

Fabrication and Characterization of Electrospun Sr/Zn-Doped Nano-Hydroxyapatite-Collagen-PLGA Nanofibrous Scaffolds for Bone Tissue Engineering

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Introduction: Bone tissue engineering (BTE) presents a promising strategy for addressing critical-size bone defects that surpass the intrinsic regenerative capacity of bone tissue. This study presents the fabrication and characterization of biomimetic electrospun scaffolds composed of strontium/zinc co-doped nano-hydroxyapatite (Sr/Zn-nHAp), collagen, and poly(lactic-co-glycolic acid) (PLGA) polymer for BTE applications.

Methods: Electrospinning was employed to fabricate the scaffolds, which were subsequently analyzed for their structural, mechanical, bioactivity, biodegradation, and ion release characteristics.

Results and Discussion: X-ray diffraction (XRD) confirmed the successful incorporation of Sr/Zn into the nHAp lattice, reducing crystallinity and enhancing bioactivity. Fourier-transform infrared spectroscopy (FTIR) revealed characteristic peaks corresponding to nHAp, collagen, and PLGA, with clear evidence of chemical interactions between the constituents. Mechanical testing using nanoindentation demonstrated superior mechanical performance in the 4% Sr/Zn-doped scaffolds, with a Young's modulus of 9.91 ± 1.7 GPa and hardness of 0.30 ± 0.08 GPa, values comparable to those of cancellous bone. Scanning electron microscopy (SEM) showed a uniform nanofibrous structure with interconnected pores (2.9 ± 0.076 μ m). In vitro assay confirmed substantial bioactivity, with apatite formation after 14 days of immersion in simulated body fluid (SBF). The ion release study indicated a sustained release of Ca^{2+} , Sr^{2+} , and Zn^{2+} , which can promote osteogenesis and antibacterial activity. Biodegradation studies demonstrated a controlled degradation profile with minimal pH fluctuations.

Conclusion: Collectively, these findings suggest that the 4% Sr/Zn-nHAp-collagen-PLGA electrospun scaffolds possess favorable structural, mechanical, and biological properties, making them promising candidates for orthopedic applications.

Keywords: nanohydroxyapatite, electrospinning, strontium, zinc, collagen, PLGA, nanofibers, bone scaffolds

Introduction

Bones possess a remarkable ability to remodel and heal; however, this regenerative potential becomes insufficient when critical-size bone defects (typically larger than 2.5 cm) are present.¹ Such defects can result from trauma, bone tumor resection, or age-related bone loss and may exceed the bone's natural healing capacity, causing delayed healing or non-union fractures.² Moreover, the increasing population and aging demographics have led to a growing incidence of bone fractures.³ To address these challenges, bone tissue engineering (BTE) has emerged as a multidisciplinary field focused on developing innovative approaches for bone regeneration. Its primary goal is to create bioengineered scaffolds, often in combination with cells, growth factors, and biomaterials, to support the natural bone healing process.^{4,5} BTE aims to overcome the shortcomings of gold standard treatments, such as autografts and allografts, which can be associated with

complications like donor site morbidity, limited availability, and potential immune rejection.⁶ The success of BTE fundamentally relies on the strategic selection of biomaterials, as scaffolds must provide an optimal environment for bone cell attachment, proliferation, and migration, which are hallmarks of osteoconductive properties.⁷ Additionally, these biomaterials should promote the differentiation of osteoprogenitor cells into osteoblasts, demonstrating osteoinductive properties, while offering adequate mechanical strength and optimal pore size to promote vascularization and facilitate the efficient delivery of nutrients and oxygen to the newly formed bone tissue.^{7,8} This highlights the critical need for advanced biomaterial platforms capable of replicating the structural and functional characteristics of natural bone while meeting the complex demands of the bone regeneration process.

