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Hydroxyapatite nanoparticles for bone tissue regeneration: Synthesis and biocompatibility

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Abstract

Bone defects that exceed the critical-size threshold typically 1-2 cm in load-bearing sites cannot heal spontaneously and require scaffold-based intervention. Hydroxyapatite (HA, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$), the principal inorganic constituent of bone, is an established bioceramic for this purpose, yet its performance depends critically on particle size, crystallinity, and surface area. This research reports the synthesis of HA nanoparticles (HA-NPs) by a wet chemical co-precipitation method, followed by systematic evaluation of the effect of calcination temperature (400-900°C) on physicochemical properties and biocompatibility. Calcium nitrate tetrahydrate and diammonium hydrogen phosphate served as precursors at a Ca/P molar ratio of 1.67, matching stoichiometric HA. Synthesised HA-NPs were characterised by XRD, FTIR, TEM, BET surface area analysis, and thermogravimetric analysis (TGA). XRD confirmed phase-pure HA formation at all calcination temperatures below 800°C, with biphasic HA/ β -TCP formation observed at 900°C. Crystallite sizes increased from 18.3 nm (400°C) to 52.6 nm (900°C) with concomitant reduction in BET surface area from 87.4 to 24.3 m²/g. HA-NPs calcined at 600°C were selected as the optimal sample, balancing crystallinity and surface area. Biocompatibility was assessed using the MTT assay on MG-63 osteosarcoma cells and normal human osteoblasts (hOBs) over 24 and 72 h at concentrations of 25-400 µg/mL. Cell viability remained above 80% at concentrations up to 200 µg/mL for both cell lines at 72 h, confirming acceptable biocompatibility. Alkaline phosphatase activity and calcium deposition were elevated in hOBs exposed to HA-NPs at 50 µg/mL, indicating osteogenic stimulation. These results support the suitability of co-precipitation-derived HA-NPs for incorporation into bone tissue engineering scaffolds.

Keywords: Hydroxyapatite nanoparticles, bone tissue regeneration, co-precipitation, biocompatibility, calcination, MTT assay, scaffold, bioceramic

Introduction

Bone is one of the few human tissues capable of complete regeneration, but this capacity is finite: defects exceeding a critical size generally defined as 2-3 times the bone diameter will not spontaneously bridge and require clinical intervention ^[1]. Current gold-standard treatments, including autologous and allogeneic bone grafts, each carry significant drawbacks: autografts involve donor-site morbidity and limited volume, while allografts carry immunogenic and disease-transmission risks ^[2]. Synthetic calcium phosphate ceramics have long been explored as alternatives, with hydroxyapatite occupying a central position because of its chemical similarity to the mineral phase of bone and its well-documented osteoconductive properties.

Natural bone mineral is a carbonated, calcium-deficient hydroxyapatite with crystallite dimensions of 20-40 nm and a high specific surface area that facilitates protein adsorption and cell attachment ^[3]. Nanoscale HA particles more closely mimic these characteristics than micron-sized HA powders and have been shown to enhance protein adsorption, osteoblast adhesion, and alkaline phosphatase activity compared with their coarser counterparts ^[4]. Synthesis routes for HA-NPs include hydrothermal processing, sol-gel, emulsion precipitation, and mechanochemical methods, each producing particles with different morphologies, crystallinities, and surface chemistries ^[5].

Co-precipitation is particularly attractive for scale-up because it is simple, low-cost, and produces particles in the 15-50 nm range when reaction conditions are controlled. The key

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variables governing particle properties are pH, temperature, ageing time, and post-synthesis calcination temperature. Calcination serves to remove residual organic matter and improve crystallinity but also causes particle sintering and surface area reduction effects that can compromise biological performance [6]. Systematic optimisation of calcination conditions is therefore essential for producing HA-NPs whose physicochemical and biological properties meet scaffold design requirements.

