

Yael del Carmen Suárez-López, ^{a,b} Reshma Vasantha Ramachandran, ^a
Varvara Platania, ^c Nikoleta N. Tavernaraki, ^c Alexandra Teleki, ^b
Maria Chatzinikolaïdou ^{*c,d} and Georgios A. Sotiriou ^{*a,e}

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Physiological bone consists of a tridimensional (3D) and porous scaffold made of collagen fibers that are 50 to 500 nm in diameter and have 40 nm gaps between the ends of the molecules, which accommodate hydroxyapatite crystals.¹ Hydroxyapatite is an inorganic crystalline structure composed of carbonated calcium phosphate that provides rigidity and roughness to the bones.^{2,3} With more than 2 million grafts done annually in the world, bone is the second most often transplanted tissue in the human body, after blood transfusions.² Bone transplants can be done as autografts, where tissue is grafted from the same individual. However, up to

8.6% of patients suffer from donor site complications, such as chronic pain, infections, fractures, or tissue morbidity, and the quantity of tissue that can be grafted is limited.^{4,5} An alternative to autografts are allografts, where the tissue is grafted from a different individual other than the patient, usually cadaveric tissue donors. However, there is the risk of immune rejection, potential disease transmission, and slower integration and healing. To address these issues, the development of synthetic materials able to replace natural bone tissue is needed.

Synthetic materials can facilitate cell migration, attachment, and formation of new bone tissue by mimicking the natural bone composition. This would in turn facilitate the integration of the synthetic material to the existing bone, avoid the triggering of adverse immune responses, and match the mechanical properties of the natural tissue. Considering this, recent research has focused on various materials and material structures to develop synthetic alternatives that replicate the structure of natural bone tissue.⁶⁻⁸

Calcium phosphate nanoparticles (CaP NPs) resemble natural bone hydroxyapatite. The large surface area of the NPs allows for cells to adhere to the material and aid in adsorption and retention of osteogenic components. Also, the roughness

^aDepartment of Microbiology, Tumor and Cell Biology, Karolinska Institute, 17177 Stockholm, Sweden. E-mail: georgios.sotiriou@su.se

^bScience for Life Laboratory, Department of Pharmacy, Uppsala University, 75123 Uppsala, Sweden

^cDepartment of Materials Science and Engineering, University of Crete, 70013 Heraklion, Greece. E-mail: mchatzin@materials.uoc.gr

^dFoundation for Research and Technology Hellas-IESL, 70013 Heraklion, Greece

^eScience for Life Laboratory, Department of Chemistry, Stockholm University, 11418 Stockholm, Sweden

tyl phosphate (Sigma-Aldrich, Sweden) was added to yield a solution with a 0.4 M total metal concentration. To obtain calcium phosphate nanoparticles (CaP NPs) with different carbonate content, the nominal ratio of calcium to phosphorus was either Ca/P 2.19 or Ca/P 1.67. To obtain CaP NPs with different crystallinities, the liquid precursor was fed through a capillary and dispersed by oxygen at two different rates, according to Table 1 (where $X:Y$ represents the precursor flow in mL min^{-1} (X) and the dispersion oxygen flow in L min^{-1} (Y)) with a pressure drop at the nozzle of 1.8 bar. The spray was ignited using a pre-mixed methane/oxygen flamelet with flow rates of 1.5 L min^{-1} and 3.2 L min^{-1} , respectively.

Particle characterization

X-ray diffraction (XRD) patterns were collected using a Rigaku MiniFlex 600, CuK α radiation with a 0.02° step and a 2° per min speed and analyzed using PDXL2 software (Rigaku, Germany). Fourier Transform Infrared (FTIR) spectroscopy was performed using a Cary 630 FTIR spectrometer (Agilent, Sweden) with a resolution of 4 cm⁻¹ in a 400–4000 cm⁻¹ spectral range. The specific surface area (SSA) was determined by the nitrogen adsorption–desorption isotherms (Brunauer–Emmett–Teller (BET) method) in liquid nitrogen at 77 K using a Tristar II Plus (Micromeritics, Norcross, GA, USA) instrument after degassing with N₂ for at least 3 h at 110 °C. The nanoparticle diameter (d_{BET}) in nm was then obtained by using eqn (1).