Recent advancements in BTE have highlighted nano-hydroxyapatite (nHAp) and collagen as promising biomaterials for bone regeneration due to their structural and compositional resemblance to the natural bone extracellular matrix (ECM), which consists of nHAp crystals embedded within collagen fibers.^{9–11} While nHAp provides superior bioactivity and osteoconductivity, collagen contributes to enhanced biocompatibility and biodegradability.¹⁰ However, their composite often lacks the mechanical strength required for surgical handling, fixation, and the support of osteogenic loads during the healing process.^{12–14} Numerous studies have explored the potential of nHAp-collagen scaffolds for BTE. Villa et al demonstrated their ability to support cell attachment and bone formation in vivo.¹⁵ While Yu et al reported enhanced osteogenic differentiation and bone formation with mineralized nHAp-collagen scaffolds.¹⁶ The mechanical properties of nHAp-collagen composites are often suboptimal. For example, Kikuchi et al reported that nHAp-collagen scaffolds prepared at 40°C exhibited a Young's modulus of 2.5 GPa, whereas those prepared at room temperature showed a significantly lower modulus of 0.54 MPa.¹⁷ Similarly, Ribeiro et al observed an elastic modulus ranging from 0.3 to 2.0 GPa using the nanoindentation technique.¹³ However, increasing the nHAp content beyond 30% can compromise scaffold integrity, as noted by Stanishevsky et al.¹² In contrast, natural bone exhibits a wide range of mechanical properties influenced by factors such as density, anatomical location, and testing conditions. Nanoindentation studies have reported Young's modulus values for cancellous bone between 1.3 and 22.3 GPa, while compressive testing indicates values ranging from 10 to 20 GPa.^{18–20}

The incorporation of trace elements such as strontium (Sr^{2+}) and zinc (Zn^{2+}) into the nHAp lattice has demonstrated considerable potential in improving scaffold biological properties. Sr^{2+} is well-documented for its dual effect of promoting bone formation while inhibiting bone resorption, thereby enhancing the scaffold osteoconductivity.²¹ Zn^{2+} , on the other hand, plays a vital role in bone metabolism and contributes antimicrobial properties.^{22,23} However, research on the co-doping of nHAp with both Sr^{2+} and Zn^{2+} on scaffold characteristics and properties remains limited. Moreover, the optimal doping concentrations of these elements must be determined to achieve maximal biological performance.

Synthetic polymers, such as poly(lactic-co-glycolic acid) (PLGA), are extensively utilized in scaffold fabrication due to their favorable mechanical properties and tunable degradation profile.^{7,24} However, PLGA inherently lacks bioactivity.²⁵ The incorporation of nHAp into PLGA can produce a composite scaffold that effectively integrates the structural integrity of PLGA with the bioactive characteristics of nHAp, thereby enhancing its potential for bone regeneration.²⁶

Bone's remarkable mechanical properties result from its complex architecture, where mineralized collagen nanofibrils align into hierarchical structures that provide strength.²⁷ Electrospinning has recently gained attention in BTE due to its simplicity, efficiency, and ability to create nanofibrous scaffolds with a high surface-to-volume ratio, closely mimicking the bone's ECM at both the micro- and nanoscale.²⁸ These nanofibrous structures promote cell attachment, proliferation, and osteogenic differentiation,²⁹ offering a more physiologically relevant 3D environment compared to conventional 2D scaffolds.³⁰ Electrospinning operates by applying a high voltage to a polymer solution, causing the formation of a Taylor cone. As the charged jet extends, it undergoes thinning and bending under the influence of the electric field before solidifying as fibers on a grounded collector.³¹ The standard electrospinning setup includes a high-voltage power supply, syringe pump, spinneret, and conductive collector. This versatile technique holds significant potential for fabricating biomimetic scaffolds for BTE applications.³²

Research has demonstrated the effectiveness of nanofibrous scaffolds in promoting bone regeneration. Yang et al reported that HA/Collagen/PLGA fibrous scaffolds significantly enhanced mesenchymal stem cell (MSC) attachment and osteogenic gene expression.³³ Similarly, another study found that nanofibrous collagen scaffolds improved osteogenic

differentiation and facilitated osteochondral defect repair in rabbit models, outperforming bulk collagen scaffolds.³⁴ Additionally, Telemeco et al observed that electrospun collagen scaffolds with cylindrical architecture supported endothelial cell infiltration and accelerated neovascularization in rat muscle tissue.³⁵ These findings underscore the potential of nanofibrous scaffolds to provide a bioactive and cytocompatible microenvironment for osteogenesis.

