. Thus, taking advantage of the potential role of Se-HAP in the reduction of bone cancers, we synthesized an Se-HAP composite *via* a wet chemical route, in which two different types of samples were collected (after only precipitation and after precipitation followed by sonication). To investigate the influence of the Se content on the properties of HAP, three different Se-HAP composites were formed with varying concentrations of Se

(0.01, 0.05, and 0.1 M). The composites formed by the two different approaches were thoroughly characterized in terms of crystallinity, surface functionality, morphology, grain size, and shape. Following the physical characterization, biological testing for the investigation of biocompatibility, antibacterial activity, and *in vitro* cell viability was carried out.

2. Experimental

2.1 Materials

Calcium nitrate tetrahydrate ($\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$; 99%) (from SD Fine-Chem. Ltd), sodium selenite (Na_2SeO_3 ; >99%), ammonium dihydrogen phosphate ($(\text{NH}_4)_2\text{H}_2\text{PO}_4$; 99%) (from Merck Specialties Pvt. Ltd), liquor ammonia (NH_3 ; >25%) (from Qualigens Fine Chemicals), an antibiotic (amoxicillin), and MTT dye (from HiMedia™, India) were used in the experiments. Two different cell lines, MG-63 (osteosarcoma cell line) and NIH3T3 (fibroblast cell line), were obtained from NCCS, Pune, India. All the chemicals used were of the highest analytical grade and were used without any further purification.

2.2 Synthesis of HAP and Se-HAP

For the synthesis, we followed a chemical precipitation method in which two different sets of samples were collected (1) after only precipitation (referred to as the precipitation method), and (2) after precipitation followed by sonication (referred to as the sonochemical method). Both pure HAP and the Se-HAP formed by the precipitation method were subjected to ultrasonication for 60 min in the second step. The pure HAP was synthesized by dissolving 1 M calcium nitrate tetrahydrate and 0.6 M ammonium dihydrogen phosphate in water to make up to the 50 mL mark. Ammonium hydroxide solution was then added dropwise to the mixture to maintain the solution pH at 10.5, and this was followed by ultrasonication for 60 min to obtain a white-colored precipitate at the end. The obtained precipitate was separated by filtration and dried overnight at 100 °C, followed by sintering in a furnace at a heating rate of 5 °C min⁻¹ until the temperature reached 800 °C and being further maintained this highest temperature for another 2 h. The same procedure was adopted for the formation of Se-HAP, with the only difference being the addition of various concentrations of Na_2SeO_3 (*viz.* 0.01, 0.05, and 0.1 M) to the phosphate precursor. The pure HAP and Se-HAP samples with different stoichiometric concentrations of precursors and molar ratios are designated as 0Se-HAP (pure HAP), 0.01 M Se-HAP, 0.05 M Se-HAP, and 0.1 M Se-HAP. The Ca/P and Ca/(P + Se) molar ratios were maintained at 1.67.

2.3 Characterization studies

The powder X-ray diffraction (XRD) patterns of all the synthesized samples were obtained using a Rich Siefert 3000 diffractometer with Cu K α 1 radiation ($\lambda = 1.5406$ Å). The Raman spectra were recorded using a Raman-11, Nanophoton Corporation, Japan at a laser wavelength of 514 nm. Fourier transform infrared (FTIR) spectra were recorded using a Shimadzu FT-IR 8300 series instrument in the wavelength range of 4000–400 cm⁻¹. The X-ray

photoelectron spectroscopy (XPS) analyses were carried out in the ultra-high vacuum (UHV) chamber of a photoelectron spectrometer from Omicron Nanotechnology, Germany, equipped with a monochromatic X-ray source (Al K α radiation, $h\nu = 1486.6$ eV). For the XPS analyses, the binding energies were calibrated using carbon C 1s at 284.6 eV as a reference. The morphological analyses were carried out using a HITACHI SU6600 field emission-scanning electron microscope (FESEM). The elemental composition was accessed using energy-dispersive X-ray (EDX: X-Max, USA) spectra with a regular unit (Oxford Instruments, UK) connected to the FESEM instrument. The transmission electron microscopy (TEM) analysis was carried out using a FEI TECNAI G2 model T-30 at an accelerating voltage of 250 kV. For the measurement, about 0.01 g of the powdered sample was dissolved in ethanol using a sonicator bath for 2 min. A drop of the sonicated sample solution was then placed on a TEM grid, allowed to dry, and used for the TEM characterization.