$$d_{\text{BET}} = \frac{6}{\rho \cdot \text{SSA}} \quad (1)$$

where ρ is the theoretical density of the sample (3.16 g cm^{-3}) and the SSA is given in $\text{m}^2 \text{ g}^{-1}$. The CO_3 content of the nanoparticles was calculated by taking the area under the curve (AUC) of the carbonate bands ($1350\text{--}1450 \text{ cm}^{-1}$) and dividing it by the AUC correspondent to the phosphate stretching ($1050\text{--}1150 \text{ cm}^{-1}$).²⁰ Furthermore, additional characterization to quantify the carbonate content based on thermogravimetric analysis (TGA) was carried out.²² In short, approximately, $85 \pm 10 \text{ mg}$ of each nanoparticle powder were placed in a platinum pan and analyzed using a TGA550 (TA Instruments, USA) under a nitrogen atmosphere with gas purge of 25 mL min^{-1} . The samples were first heated from room temperature to $150 \text{ }^\circ\text{C}$ at $20 \text{ }^\circ\text{C min}^{-1}$ followed by an isothermal period of 30 min to remove moisture. Then, the samples were heated to $550 \text{ }^\circ\text{C}$ at $20 \text{ }^\circ\text{C min}^{-1}$ and held at an isothermal period for 30 min to ensure complete combustion of organic matter. Finally, the samples were heated to $950 \text{ }^\circ\text{C}$ and held for 30 min

Particle synthesis

CaP nanoparticles were produced by flame spray pyrolysis (FSP) as previously described.¹⁶ A liquid precursor solution was prepared by dissolving calcium acetate hydrate (Sigma-Aldrich, Sweden) in a mixture of 1:1 propionic acid and 2-ethylhexanoic acid under reflux at 70 °C for 1 h. Then, tribu-

Table 1 Conditions used for flame spray pyrolysis

Nanoparticle	10 : 5 Ca/ P 2.19	10 : 5 Ca/ P 1.67	3 : 8 Ca/ P 2.19	3 : 8 Ca/ P 1.67
Ca/P ratio	2.19	1.67	2.19	1.67
Precursor rate (mL min ⁻¹) (<i>X</i>)	10	10	3	3
Dispersion oxygen rate (mL h ⁻¹) (<i>Y</i>)	5	5	8	8



To determine the number of nanoparticles originally loaded in the fibers, the 12 mm discs were dissolved in HFIP to remove the polymer, and the solution was centrifuged at 22 000g for 10 minutes to collect the CaP nanoparticles. These nanoparticles were then dissolved in 2% nitric acid and analyzed by ICP-OES. Calcium release results after 24 h were normalized to the nanoparticle loading in the fibers.

Biom mineralization characterization

For each sample of electrospun fibers containing calcium phosphate, 12 mm diameter circles were cut with a punch and hammer. Then, the fiber disks were sterilized in UV for 1 h (30 min on one side and 30 min on the other). Samples were placed separately on wells containing 4 mL of sterile simulated body fluid (SBF) at pH 7.4 and incubated for different intervals (0, 24, and 100 h) at 37 °C. Every 48 h, the SBF was completely replaced by 4 mL of a new sterile SBF to guarantee a constant liquid composition.¹⁵ Each experiment was carried out using 3 disks from the same scaffold. At the end of each time point, the SBF was removed, the disks were washed with MilliQ water, and the samples were left to dry in a hood overnight. After the samples were completely dry, they were placed in a metal mount with carbon tape to obtain SEM micrographs as previously described. In parallel, CaP NP powder in a concentration of 39 mg mL⁻¹, corresponding to the nanoparticles as integrated in the fibers, was dispersed in a sterile SBF and samples were taken at 0, 24, and 100 h intervals. Then, the powder was freeze-dried and XRD diffractograms were recorded as previously described.