This study aims to develop and evaluate biomimetic composite scaffolds consisting of Sr/Zn-doped nHAp, collagen, and PLGA, fabricated using electrospinning. The research systematically investigates the impact of Sr/Zn co-doping on scaffold properties, with a comprehensive characterization of scaffold structural features, morphology, porosity, mechanical performance, bioactivity, biodegradation, and ion release. Collagen was added to Sr/Zn-nHAp to enhance biocompatibility, while PLGA was selected to improve structural stability and provide controlled degradation. Electrospinning, recognized for producing nanofibrous structures with interconnected pores, was employed to create scaffolds that closely mimic the native bone ECM. Unlike previous studies on Sr- or Zn-doped hydroxyapatite in bulk scaffolds or coatings, the present work integrates Sr/Zn co-doped nHAp with collagen and PLGA in an electrospun nanofibrous platform, closely mimicking the bone extracellular matrix. To our knowledge, this is the first systematic evaluation of Sr/Zn-nHAp-collagen-PLGA electrospun scaffolds, clearly distinguishing this work from earlier reports using single dopants, alternative polymers, or different fabrication methods. By fine-tuning the concentrations of Sr^{2+} and Zn^{2+} and optimizing fabrication parameters, the study aims to develop a multifunctional scaffold that supports bone regeneration and exhibits antibacterial effects. The findings are expected to advance the development of next-generation biomaterials for orthopaedic applications and offer valuable insights for clinical translation.

Materials and Methods

Sr/Zn-nHAp-Collagen-PLGA Scaffold Fabrication Using Electrospinning

Preparation of Electrospinning Solution

Sr/Zn-nHAp powders with different Sr/Zn concentrations (1%, 2.5%, and 4%) were prepared according to our previously published protocol.^{23,36} Briefly, nHAp was synthesized by chemical precipitation using calcium nitrate tetrahydrate (0.1 M) and ammonium phosphate dibasic (0.06 M) at a Ca/P ratio of 1.67. The phosphate solution was added to the calcium solution and aged overnight, then filtered and calcinated. For Zn- and/or Sr-substituted nHAp, zinc nitrate and/or strontium nitrate were incorporated into the calcium solution at 1, 2.5, or 4 mol% before precipitation, and the same synthesis and post-treatment steps were followed (Figure 1A). To prepare the electrospinning solution, 2.25 g of Sr/Zn-nHAp was dispersed in 10 mL of 1,1,1,3,3,3-Hexafluor-2-propanol [HFP, 99%, Sigma-Aldrich] and sonicated for 30 minutes to break up any potential agglomerates, then stirred for 1 hour. Type I collagen from rat tail [3 mg/mL, Gibco, NY, USA] and PLGA 75:25 pellets were added to the Sr/Zn-nHAp/HFP dispersion to create a 20% (w/v) polymer solution, followed by stirring until the polymer was fully dissolved (Figure 1B).

Electrospinning Process

The electrospinning SKE Research Equipment, model (E-Fiber EF100-HV4PSKe, MI, Italy), was used to create a fibrous scaffold. Electrospinning parameters, including voltage, flow rate, and tip-to-collector distance, were optimized to ensure uniform fiber morphology and scaffold consistency. The polymer multicomponent solution was loaded into a 10 mL syringe equipped with a 20-gauge blunt stainless-steel needle, and the solution was electrospun onto a rotating collector, covered with an aluminum foil, at 500 RPM, with an 18 kV voltage applied to the needle. The syringe pump operated at a flow rate of 1 mL/h, and the collecting drum was positioned 12.5 cm from the needle tip (Figure 1C). The process was carried out at room temperature. After drying the electrospun mat for 24 hours, it was removed and cut into squares. These squares were then stacked and pressed with a spatula to form a 3D scaffold (Figure 1D) of 10 mm (width) $\times$ 2 mm (thickness).³⁷

Scaffolds Crosslinking

The crosslinking protocol using 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide hydrochloride [EDC, Apollo Scientific Ltd, UK] was adapted from Barnes et al.³⁸ The scaffolds were cross-linked for 18 h in a freshly prepared 20 mM EDC/ethanol solution. Afterward, they were rinsed and soaked in sterile PBS for 2 h to hydrolyze unreacted O-isoacylurea