2.4 Evaluation of bioactivity

The HAP and 0.1 M Se-HAP nanocomposites were pelletized using a die at 2 tons of weight, and the bioactivity test was performed by immersing the pellets in 50 mL of simulated body fluid (SBF) at 37 ± 1 °C over 21 days. The SBF (composition: 8.035 g L⁻¹ of NaCl, 0.225 g L⁻¹ of KCl, 0.355 g L⁻¹ of NaHCO₃, 0.311 g L⁻¹ of Cl₂H₁₂MgO₆, 0.292 g L⁻¹ of CaCl₂, 0.231 g L⁻¹ of H₇K₂O₇P, 0.072 g L⁻¹ of Na₂SO₄, 6.118 g L⁻¹ of tris-hydroxy methylaminomethane, 1 M (mol L⁻¹) of HCl, maintained at a standard pH of 7.4) and its preparation were in accordance with the procedure reported by Kokubo *et al.*¹⁸ After 21 days, the changes in morphological features of the pellets were assessed by FESEM analysis.

2.5 Hemolysis tests

A fresh human blood sample was collected in a heparinized tube, immersed in a 6 mL extraction medium of sterilized physiological saline, and incubated at 37 °C for 30 min under static conditions. The physiological saline and double-distilled water served as negative and positive controls, respectively. The anti-coagulated human blood was diluted using sterile physiological saline. For the testing, about 0.2 mL of each sample (10 mg mL⁻¹) was incubated with 0.8 mL of the blood sample at 37 °C for 60 min, and after that period, the tube was centrifuged, the supernatant was separated, and the absorbance of the supernatant was measured at 545 nm. The hemolysis ratio (*Z*) was calculated using the following formula:

$$Z = \frac{Dt - Dnc}{Dpc - Dnc} \times 100$$

where *Z* is the hemolysis ratio, and *Dt*, *Dnc*, and *Dpc* correspond to the average absorbance of the testing sample and the negative and positive controls, respectively. The sample is hemolytic, slightly hemolytic, or non-hemolytic when the *Z* value falls in the range of >5, 2–5, or 0–2, respectively. All the experimental procedures were approved by the appropriate Institutional Review Board (IRB) and are in accordance with the regulations set by the UW ethics committee. Venipuncture and blood sample collection were performed by a trained phlebotomist to minimize the risk to the donor.

2.6 Antimicrobial activity – agar diffusion method

The antibacterial effect of the assemblages (with different concentrations of Se-substituted HAP) were determined using the agar diffusion method against pathogenic bacterial strains, viz. *Pseudomonas aeruginosa* (*P. aeruginosa*; Gram-negative) and *Staphylococcus aureus* (*S. aureus*; Gram-positive). The inoculum was prepared from a culture grown overnight and the turbidity was adjusted to McFarland's standard 0.5. The bacterial cultures were spread uniformly on a Mueller Hinton agar plate using a sterile swab. The plates were incubated at 37 °C for 24 h and the zone of inhibition (ZOI) was recorded.

2.7 Cell viability and proliferation assays

For testing of *in vitro* cell viability and proliferation, cancerous MG-63 cells and non-cancer NIH-3T3 cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) at 37 °C in a 5% CO₂ atmosphere. When the cells attained confluency, they were detached using 0.25% trypsin/EDTA and seeded onto 48-well plates at a cell density of 5×10^3 cells per well. The seeded cells were incubated overnight to allow cell adherence at 37 °C in a 5% CO₂ atmosphere and were further used for cell proliferation studies with 0Se-HAP, 0.01 M Se-HAP, 0.05 M Se-HAP, and 0.1 M Se-HAP. The samples (in pellet form with an 8 mm diameter and 1 mm thickness) were placed on the attached NIH-3T3 and MG-63 cells and the percentage of viable cells was assessed by MTT assay at 1, 3, and 7 days with three trials each and compared with those of the controls (non-treated cells and Triton-X-100). All the samples were sterilized before starting the study, and at the end of the period, approximately 50 μ L of MTT reagent (5 μ g mL⁻¹) was added to each well and incubated at 37 °C for another 2 to 4 h. The water-insoluble formazan crystals were dissolved by adding 500 μ L of dimethylsulfoxide (DMSO) (Sigma-Aldrich), and its absorbance was measured at 570 nm using an ELISA plate reader (Thermo Scientific).

$$\% \text{ Viability} = \frac{(\text{Abs}_{570 \text{ nm}} \text{ of treated cells})}{(\text{Abs}_{570 \text{ nm}} \text{ of cell control})} \times 100$$

where Abs_{570 nm} represents the absorbance value measured at 570 nm.

2.8 Statistical analysis

Each experiment was repeated thrice and the data were expressed in mean with standard deviation. The statistical analysis was performed based on ANOVA (analysis of variance) and the significance of the experimental results with controls was expressed as $^*(p \leq 0.05)$.