Pre-osteoblastic cell culture maintenance and cell seeding

For the viability and morphology evaluation experiments, MC3T3-E1 pre-osteoblastic cells were cultured in alpha-MEM (minimum essential media) (PAN Biotech, Germany), supplemented with 10% FBS (PAN Biotech, Germany), 1% of stock solution penicillin/streptomycin (10.000 U mL⁻¹ pen, 10 mg mL⁻¹ strep, PAN Biotech, Germany), 1% L-glutamine (PAN-Biotech, Germany) and 1% amphotericin (Gibco, USA). Cells between passages 7 to 11 were used for the experiments. The cell-loaded membranes were maintained under a humidified atmosphere under 5% CO₂ at 37 °C. Culture media were changed every three days. For the osteogenic differentiation experiments, 10 nM dexamethasone, 10 mM β-glycerophosphate, and 50 μg mL⁻¹ L-ascorbic acid were added to the primary culture medium.

Cell viability and proliferation assay

The viability and proliferation of MC3T3-E1 pre-osteoblasts cultured in direct contact to the different fibrous scaffolds were quantitatively assessed. The resazurin-based metabolic assay PrestoBlue® (Invitrogen, USA) was conducted as previously described. Briefly, the membranes were cut into circular pieces, with a 10 mm diameter and placed into 48-well plates and each sample was seeded with 2.5 × 10⁴ cells. After 3, 6, and 14 days in culture, the PrestoBlue® reagent was added directly to the wells at a 1:10 dilution in a culture medium

and incubated at 37 °C for 60 min. The absorbance was measured at 570 and 600 nm using a spectrophotometer (Synergy HT, BioTek, USA). All samples were analyzed in quadruplicate.

Cell morphology via SEM

The morphology of MC3T3-E1 pre-osteoblasts seeded onto the fibrous scaffolds was observed using SEM (JEOL JSM-6390 LV), after 3 and 10 days in culture. For the monitoring of osteogenic differentiation of the MC3T3-E1 cells, the mineralization process was observed also *via* SEM, on day 21 of differentiation. Seeded scaffolds with 2.5 × 10⁴ cells per sample for the morphology evaluation and 4 × 10⁴ for the mineralization experiment were placed in a CO₂ incubator at 37 °C. In pre-determined time points, samples were removed from the incubator and rinsed three times with phosphate-buffered saline (PBS). Fixation was ensured by submersing the samples in 4% v/v paraformaldehyde for 20 min. Following that, samples were dehydrated in increasing concentrations (30–100% v/v) of ethanol. The cell-seeded fibrous scaffolds were finally dried using hexamethyldisilazane (HMDS), sputter-coated with a 20 nm thick layer of gold (Baltec SCD 050) and observed using a SEM at an accelerating voltage of 15 kV.

Measurement of secreted calcium

Secretion of calcium by cells is considered a late marker for osteogenesis and formation of the bone extracellular matrix. The secreted calcium concentration in culture supernatants has been determined by means of the *O*-cresolphthalein complexone (CPC) method, as previously described.²⁴ Briefly, a volume of 5 μL of the culture medium from the collected supernatants from each sample type was mixed with 50 μL of calcium buffer and 50 μL of the calcium dye. The solutions were transferred into the wells of a 96-well plate and the absorbance was measured at 570 nm (Synergy HTX Multi-Mode Microplate Reader, BioTek, USA). Samples were analysed in quadruplicate.

Alkaline phosphatase (ALP) activity measurement

Alkaline phosphatase activity was assessed on days 3, 7, and 14. Fibrous scaffolds were seeded with MC3T3-E1 pre-osteoblasts, and underwent thorough washing with PBS and subsequent submerging in 100 μL of lysis buffer (0.1% Triton X-100 in 50 mM Tris-HCl, pH 10.5). After that, samples were subjected to two freeze-thaw cycles, each for 10 min, from -20 °C to room temperature. Then, 100 μL of a 2 mg mL⁻¹ *p*-nitrophenyl phosphate (pNPP, Sigma, USA) diluted in 50 mM Tris-HCl at pH 10 with 2 mM MgCl₂ were added to each sample and incubated at 37 °C for 60 min. The absorbance was measured at 405 nm using a Synergy HTX plate reader (BioTek, USA) and was correlated to equivalent amounts of *para*-nitrophenol using a calibration curve. The enzymatic activity was calculated using the equation [units = nmol *p*-nitrophenol min⁻¹] and normalized by the total protein. All samples were analyzed in quadruplicate.