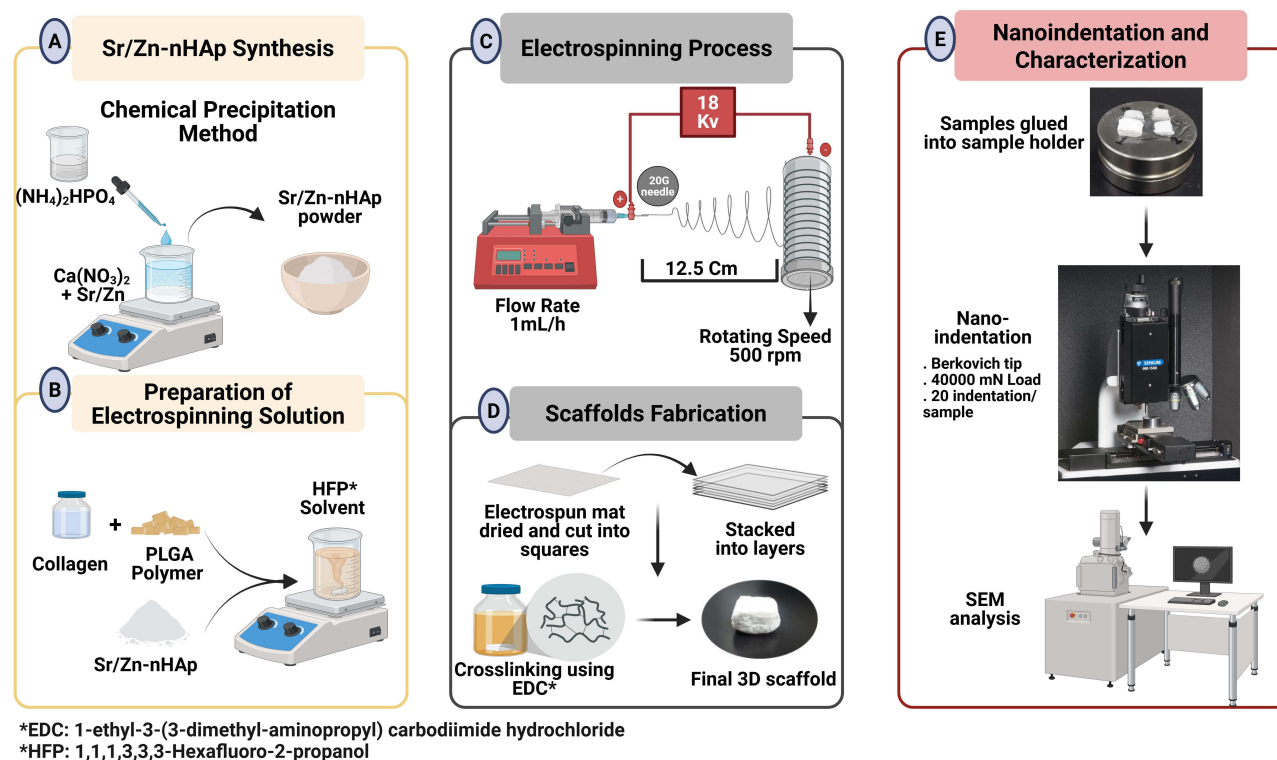


Figure 1 (A–E) Step wise procedure followed for the fabrication of Sr/Zn-nHAp-collagen-PLGA scaffolds using electrospinning (created using BioRender.com). **(A)** Chemical precipitation method **(B)** Preparation of electrospinning solution **(C)** Electrospinning process **(D)** Fabrication of scaffolds **(E)** Nanoindentation and characterization.

intermediates. Finally, the samples were dried at 40 °C for 2 h and air-dried overnight at room temperature before further testing (Figure 1D).

Electrospun Scaffold Characterization

Spectroscopic Characterization

The scaffolds were characterized using X-ray diffraction (XRD) and Fourier-transform infrared spectroscopy (FTIR). The structure of the prepared material, including phase composition and crystallinity, was analyzed with an X-ray diffractometer (Shimadzu Lab X, XRD-6100 Diffractometer, Kyoto, Japan). XRD data were obtained at 2θ angles ranging from 20° to 80°, with a scanning speed of 0.02°/min, 40 kV, and 30 mA, and the resulting patterns were compared with standards established by the Joint Committee on Powder Diffraction and Standards (JCPDS).³⁹

Fourier transform infrared (FTIR) spectroscopy (Thermo-Fischer Nicolet Nexus 470 FT-IR, Waltham, MA, USA) was used to determine the structure and chemical bonds of strontium/zinc co-doped nano-hydroxyapatite (Sr/Zn-nHAp), collagen, and poly(lactic-co-glycolic acid (PLGA) polymer. Scaffolds were crushed and mixed with potassium bromide (KBr) in a 1:100 ratio. The mixture was ground into a fine powder and then pressed into a circular wafer using a hydraulic press. Spectra were recorded with a resolution of 4 cm⁻¹ over a scan range of 400 cm⁻¹–4000 cm⁻¹, averaging 50 scans.