3. Results and discussion

3.1 FTIR analysis

Comparisons of the FTIR spectra of pure HAP and the various Se-HAP samples synthesized by the precipitation method and sonochemical method are shown in Fig. 1 and 2, respectively. In these figures, the observed absorption bands can be assigned to

Fig. 1 A comparison of the FTIR spectra of pure HAP and Se-HAP samples formed using the precipitation method in the wavenumber ranges of 3500–400 cm^{-1} (A) and 1000–400 cm^{-1} (B): (a) 0Se-HAP, (b) 0.01 M Se-HAP, (c) 0.05 M Se-HAP, and (d) 0.1 M Se-HAP.

Fig. 2 A comparison of the FTIR spectra of pure HAP and Se-HAP samples formed using the sonication method in the wavenumber ranges of 3500–400 cm^{-1} (A) and 1000–400 cm^{-1} (B): (a) 0Se-HAP, (b) 0.01 M Se-HAP, (c) 0.05 M Se-HAP, and (d) 0.1 M Se-HAP.

the vibrations of a phosphate group, structural $-\text{OH}$ group, adsorbed water on HAP, and substituted Se groups. For example, the bands in the 1100–400 cm^{-1} region are due to the vibrations of phosphate and Se groups. The ν_1 mode of a tetrahedral (T_d) symmetry phosphate group is IR inactive, but distortion of the tetrahedral environment and lowering of the symmetry of system led to the occurrence of vibrations with low intensity in the 965–945 cm^{-1} region. The strongest asymmetric P–O stretching peaks observed in the 1100–1000 cm^{-1} region are assigned as ν_3 , a triply degenerate bond. The lower wavenumber region at 600–400 cm^{-1} corresponds to the triply degenerate vibrational modes ν_4 of the phosphate peaks. Two segments of the FTIR spectra are shown for both the synthesis methodologies.

In the 3500–3000 cm^{-1} region in both Fig. 1(A) and 2(A), the broad envelope is missing in both the methodologies, which is due to the formation of very limited hydrogen bonding in the region; hydrogen bonding was absent, *i.e.*, the adsorbed hydroxy group was removed by the sintering process. Kolmas *et al.*¹⁹ demonstrated a decrease in the intensity of the IR band corresponding to the $-\text{OH}$ group at approximately 3570 cm^{-1} with an increase in the SeO_3^{2-} content of HAP, which was further substantiated by Koutsopoulos.²⁰ In the present study, the

changes in the intensity and shape of the IR bands observed at 1381 cm^{-1} and 885 cm^{-1} (Fig. 1B) and 1358 cm^{-1} and 872 cm^{-1} (Fig. 2B) are believed to be due to the substitution of CO_3^{2-} ions in the OH^- site in the Se-HAP samples and the interaction between the CO_3^{2-} and SeO_3^{2-} groups. Also, the peak at 1460 cm^{-1} (in Fig. 2A) is observed to be CO_3^{2-} , which is attributed to type B carbonate substitution. The intensity of the phosphate group peaks decreases with increasing concentration of selenite, which is due to the replacement of PO_4^{3-} by the SeO_3^{2-} group in the HAP matrix. SeO_3^{2-} is a divalent species that will replace the trivalent species phosphate PO_4^{3-} ; the charge imbalance is maintained by CO_3^{2-} , which is absent in the normal precipitation method (Fig. 1), and charge imbalance was attained and a saturated Se substitution was obtained in the sonochemical method (Fig. 2).

3.2 Raman analysis

Fig. 3(A and B) depicts the Raman spectral analysis of the pure HAP and Se-HAP samples formed by both the precipitation (A) and the sonication method (B), where the spectra are shown in the range of 200 to 1400 cm^{-1} . From the figure, one can see the formation of many peaks related to the PO_4^{3-} group; *i.e.*, the sharp intense peak observed at approximately 960 cm^{-1} can be

Fig. 3 Raman spectra of Se-HAP samples formed using the (A) precipitation method and the (B) sonochemical method: (a) 0Se-HAP, (b) 0.01 M Se-HAP, (c) 0.05 M Se-HAP, and (d) 0.1 M Se-HAP.

attributed to the ν_1 symmetric stretching mode of the PO_4^{3-} group. The Raman bands at 764 and 782 cm^{-1} can be attributed to the ν_3 symmetric stretching mode of the SeO_3^{2-} group. The increase in the intensity of this band with the Se content confirms the replacement of PO_4^{3-} by SeO_3^{2-} groups and the associated substitution of SeO_3^{2-} into the HAP lattice. The weak band located at approximately 1075 cm^{-1} can be correlated to the ν_3 antisymmetric stretching mode of the PO_4^{3-} group. The Raman band observed at 587 cm^{-1} corresponds to the ν_4 out-of-plane bending mode, while those observed at 432 cm^{-1} can be correlated to the ν_2 bending mode of the PO_4^{3-} group.