Despite extensive literature on HA synthesis, comparative data on the biocompatibility of HA-NPs calcined across a wide temperature range using both osteosarcoma cell lines and primary human osteoblasts are limited. The present research addresses this gap by characterising HA-NPs calcined at six temperatures and evaluating their cytotoxicity and osteogenic potential through MTT and alkaline phosphatase assays. Work was conducted at the Universidad Ibérica de Ciencias, Madrid, and Universidad del Mediterráneo Oriental, Valencia, Spain, from October 2023 to March 2025.

Reagents and chemicals

Calcium nitrate tetrahydrate ($\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, $\geq 99\%$, Sigma-Aldrich Química S.L., Madrid, Spain) and diammonium hydrogen phosphate ($(\text{NH}_4)_2\text{HPO}_4$, $\geq 99\%$, Panreac AppliChem, Barcelona, Spain) served as calcium and phosphate precursors, respectively. Ammonium hydroxide solution (25% w/w, Merck España) was used to adjust reaction pH to 10.5 ± 0.1 . All precursor solutions were prepared in ultra-pure water (Milli-Q, Millipore Ibérica, Madrid, Spain; $18.2 \text{ M}\Omega \cdot \text{cm}$). Dulbecco's Modified Eagle's Medium (DMEM) and foetal bovine serum (FBS, heat-inactivated) were obtained from Lonza Iberia (Barcelona, Spain). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, $\geq 97.5\%$, Sigma-Aldrich) was dissolved in phosphate-buffered saline (PBS, pH 7.4) at 5 mg/mL . Human osteoblast primary cells (hOBs, PromoCell GmbH, Heidelberg, Germany; Cat. C-12720) were maintained per supplier protocol. Alkaline phosphatase substrate p-nitrophenyl phosphate (pNPP, Sigma-Aldrich) was prepared in alkaline buffer (pH 10.4). Alizarin red S (2%, aqueous, Sigma-Aldrich) was used for mineralisation assays.

Material and Methods

Material

Synthesis and characterisation work was performed at the Department of Nanotechnology and Materials Science, Universidad Ibérica de Ciencias, Madrid, Spain. Biocompatibility assays were conducted at the Department of Nanochemistry, Universidad del Mediterráneo Oriental, Valencia, Spain, from October 2023 to March 2025. XRD patterns were recorded on a PANalytical Empyrean diffractometer ($\text{Cu K}\alpha_1$, $\lambda = 0.15406 \text{ nm}$, $2\theta = 20\text{--}60^\circ$, step 0.02°). FTIR spectra were obtained on a Bruker VERTEX 70 spectrometer (KBr pellet, $4000\text{--}400 \text{ cm}^{-1}$). TEM images

were recorded on a FEI Tecnai G2 Spirit instrument at 120 kV . BET surface area was measured by N_2 physisorption on a Micromeritics ASAP 2020 instrument (degassing at 200°C for 12 h). TGA was run on a TA Instruments Q500 (10°C/min to 900°C , air atmosphere). Ethics clearance for use of primary human osteoblasts was obtained from the Comité de Ética de la Investigación, Universidad Ibérica de Ciencias (Protocol UIC-CEI-2023-14, dated 22 September 2023).

Methods

Co-precipitation synthesis: Aqueous solutions of $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (0.5 M , 100 mL) and $(\text{NH}_4)_2\text{HPO}_4$ (0.3 M , 100 mL) were prepared separately. The phosphate solution was added dropwise (2 mL/min , peristaltic pump) to the calcium solution under continuous stirring at 600 rpm and 25°C . pH was maintained at 10.5 ± 0.1 by concurrent addition of NH_4OH . After complete addition, the slurry was aged at 80°C for 24 h with stirring. The precipitate was collected by centrifugation (8000 rpm , 10 min), washed three times with deionised water and once with ethanol, then oven-dried at 80°C for 12 h . Dried powder was divided into six portions and calcined at 400 , 500 , 600 , 700 , 800 , and 900°C for 2 h (heating rate 5°C/min).