Mechanical Properties Using Nanoindentation

Samples were glued on a sample holder (Figure 1E) to secure the fixation on the nanoindenter stage and cleaned with soft tissue before each manipulation. Testing was performed using a nanoindenter device (IND-1500; Semilab Co. Ltd, Budapest, Hungary) equipped with a Berkovich diamond indenter tip and applying a high load of 40,000 mN. A total of 20 indentations were made per specimen, and the scaffold microstructure at each indentation site was examined using an optical microscope. The test was conducted in triplicate, and results were analyzed using IBIS software (Fischer-Cripps

Laboratories Pty Ltd, Australia) to obtain Young's modulus (E) and hardness (H) values from the loading-unloading curves.

Morphological Characterization

Scanning electron microscopy (SEM; JSM-6010PLUS, JEOL, Tokyo, Japan) was performed to examine the morphological features of the scaffold samples. Before imaging, the samples were mounted on aluminum stubs and sputter-coated with gold to enhance conductivity. SEM images were analyzed using ImageJ software to assess fiber diameter and pore size. Fiber diameter measurements were obtained by measuring three distinct points along the length of 50 randomly selected fibers, with the average recorded as the fiber diameter. Pore size analysis was conducted on three independent SEM images to calculate the average pore size.⁴⁰

Scaffolds Biodegradation and Bioactivity

The bioactivity of the scaffolds was assessed by observing the formation of an apatite-like phase after soaking in modified simulated body fluid (SBF) for 2 weeks. SBF was prepared according to the previous study.⁴¹ The composite scaffold samples were weighed, and the required amount of SBF for each sample was calculated using the equation: Volume of SBF needed = sample weight (g) $\times$ 50 mL/0.075 g.⁴⁰ Samples were placed in an agitator at 37°C, with the pH adjusted to 7.4. The solution was filtered, and the filtered scaffold material was sterilized by washing it with ethanol. SEM analysis was then performed to assess bioactivity through apatite crystal formation.⁴⁰

The biodegradation behaviors of different Sr/Zn-nHAp-collagen-PLGA scaffolds were determined by monitoring pH changes and the percentage of weight loss in SBF. The samples were incubated at 37°C for 35 days, and scaffolds were removed from SBF at various time points, then dried and weighed. The percentage of weight loss was determined using the following formula: Weight loss = $[W_0 - W_t] / W_0 \times 100$, where W_0 was the initial weight and W_t represented the dry weight at time t . The pH of the SBF was measured with a pH meter (Thermo Scientific, Orion Star, A211, Waltham, MA, USA).

Ions Release Kinetics

To assess the release kinetics of Ca^{2+} , Sr^{2+} , and Zn^{2+} ions from the fabricated scaffolds, scaffold samples were accurately weighed and immersed in SBF at a ratio of 50 mL per 0.075 g of scaffold material. The samples were incubated at 37 °C under gentle agitation. At predetermined intervals, aliquots of the SBF were collected and filtered to remove particulate matter. The concentrations of Ca^{2+} , Sr^{2+} , and Zn^{2+} ions in the collected samples were quantified using inductively coupled plasma mass spectrometry (ICP-MS; NexION 300X, PerkinElmer, Waltham, MA, USA). Calibration standards were prepared in SBF to match the ionic strength of the samples. Before analysis, samples were diluted 1:10000 with 2% nitric acid, which was prepared in deionized water to prevent precipitation during ICP-MS analysis.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism (version 8; GraphPad Software, San Diego, CA, USA). Data are presented as mean $\pm$ standard deviation (SD) unless otherwise stated. One-way analysis of variance (ANOVA) was conducted to compare the mechanical properties (Young's modulus and hardness) among the different scaffold groups. Bonferroni's post-hoc test was applied to identify significant differences between groups. Statistical significance was set at $p < 0.05$, with asterisks indicating significance levels as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Results and Discussion