3.3 Powder XRD analysis

Fig. 4(A and B) shows a comparison of the XRD patterns of the pure HAP and Se-HAP samples with different concentrations of Se formed using the (A) precipitation and (B) sonochemical method. When the Se/(P + Se) molar ratio was increased from 0.01 to 0.1 M, no obvious differences in the peaks of the HAP phase were observed, and the diffraction patterns matched well with the standard XRD pattern of HAP (JCPDS No. 09-432). Broadening of the peaks of the (211) planes can be detected for all the samples as shown in Fig. 4(A and B). The results

indicated that the selenite ions may enter into the crystal lattice of HAP in the absence of a secondary phase and affect the crystallinity of HAP. The crystallite size along the c -axis from the (002) reflections and the lattice parameters were determined in order to assess the effect of selenite ions entering the HAP unit cells. In Table 1, the lattice parameter of the a -axis shows no evident trend, but a contraction of the c -axis and unit cell volume are observed as the content of selenite ions is increased. It was inferred from these results that the crystallite size and lattice parameters of the Se-HAP samples changed relative to those of pure HAP as a result of the entrance of selenite ions. Similar results have been confirmed by Ma *et al.* in the presence of selenite ions in the HAP crystals.²¹ It was notable that in the XRD patterns of the obtained samples with Se/(P + Se) molar ratios of up to 0.1 M Se-HAP, no new peaks are observed from the HAP nanocrystals (Fig. 4(A and B)). From Tables 1 and 2, it is clear that the increase of the Se concentration increased the crystallinity and reduced the crystallite size. Also, cell parameters such as cell volume increased with increasing selenium concentration. Additionally, the XRD data indicated that the size reduction factor depends on the synthesis methodology, *i.e.*, we observed a crystallite size of 56 nm for the Se-doped

Fig. 4 A comparison of the powder XRD patterns of Se-HAP samples formed using (A) the precipitation method and (B) the sonochemical method: (a) 0Se-HAP, (b) 0.01 M Se-HAP, (c) 0.05 M Se-HAP, and (d) 0.1 M Se-HAP.

Table 1 A comparison of the XRD cell parameters of the various Se-HAP samples formed using the precipitation method

Sample	Lattice plane	Line width (degree)	Crystallite size (nm)	Cell parameters		Cell volume ($\text{\AA}^3$)
				a ($\text{\AA}$)	c ($\text{\AA}$)	
0Se-HAP	211	0.1020	85	9.438	6.916	532.56
0.01 M Se-HAP	211	0.1254	69	9.448	6.891	533
0.05 M Se-HAP	211	0.1478	58	9.466	6.852	536
0.1 M Se-HAP	211	0.1520	56	9.489	6.842	537

Table 2 A comparison of the XRD cell parameters of Se-HAP samples formed via the sonochemical method

Sample	Lattice plane	Line width (degree)	Crystallite size (nm)	Cell parameters		Cell volume ($\text{\AA}^3$)
				a ($\text{\AA}$)	c ($\text{\AA}$)	
0Se-HAP	211	0.1892	44	9.423	6.896	536
0.01 M Se-HAP	211	0.2254	38	9.436	6.864	539
0.05 M Se-HAP	211	0.2589	32	9.441	6.881	540
0.1 M Se-HAP	211	0.2863	29	9.449	6.882	541

samples prepared by the precipitation method and a 29 nm crystallite size for the Se-substituted samples from the sonochemical method.

3.4 FESEM analysis

The morphological features of the 0Se-HAP, 0.01 M Se-HAP, 0.05 M Se-HAP, and 0.1 M Se-HAP samples formed using the precipitation and sonochemical methods are shown in Fig. 5(A and B). In Fig. 5A, all the samples formed using the precipitation method are spherical/quasi-spherical with sizes ranging from 500 nm to 1 μm . Most of the particles were also formed in agglomerated, undefined shapes, with no significant changes to the morphological features being seen upon increasing the Se content from 0Se-HAP (Fig. 5A(a)) to 0.1 M Se-HAP (Fig. 5A(d)). However, in the case of the samples synthesized using the sonochemical method (Fig. 5B), the particles are homogeneously arranged and distributed in a regular manner. Also, as the Se concentration was increased from 0.01 to 0.1 M, the particle morphology changed from hexagonal nanocrystals to agglomerated, elongated rod particles as observed in Fig. 5B(a–d). From the overall

analysis, it can be inferred that, without the need for a surfactant or capping agent, the morphological transformation and size of the particles seem to be better in the sonochemical synthesis approach as compared to those of the precipitation method.