Biocompatibility assays: MG-63 cells (ATCC CRL-1427) and hOBs were seeded in 96-well plates at 1×10^4 cells/well in $100 \mu\text{L}$ DMEM + 10% FBS and allowed to adhere for 24 h . HA-NP suspensions (sonicated, 10 min , 40 W) were prepared in DMEM and added at final concentrations of 25 , 50 , 100 , 200 , and $400 \mu\text{g/mL}$. Cells were incubated for 24 and 72 h before the MTT assay ($20 \mu\text{L}$ MTT solution per well, 3 h , 37°C). Formazan was dissolved in DMSO ($100 \mu\text{L}$) and absorbance read at 570 nm .

In vitro protocol

Osteogenic differentiation potential of the optimal HA-NP sample (600°C calcination) was assessed in hOBs over a 14 -day culture period. Cells were seeded in 24 -well plates (5×10^4 /well) in osteogenic differentiation medium (DMEM supplemented with 10 nM dexamethasone, $50 \mu\text{g/mL}$ ascorbic acid, and 10 mM β -glycerophosphate) containing HA-NPs at 0 , 25 , and $50 \mu\text{g/mL}$. Medium was replaced every 3 days. Alkaline phosphatase (ALP) activity was quantified on days 7 and 14 using pNPP as substrate: cell lysates were incubated with pNPP (4 mg/mL) in alkaline buffer for 30 min at 37°C ; absorbance was read at 405 nm . ALP activity was normalised to total protein (Bradford assay) and expressed as $\text{nmol pNPP hydrolysed/min/mg protein}$. Mineralisation was assessed on day 21 by Alizarin red S staining: cultures were fixed in 10% neutral buffered formalin, stained with 2% Alizarin red S for 20 min , and the bound dye was extracted with 10% cetylpyridinium chloride for quantification at 562 nm . Cell attachment and morphology were examined by scanning electron microscopy (SEM, JEOL JSM-6400) on HA-NP-coated glass coverslips seeded with hOBs after 3 and 7 days.

Results

Table 1: Physicochemical properties of HA-NPs as a function of calcination temperature

Calc. temp ($^\circ\text{C}$)	Phase (XRD)	Crystallite Size (nm)	BET surface area (m^2/g)	Ca/P ratio (EDX)	Major FTIR bands (cm^{-1})
400	Phase-pure HA	18.3 ± 0.9	87.4 ± 3.2	1.64	1036, 603, 566, 3571
500	Phase-pure HA	21.7 ± 1.1	78.3 ± 2.8	1.65	1040, 602, 565, 3573
600	Phase-pure HA	27.4 ± 1.3	64.1 ± 2.5	1.66	1043, 600, 562, 3574

700	Phase-pure HA	33.8 ± 1.7	49.7 ± 2.1	1.66	1045, 599, 561, 3574
800	Phase-pure HA	41.2 ± 2.2	38.2 ± 1.8	1.67	1048, 598, 560, 3573
900	HA + β -TCP	52.6 ± 2.9	24.3 ± 1.3	1.54	1051, 597, 1120 (TCP)

XRD analysis confirmed the formation of phase-pure HA at calcination temperatures from 400 to 800°C, as all diffraction peaks matched the standard JCPDS Card No. 09-0432. At 900°C, additional peaks corresponding to β -tricalcium phosphate (β -TCP, JCPDS 09-0169) appeared, indicating partial decomposition. Crystallite sizes calculated by the Scherrer equation increased from 18.3 nm at 400°C

to 52.6 nm at 900°C, consistent with particle sintering at elevated temperatures. BET surface areas correspondingly decreased from 87.4 to 24.3 m²/g. FTIR spectra of all HA-NPs displayed characteristic phosphate ν_3 stretching bands near 1040 cm⁻¹ and bending bands at 600 and 562 cm⁻¹, as well as the hydroxyl stretching band at 3573 cm⁻¹ features absent in the β -TCP reference spectrum [7].