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CO₃/P ratio calculated by the area below the CO₃ and the P bands for each type of nanoparticle, and the CO₃% by thermogravimetric analysis,²² as well as the nanoparticle diameter (d_{BET} and d_{TEM}).

The electrospinning conditions that produced the most stable fibers without nanoparticles were assessed by a Design-of-Experiments (DoE) approach. A risk assessment through an Ishikawa diagram was constructed to identify variables that may influence critical quality attributes (Fig. S2a) and critical process parameters (Fig. S2b), ultimately impacting the electrospinning process and fibers in bone tissue engineering. Afterwards, the variables were ranked on severity, occurrence, and detectability using failure mode and effects analysis (Table S1). The polymer flow rate, polymer concentration, and voltage applied are known to influence fiber thickness during

Furthermore, the amorphous nanoparticles exhibited a higher CO₃ content than their crystalline counterparts with the same Ca/P ratio (Fig. 1c and Table 2). Controlling the carbonate content in flame-made CaP NPs allows for a systematic investigation of its effect in biomineralization, as it has been suggested that it plays a crucial role.^{30–32} Table 2 details the

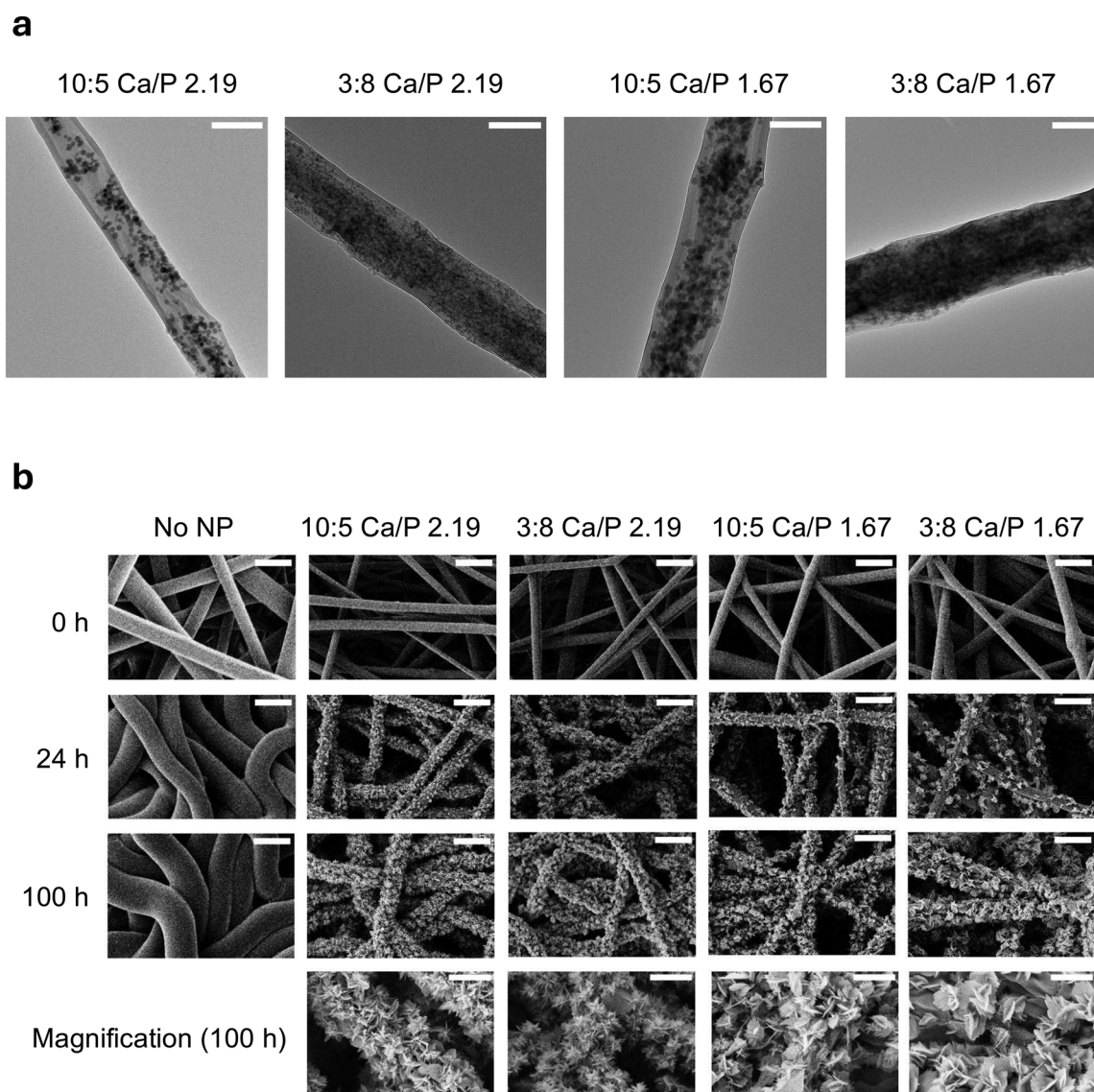
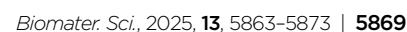