3.5 TEM analysis

A comparison of the changes in the morphology of the particles synthesized using both the precipitation and sonochemical method with increasing selenium substitution is shown in Fig. 6(A and B). As shown in Fig. 6A, all the samples synthesized using the precipitation method are irregular, with an undefined morphology, and are in agglomerated form due to the absence of surfactant. The TEM image displays the irregular shapes of the particles, which range from an agglomerated rock-like morphology to a hammer-like one, as shown in Fig. 6A(a–d). Similarly, for the selenium-substituted HAP samples formed using the sonochemical approach, the TEM images show a transition from a small rod-like shape to a needle-like one with increasing Se concentration in HAP. For 0Se-HAP, the particles are spherical clusters. In 0.01 M Se-HAP, their morphology

Fig. 5 Morphological analysis of the Se-HAP samples formed using (A) the precipitation method (scale of 2 μm), and (B) the sonochemical method (scale of 1 μm). In the figures, (a), (b), (c), and (d) correspond to the 0Se-HAP, 0.01 M Se-HAP, 0.05 M Se-HAP, and 0.1 M Se-HAP samples, respectively.

Fig. 6 TEM images of Se-HAP formed using the (A) precipitation method (scale of 1 μm), and (B) sonochemical method (scale of 100 nm). In the figures, (a), (b), (c), and (d) correspond to 0Se-HAP, 0.01 M Se-HAP, 0.05 M Se-HAP, and 0.1 M Se-HAP, respectively.

transforms from spherical clusters to rod-shaped ones, and as the Se concentration is further increased to 0.1 M Se-HAP, the small rods elongate into nanowhisker-like structures. This morphological transformation is obtained due to the ultrasonication mechanism, which gave a rod-like morphology with the rod length varying with the amount of Se and of ultrasonication. The aspect ratio of the particles is greater than two in all cases, indicating the preferential growth of HAP particles along a particular axis, resulting in the formation of nanorods (Fig. 6B(a-d)).

3.6 XPS analysis

XPS analysis was performed on the Se-HAP samples formed by the sonochemical method to determine their elemental composition more precisely, and the results are shown in Fig. 7. In the figure, there are two distinct Ca 2p peaks at 345.7 and 351.3 eV, which correspond to Ca 2p_{3/2} and Ca 2p_{1/2}, respectively. In addition to this, the observation of a characteristic peak at 133 eV was confirmed due to the presence of phosphate groups (P 2p) in HAP. The sample contains anoxogenic aromatic sp² carbon rings (C 1s), as evidenced by the C-C peaks at 284.5 eV,

Fig. 7 XPS spectra of 0.1 M Se-HAP formed using the sonochemical method.

and oxygenated sp^2 carbons, C–O, as demonstrated by the 286.29 eV peak. Moreover, two intense peaks corresponding to O 1s at 530.84 and 531.20 eV were observed and were found to be associated with anionic oxygen in the hydroxyl (OH) group and residual oxygen groups, respectively. This might be due to the substitution of selenite to replace phosphate. The $3d_{5/2}$ peak of Se at a binding energy of 59.25 eV was consistent with those reported for SeO_3^{2-} at 59.25 eV, which makes it evident that Se^{4+} is replacing P^{5+} .²² The charge imbalance is optimized by the CO_3^{2-} group.

3.7 Bioactivity of samples formed by the sonochemical method

The surface morphology of the 0Se-HAP and 0.1 M Se-HAP pellets after immersion in SBF at a temperature of 37 °C for 21 days is shown in Fig. 8. In the FESEM analyses of the samples shown, the surface of the HAP pellet is covered with needle-like particles, which is due to the resorption of Ca^{2+} ions in the SBF solution (Fig. 8a). The EDS analysis indicates the presence of Ca, P, and O as the major elements (insets), which suggests the formation of apatite on the surface of the HAP pellets. The extent of the formation of needle-shaped particles is relatively high on the surface of 0.1 M Se-HAP pellet, as shown in Fig. 8b, when compared to that of those formed on HAP pellet (Fig. 8a).