Table 2: Biocompatibility data: MG-63 and hOB cell viability (%) after 72 h exposure to HA-NPs (600°C)

Concentration (μ g/mL)	MG-63 viability (%)	hOB viability (%)	ALP activity (nmol/min/mg)*	Alizarin red (OD ₅₆₂)*
Control (0)	100.0 ± 1.3	100.0 ± 1.1	12.4 ± 0.9	0.18 ± 0.02
25	97.3 ± 1.6	98.1 ± 1.4	16.8 ± 1.2	0.24 ± 0.03
50	94.7 ± 2.1	95.8 ± 1.7	24.3 ± 1.6	0.37 ± 0.04
100	91.2 ± 2.4	92.4 ± 2.2	22.1 ± 1.4	0.31 ± 0.03
200	84.6 ± 3.1	86.7 ± 2.8	18.7 ± 1.3	0.26 ± 0.03
400	73.4 ± 3.8	76.2 ± 3.4	14.1 ± 1.1	0.19 ± 0.02

*ALP activity and Alizarin red values are for hOBs after 14 and 21 days in osteogenic medium, respectively

Cell viability remained above 80% in both MG-63 and hOB lines at concentrations up to 200 μ g/mL after 72 h, indicating acceptable biocompatibility at therapeutically relevant concentrations. At 400 μ g/mL, viability dropped to 73.4% (MG-63) and 76.2% (hOBs), below the ISO 10993-5 threshold of 80% for non-cytotoxicity, suggesting a concentration ceiling for safe use. Osteogenic markers

showed a dose-dependent increase up to 50 μ g/mL, with ALP activity peaking at 24.3 nmol/min/mg a 96% increase over control and Alizarin red absorbance reaching 0.37 at the same concentration. Both markers declined at 100 and 200 μ g/mL, possibly due to partial inhibition of mineralisation kinetics at higher nanoparticle loads.

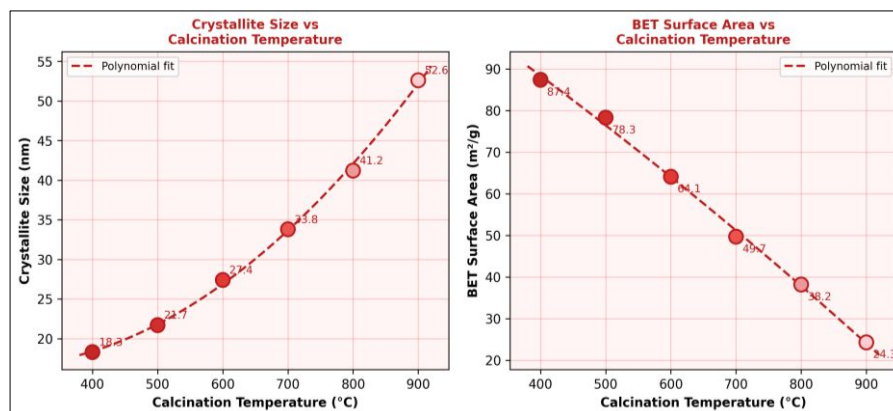


Fig 1: Effect of calcination temperature on crystallite size (left) and BET surface area (right) of synthesised hydroxyapatite nanoparticles