Fig. 2 (a) TEM micrographs of the fibers containing CaP NPs. Scale bar is 200 nm. (b) SEM micrographs of the fibers incubated in SBF for 24 and 100 h. Scale bar is 3 μm , except for the magnified micrographs, where the scale bar corresponds to 1 μm .

Transmission electron microscopy (TEM) micrographs of the CaP NP containing fibers are presented in Fig. 2a. Uniform distribution of the particles could be observed in all fibers, with the nanoparticles produced at bigger flames (10:5) appearing larger than the particles produced at a smaller (3:8) flame, in accordance with Fig. S4 as well. Ca^{2+} release was within 0.1–0.7% for all fiber samples (Fig. S7). Fig. 2b presents scanning electron microscopy (SEM) images from the biomineralization experiments at various time points, showing sig-



nificant CaP crystal deposition on the fibers, which increases with incubation time. Ca^{2+} release ranged from 0.1% to 0.7% across all fiber samples. The slight variations may be related to fiber thickness, as the 10:5 Ca/P ratio 2.19 sample also contained the thinnest fibers (Fig. S6 and Table S4). XRD analysis of SBF-incubated CaP NPs revealed a trend towards a hydroxyapatite crystalline phase, regardless of the initial crystallinity of the nanoparticles (Fig. S8). These observations indicated that hydroxyapatite deposition on fibers significantly increased over time, with longer incubation periods enhancing this effect. These results could be attributed to the role of CaP nanomaterials as sources of Ca^{2+} and PO_4^{3-} ions.⁹ The release of Ca^{2+} , PO_4^{3-} , and HPO_4 ions into the surrounding tissue

leads to local supersaturation, promoting hydroxyapatite reprecipitation on the scaffold.^{9,15} In solution, initial hydroxyapatite precipitation from CaP materials forms spherulites composed of radially oriented platelet crystallites. The carbonate content and initial crystallinity of the samples did not affect scaffold bioactivity, as hydroxyapatite crystals continued to grow over time in all four samples (Fig. 2).

The cytotoxicity of fibers incorporated with CaP NPs was determined by the PrestoBlue™ viability assay using the mouse calvaria-derived pre-osteoblastic cells MC3T3-E1.⁴⁰ Cell morphology was visualized *via* SEM. Fig. 3a and b show the viability after 3, 6, and 10 days of incubation with the fibers, as well as their morphology on day 6. The values on day 10