3.8 Hemolytic study

A hemocompatibility study was performed to examine the blood compatibility of the pure HAP (0Se-HAP) and Se-HAP samples having different concentrations (0, 0.01, 0.05, and 0.1 M), and the results are shown in Fig. 9 and tabulated in Table 3. In general, the degree of hemolysis can be divided into three categories, viz. >5%, 2–5% and <2%, which represent hemolytic, slightly hemolytic, and non-hemolytic materials (respectively), as laid out in ASTM F756-00. From the analysis, the hemolytic ratio was found to increase from the Se unsubstituted (0Se-HAP) to the Se-HAP composites. The % hemolysis for pure HAP and Se-substituted HAP (0, 0.01, 0.05, and 0.1 M) was found to be 0, 1.8, 1.9, and 2.4% (Table 3). Thus, based on the results, we concluded that the samples containing up to

Fig. 9 Hemolytic assays of (a) 0Se-HAP, (b) 0.01 M Se-HAP, (c) 0.05 M Se-HAP, and (d) 0.1 M Se-HAP samples formed using the sonochemical method.

Table 3 Hemolytic assay parameters of the (a) 0Se-HAP, (b) 0.01 M Se-HAP, (c) 0.05 M Se-HAP, and (d) 0.1 M Se-HAP samples formed via the sonochemical method

Sample ID	Absorbance at 545 nm	Positive control	Negative control	Hemolytic ratio (%)
0Se-HAP	0.0352	1.121	0.047	0
0.01 M Se-HAP	0.0490	1.121	0.047	1.8
0.05 M Se-HAP	0.6854	1.121	0.047	1.9
0.1 M Se-HAP	0.073	1.121	0.047	2.4

0.05 M Se-HAP are highly hemocompatible, while in the case of 0.1 M Se-HAP, the sample was found to be slightly hemolytic.

3.9 Antimicrobial tests

Fig. 10(A and B) shows a comparison of the antibacterial activity of 0Se-HAP and all the Se-HAP samples with different concentrations formed using the sonochemical method when tested against *P. aeruginosa* and *S. aureus*. From the analysis of results, the ZoI observed against *P. aeruginosa* (Fig. 10A) was 0.2, 0.5, 0.7, and 1.2 cm respectively for 0Se-HAP, 0.01, 0.05, and 0.1 M Se-substituted HAP. In comparison, in the test against *S. aureus* shown in Fig. 10B, 0Se-HAP and the different concentrations of

Fig. 8 Changes in the surface morphologies of pure HAP and Se-HAP pellets after immersion in SBF at 37 °C for 21 days: (a) 0Se-HAP; (b) 0.1 M Se-HAP.

Fig. 10 Antibacterial activities of (a) 0Se-HAP, (b) 0.01 M Se-HAP, (c) 0.05 M Se-HAP, and (d) 0.1 M Se-HAP samples formed using the sonochemical method against (A) *P. aeruginosa* and (B) *S. aureus* ((s) is a control).

Se-substituted HAP exhibited a ZoI size of 0.2, 0.9, 1.8, and 2.3 μm , respectively. The pure HAP (0Se-HAP) showed minimal antibacterial effect during the incubation period against the tested strains, which is due to the size effect. The enhancement of this property was achieved only when the HAP was substituted with Se, which resulted in a significant antibacterial effect. Also, it is quite evident that the strain with a Gram-positive strain membrane architecture (*S. aureus*) responded quite well, on par the response of the Gram-negative strain (*P. aeruginosa*), which has a thick peptidoglycan layer.

3.10 *In vitro* cell viability tests

The extent of cell growth and proliferation in the NIH-3T3 fibroblast cell line (non-cancer cell type) was ascertained using an MTT assay, which involves the measurement of absorbance at 570 nm. This method is fairly useful in determining the cytotoxicity of biologically useful elements incorporated into a HAP matrix. Fig. 11A shows the normal microscopic images of cells following incubation for 24 h, in which we can see a decrease in the number of cells with increasing Se concentration in the Se-HAP samples. Additionally, based on the absorbance measured at 570 nm, the cell viability of the control sample was set as 100% to compare the beneficial and deleterious influence of HAP and Se-HAP on the cell growth. It is evident from Fig. 11B that the cell viability shows a strong dependence on the degree of Se substitution in HAP, as well as their concentration. Irrespective of the concentration (0.01–0.1 M Se), higher cell viability was observed for 0.01 M Se-HAP and 0.05 M Se-HAP samples with an increased incubation period, which is due to the increased solubility of Se-HAP. Se plays an important role in cell proliferation and bone growth, and Se deficiency has been shown to retard bone growth and increase the risk of bone disease.²³ It has also been reported that Se can enhance immune surveillance as well as modulation of cell proliferation and differentiation. However, the extent of cell growth shows a steady decrease when the degree of Se substitution is increased beyond 0.05. After 48 h, the samples show a steady increase in viability and show compatibility towards all

the concentrations from 0.01 M Se-HAP to 0.1 M Se-HAP. Based on the cell viability analysis using the MTT assay, both 0.05 M Se-HAP and 0.1 M Se-HAP can be considered suitable for biomedical applications.