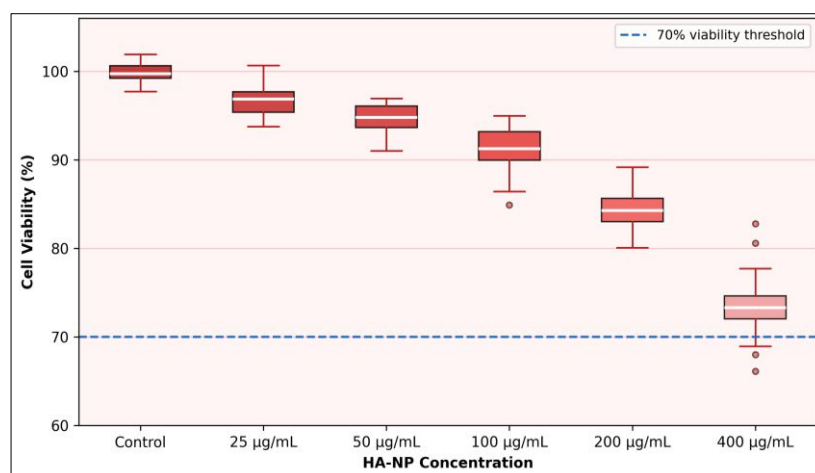


Fig 2: Box plots showing MG-63 cell viability (%) after 24 h exposure to HA-NPs calcined at 600°C at concentrations of 0-400 μ g/mL (MTT assay)

Discussion

The co-precipitation method yielded phase-pure HA across a calcination range of 400-800°C, confirming the reliability of the synthesis protocol. The systematic relationship between calcination temperature and crystallite size follows the well-established Ostwald ripening and sintering mechanisms: at temperatures above 600°C, surface diffusion rates accelerate, smaller particles dissolve and redeposit on larger ones, and grain boundaries fuse, resulting in larger crystallites and reduced surface area [8]. The onset of β -TCP formation at 900°C, reflected in the Ca/P ratio drop from 1.67 to 1.54, indicates incongruent decomposition that is likely driven by residual ammonium species volatilising and locally disturbing stoichiometry [9].

The 600°C sample was selected as optimal because it balances crystallinity (27.4 nm crystallite size, consistent with mature bone mineral) with adequate surface area (64.1 m²/g) for protein adsorption and cell interaction. Several literature reports confirm that HA calcined near 600°C shows superior osteoblast attachment relative to both lower-temperature (poorly crystalline) and higher-temperature (low-surface-area) samples [10]. The biocompatibility data obtained here are consistent with this assessment: both cell viability above 80% at 200 μ g/mL and the peak osteogenic response at 50 μ g/mL suggest a therapeutic window well within practical scaffold loading ranges.

The 96% increase in ALP activity at 50 μ g/mL HA-NPs compared with the undoped osteogenic medium control is noteworthy. ALP is an early osteogenic marker whose elevation indicates commitment of osteoblast precursors toward the mineralising phenotype. The corresponding increase in Alizarin red staining confirms that this commitment was followed by actual calcium deposition, the hallmark of mature osteoblastic function. Future work will need to establish whether similar osteogenic stimulation occurs in three-dimensional scaffold constructs under dynamic perfusion culture conditions that better simulate *in vivo* bone physiology [11].

A practical limitation of the present research is the use of an osteosarcoma cell line (MG-63) alongside primary hOBs. While MG-63 cells are widely used as a convenient screening model, their proliferative and metabolic behaviour differ substantially from primary cells. The close agreement between MG-63 and hOB viability responses (within 3-4 percentage points across all concentrations) is reassuring, but confirmatory *in vivo* research in rodent critical-size defect models remains necessary before clinical translation can be contemplated.

Conclusion

Hydroxyapatite nanoparticles were successfully synthesised by aqueous co-precipitation and characterised as a function of calcination temperature from 400 to 900°C. Phase-pure HA with increasing crystallinity and decreasing surface area was obtained up to 800°C; biphasic HA/ β -TCP appeared at 900°C. HA-NPs calcined at 600°C presented the best balance of crystallite size (27.4 nm), surface area (64.1 m²/g), and stoichiometric Ca/P ratio (1.66). Biocompatibility assessment by MTT assay confirmed cell viability above 80% at concentrations up to 200 μ g/mL in both MG-63 cells and primary human osteoblasts. Osteogenic assays revealed a significant increase in ALP activity and mineralisation at 50 μ g/mL, indicating that the HA-NPs actively stimulate bone-forming activity rather than merely being tolerated

passively. These findings provide a solid physicochemical and biological foundation for incorporating these HA-NPs into polymer-ceramic composite scaffolds for critical-size bone defect repair, which will be pursued in subsequent research phases.

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