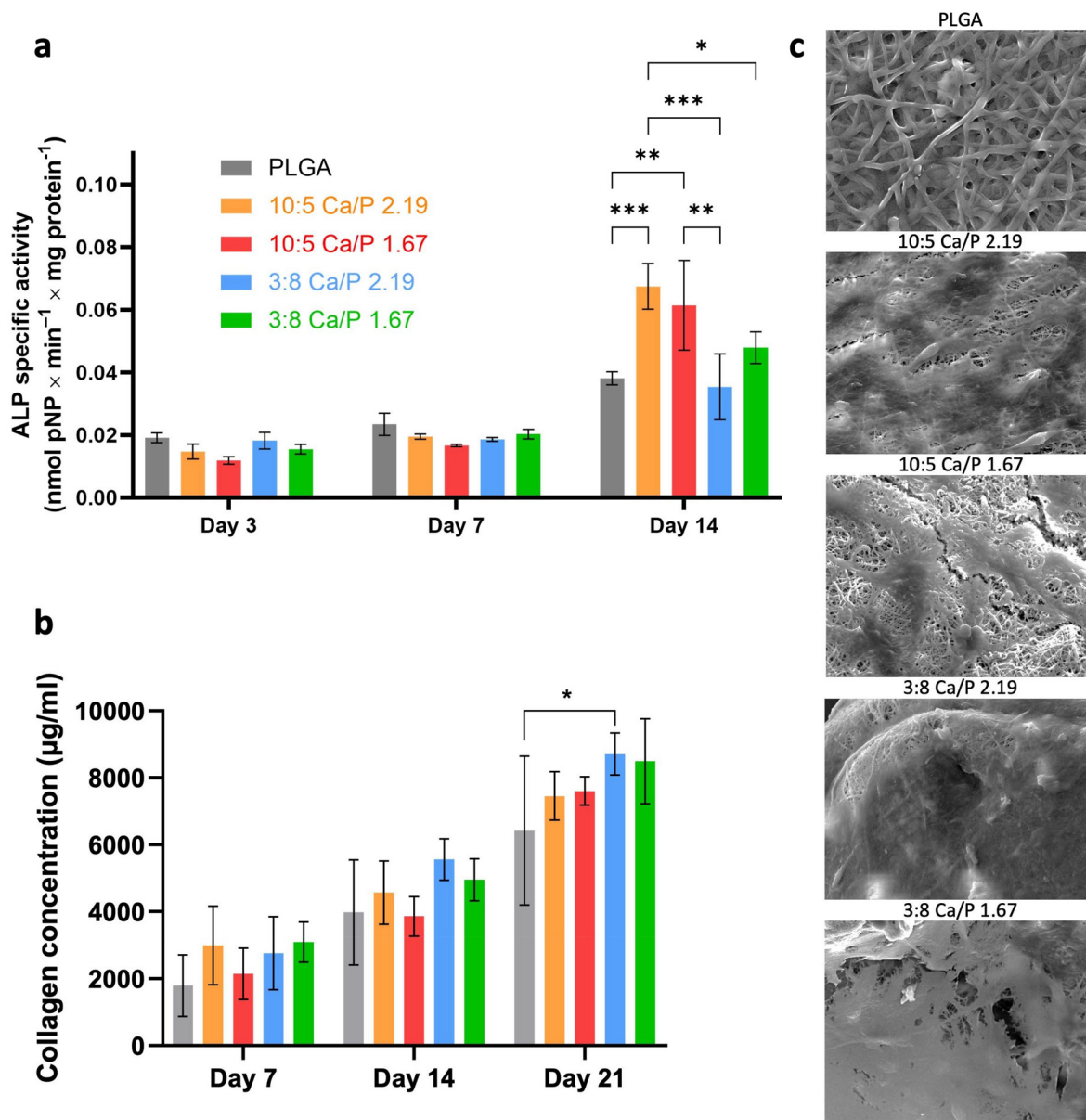


Fig. 4 (a) ALP activity and (b) collagen production of MC3T3-E1 cells in osteogenic media after 3, 7, and 14 days of incubation with PLGA fibers incorporated with flame-made calcium phosphate nanoparticles. (c) Cell morphology on fibrous scaffolds after 14 days.



showed that the fibers supported cell proliferation, with those containing crystalline CaP NPs (10 : 5 Ca/P 2.19) providing the most favourable environment. These observations were corroborated by the characteristic elongated cell morphology on day 6, across all five matrices, suggesting the absence of cytotoxic effects up to day 10 (Fig. S9). The cell layer density was higher for fibers with crystalline CaP NPs; however, cells reached confluence earlier on samples with amorphous particles (3 : 8 Ca/P 2.19 and 3 : 8 Ca/P 1.67), which may explain the observed decrease in cell proliferation on day 10 on these fibers, as shown in Fig. 3a. Thus, the cytocompatibility assessment revealed that fibers embedded with CaP NPs provided an optimal environment for the proliferation of pre-osteoblastic cells, with crystalline NPs being the preferred formulation.