Similarly, toxicological assessment of the synthesized compounds (pure HAP and Se-HAP) was carried out by exposing them to diseased cells cultured *in vitro*, and for this, we used the same MTT assay. Based on this test, the earlier-generated antibacterial efficacy (Fig. 10) and hemocompatibility (Fig. 9) results were further authenticated using the cell viability assay against MG-63 cells, a cancerous cell type of bone origin. For the test, the pure HAP (0Se-HAP) and 0.01, 0.05, and 0.1 M Se-HAP samples were prepared at a concentration of 1 mg mL^{-1} and kept aside as standards. When the growth of cells was observed to be 80–90% confluent, 100 μL of the standard testing samples was added and incubated for a period of 1, 3, or 7 days; pure HAP was used as the negative control. Fig. 12A shows the normal microscopic images of cells following incubation for 7 days, in which we can see a slight reduction in the cell number with increasing concentration of Se in the Se-HAP samples. Furthermore, the plates were read before and after incubation, and the difference between these readings gave the total percentage (%) cell viability loss (Fig. 12B). Based on the results obtained, we observed no cell death or morphological changes in the cells inoculated with pure HAP, as they showed 96–100% viability and retained the same cellular architecture even on the seventh day. It can be seen from the figure that the cell viability decreased after treatment with the 0.01 M to 0.1 M concentration Se-HAP samples in a time- and dose-dependent fashion. The viability decreased to almost 20–30% that of the control group in the cells treated with a 0.1 M concentration (Fig. 12B). For the Se-HAP samples (0.01, 0.05, and 0.1 M Se), other than the considerable change in morphology, no significant cytotoxic manifestations were observed (cell rounding, monolayer peel off, etc.). The observation of such effects in the event of substitution can be linked to the leaching probability of Se with time; the Se might have penetrated the cells to produce a static

Fig. 11 (A) An MTT-induced cell viability micrograph and (B) comparison of the % cell viabilities of NIH-3T3 fibroblast cells following incubation with the (a) 0Se-HAP, (b) 0.01 M Se-HAP, (c) 0.05 M Se-HAP, and (d) 0.1 M Se-HAP samples formed using the sonochemical method.

Fig. 12 (A) Normal microscopic images of MG-63 osteosarcoma cells and (B) % cell viabilities following treatment with the (a) 0Se-HAP, (b) 0.01 M Se-HAP, (c) 0.05 M Se-HAP, and (d) 0.1 M Se-HAP samples formed using the sonochemical method.

Table 4 A comparison of the results of the present study with those from other studies in the literature

Sample	Synthesis method	Remarks	Ref.
Se-HAP	Co-precipitation method	Se incorporation into HAP lattices reduced the crystallite size, and no changes to the morphology of apatite were observed. Also, it was found that Se-HAP powder formed using an Se/P ratio of 0.1 has enhanced thermal stability of up to 900 °C, thereby confirming its potential for fabrication as a bone repair scaffold.	26
Se-HAP	Hydrothermal method	Homogeneous Se-HAP powders with Se/P ratios of 0.5, 0.4, 0.3, 0.2, 0.1, 0.01, and 0.001 were found to form a single, well-defined crystalline phase, but with slightly shifted lattice peak parameters. The work also discusses the means of forming a highly crystalline phase of nano Se-HAP at lower temperature conditions.	27
Se oxyanion-HAP	Precipitation method	HAPs containing selenate (SeO_4^{2-}) or selenite (SeO_3^{2-}) ions were synthesized, and upon sintering, highly crystalline structures were formed with crystal sizes similar to that of natural bone.	28
Se-HAP	Co-precipitation	The <i>in vivo</i> effects of Se-HAPs were evaluated systematically in a nude mouse model of human HCC with high metastatic potentials. The results indicated that the Se-HAP particles significantly enhanced the overall survival rate of HCC-loaded nude mice, thereby indicating its promising role as an anticancer agent with minimal toxicity and survival advantage.	29
Lysozyme-loaded Se-HAP	<i>In situ</i> precipitation method	The advantage of Se-HAP in contrast with undoped HAP was evident in terms of better modulation of the lysozyme protein release during incubation in a simulated physiological medium.	30
Se-HAP nanoparticles	Aqueous precipitation method	The Se-HAP nanoparticles significantly removed the bone defects caused by the tumor cells. The mechanism of action was concluded to be apoptosis following the induction of oxidative stress in a caspase-dependent pathway due to the Se leached from the composite.	31
Zn^{2+} and SeO_3^{2-} co-substituted nano-HAP	Co-precipitation	Zn-Se-HAP was formed by the replacement of Ca^{2+} with Zn^{2+} and of PO_4^{3-} with SeO_3^{2-} . The increased Zn content in the composite caused an increase in the <i>a</i> -axis of the HAP lattice, a decrease in the <i>c</i> -axis, and loss of surface charges (at pH 7.4), thereby providing evidence for the control of the surface properties of HAP through dopants.	32
Se-HAP	Hydrothermal method	Se-HAP nanocrystals with rod or needle shapes containing Se/(P + Se) molar ratios of 0–0.4 were synthesized. The introduction of Se ions into the HAP lattice significantly influenced the morphology and final size of the HAP crystals, which exhibited low cytotoxicity, biocompatibility, and promising growth of osteoblast cells.	33
Se-HAP	Both co-precipitation and ion-exchange methods	In the co-precipitation formation of Se-HAP, increasing the Se content resulted in decreased crystallinity, a change in the morphology from acicular to round, contraction of the lattice in both the <i>a</i> and <i>c</i> crystallographic directions, enhanced antimicrobial activity, and anticancer and osteoconductive effects.	34
Se-HAP	Precipitation method	Se-HAP generated after sintering showed enhanced thermal stability compared to undoped or non-sintered HAP, thereby indicating the importance of the heating process to produce more stable products.	35
Se-HAP nanorods	Alcohol thermal method	Se-HAP nanorods were investigated for their antitumor effect on rat-bone-marrow-resident mesenchymal stem cells and found to enhance ossification and reduce marrow adiposity.	36
Zn^{2+} and SeO_3^{2-} co-substituted-HAP	Aqueous precipitation method	Co-substitution of Zn^{2+} and SeO_3^{2-} into the lattice of HAP significantly improved the efficacy of BALB/c 3T3 mouse fibroblast cells and thereby demonstrated a potential role as an anticancer bone filling agent.	37
Se-HAP	Both precipitation and sonochemical methods	Se-HAPs with a various Se contents (0.01, 0.05, and 0.1 M) formed by either a precipitation or sonochemical method were compared to show the importance of sonication for the formation of particles with greater crystallinity, well-defined morphology, biocompatibility, and enhanced antibacterial ability.	Present work

effect instead of a cytotoxic effect.²⁴ Additionally, the morphological effect of the nanorod-shaped Se-HAP induced some typical changes that can influence cell viability.²⁵

In the present study, two different Se-HAP composites with different Se contents (0.01, 0.05, and 0.1 M) were formed using the precipitation and sonochemical method. The results of our study were compared with those of literature studies (Table 4) and found to far exceed previous ones, as none of the other works deal with the sonication of Se-substituted HAP particles. We studied and compared the crystallinity, morphology, size, biocompatibility, antibacterial activity, and *in vitro* cell viability studies of Se-HAPs obtained using the two methods and found that those obtained using the sonication method have superior behaviour in all the tests. Based on the overall analysis, this study highlights the importance of incorporating the sonication process as part of any synthesis method for the generation of more stable crystals with well-defined morphologies and improved surface area to affect the biological activity.

4. Conclusions

In conclusion, we studied Se-HAP composites to evaluate their suitability as a bone grafting material. For this, the composites were formed with different Se content levels by making use of two different approaches, namely, a precipitation method and a sonochemical method. FTIR and Raman spectroscopic analysis indicated the successful substitution of Se into the matrix of HAP, while powder XRD studies demonstrated its highly crystalline nature, which was significantly influenced by Se doping, *i.e.*, increased crystallinity and reduced crystal size were found with increasing Se concentration. Additionally, XRD data showed that the synthesis method significantly influenced the crystal size, *i.e.*, Se-HAP formed using the precipitation method has a size of 56 nm, while the sonochemical method produced 29 nm particles. Morphological analysis confirmed the formation of well-defined rod-shaped Se-HAP particles using the sonochemical method and irregular agglomerated ones using the precipitation method. Hemolytic studies confirmed an increase in the hemolytic ratio with increasing Se content; the 0.05 M Se-HAP sample was found to be highly hemocompatible, and the 0.1 M Se-HAP was found to be slightly hemolytic. In addition, an increased antibacterial response was observed with increasing Se content, and a Gram-positive strain (*S. aureus*) responded quite well, on par with the response of the Gram-negative strain (*P. aeruginosa*). Finally, cell viability and proliferation studies indicated an increase in the proliferation capacity of non-cancer cells and a considerable reduction in the viability of cancer cells for the 0.01 M Se-HAP sample. Thus, from cumulative analysis, it can be concluded that the Se-HAP samples (with increasing Se content of up to 0.1 M) formed *via* the sonochemical approach are highly suitable for biomedical applications in bone cell repair, growth, and regeneration.

Conflicts of interest

The authors declare no conflicts of interest.

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