The osteogenic potential of the fibers was evaluated by measuring ALP and collagen formation, both of which are indicative of the differentiation of pre-osteoblastic MC3T3-E1 cells into mature osteoblasts.^{41,42} Additionally, a cresolphthalein complexone (CPC) assay was performed to directly quantify soluble calcium secreted by the MC3T3-E1 cells (Fig. S10). The CPC assay showed that all groups increased calcium production over 21 days. Variations in the Ca/P ratio or flame conditions had a minimal effect on the calcium production, with all Ca/P groups performing similarly (Fig. S10). Cell morphology was observed *via* SEM. Fig. 4a, b and c illustrate the ALP activity, the produced collagen following 3, 7, and 14 days of incubation on the fibrous scaffolds, and cell morphology on day 14. The ALP activity peaked on day 14 in fibers containing crystalline CaP NPs (10 : 5 Ca/P 2.19), while collagen formation remained consistent across different matrices and exceeded that in blank PLGA fibers. This finding aligns with the literature where ALP activity is low in pre-osteoblasts but increases in immature and mature-osteoblast stages, whereas collagen type I is expressed in pre-, immature- and mature-osteoblast stages.⁴²

Scanning electron microscopy images further support these findings, showing clear signs of cell maturation by day 21 (Fig. S11). Additionally, mature osteoblasts formed clusters and aggregates as they produce and deposit bone matrix, which were observed in the images as denser areas of cells with a higher concentration of extracellular matrix material. Notably, ALP activity and SEM images suggest that this differentiation occurs more rapidly on fibers integrated with crystalline CaP NPs. Thus, fibers containing crystalline CaP NPs created the most favourable environment for osteogenesis, likely due to their hydroxyapatite phase, which is similar to the inorganic component of bones and possesses osteoinductive potential.^{43,44}

Prior research has shown that hydroxyapatite (HAP)-like crystalline CaP structures provide superior osteoinductive properties compared to amorphous calcium phosphate (ACP). For instance, Hu *et al.*⁴⁵ demonstrated that well-crystallized HAP nanoparticles promoted mesenchymal stem cell adhesion, proliferation, and differentiation more effectively than amorphous CaP nanoparticles. Similarly, ter Brugge *et al.*⁴⁶ investigated the effect of CaP coating crystallinity on rat bone marrow stem cell differentiation. They found that crystalline CaP coatings

stimulated cell differentiation to a greater extent than amorphous coatings, which exhibited negative effects on cell growth and differentiation. The findings of this study were in line with these observations, showing that crystalline CaP nanoparticles enhance osteogenic differentiation, likely due to their structural similarity to the inorganic phase of bones.

Conclusions

This study advances the understanding of how calcium phosphate (CaP) nanoparticle crystallinity and carbonate content influence biomineralization and osteogenic cell responses in electrospun poly(lactic-co-glycolic acid) (PLGA) scaffolds. By leveraging flame spray pyrolysis (FSP), the ability to control nanoparticle physicochemical properties in a scalable and industrially relevant manner was demonstrated. The integration of these nanoparticles into electrospun fibers resulted in composite scaffolds that successfully promoted hydroxyapatite deposition and supported osteogenic differentiation of pre-osteoblastic MC3T3-E1 cells. Neither the carbonate content nor the crystallinity affected the biomineralization of the developed scaffolds. However, scaffolds containing crystalline CaP nanoparticles (Ca/P = 2.19) exhibited the highest alkaline phosphatase activity and collagen production, highlighting the role of nanoparticle crystallinity in enhancing osteogenesis. These findings provide fundamental insights into the design of bioactive scaffolds for bone tissue engineering and underscore the potential of FSP-derived CaP nanoparticles for industrial-scale applications. Future work should focus on further optimizing the scaffold architecture for enhanced mechanical performance and exploring *in vivo* applications to validate the clinical potential of these biomaterials. Expanding this research could pave the way for next-generation bone graft substitutes that more closely mimic the complexity of natural bone regeneration.

Conflicts of interest

There are no conflicts of interest to declare.

Data availability

Data for this article, including raw data, are available at Karolinska Institutet's Electronic Notebook platform at eln.ki.se.

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