Electrospun Scaffolds Structural and Spectral Analysis

X-Ray Diffraction

The XRD patterns of different Sr/Zn-nHAp-collagen-PLGA electrospun scaffolds are shown in Figure 2. Results revealed characteristic diffraction peaks of nHAp, with prominent peaks appearing at 25.9°, 31.8°, 32.9°, 34.2°, 39.9°, and 46.8°. These correspond respectively to the 002, 211, 112, 202, 310, and 222 crystallographic planes, as identified by

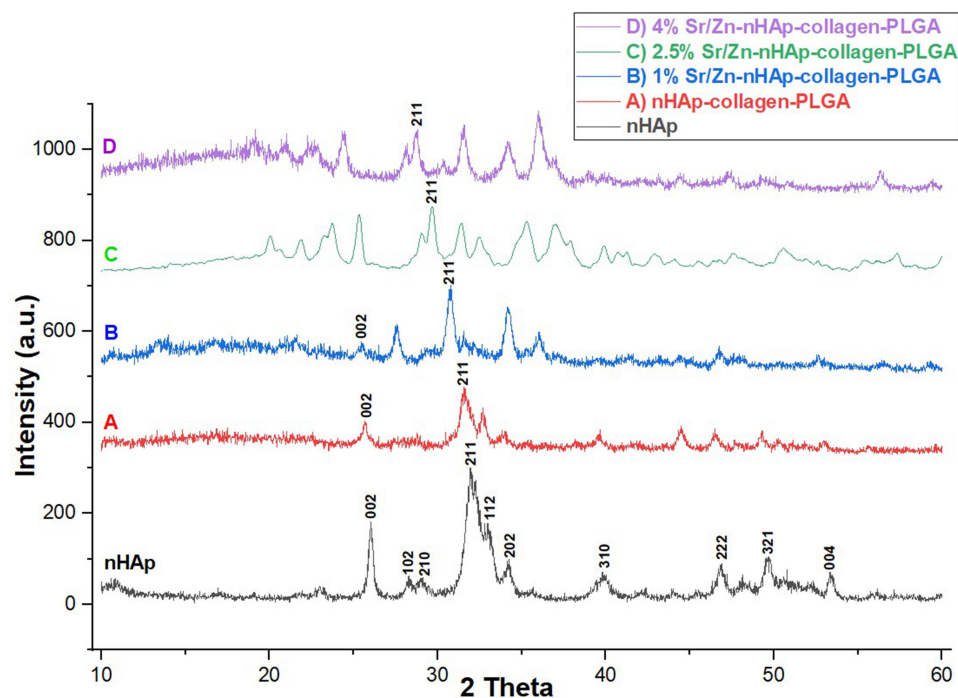


Figure 2 XRD patterns of different electrospun scaffolds.

JCPDS card no.009–0432. In Sr/Zn-nHAp-collagen-PLGA scaffolds, broadening and shifting of the peaks toward lower angles were observed, with the extent of the shift increasing proportionally to the Sr/Zn concentration, indicating decreased crystallinity. This is likely due to the larger ionic radius of Sr^{2+} compared to Ca^{2+} , causing an expansion in the nHAp lattice. It has been reported that high levels of ion substitution in nHAp resulted in peak broadening, which indicates successful Ca^{2+} substitution.^{42,43} Also, Sr^{2+} ions tend to preferentially occupy the Ca (II) sites due to their larger size, which aligns more closely with the spatial configuration of these sites. These sites are also more exposed and easier to access compared to the smaller, less accessible Ca (I) sites. This preference for Ca (II) site occupancy by Sr^{2+} may contribute to the observed reduction in crystallinity.^{43,44} Simultaneously, Zn^{2+} could also reduce the crystallinity by decreasing the Ca/P molar ratio.⁴⁵ Overall, the diffraction peak intensities of the Sr/Zn-nHAp-collagen-PLGA scaffolds were lower compared to pure nHAp. This decrease in intensity is attributed to a reduction in crystallinity, which results from the presence of amorphous collagen and PLGA, along with the co-substitution of Sr and Zn into the nHAp structure. The same characteristic peaks for Sr/Zn-nHAp-PLGA and decrease in crystallinity were identified in our earlier study (see [Supplementary Figure S1](#)).³⁶ Furthermore, Hayakawa et al demonstrated that apatite with lower crystallinity exhibited superior bone formation and osteointegration compared to highly crystalline composite scaffolds.⁴⁶ Similarly, another study showed that low crystallinity apatite coatings on collagen sponges enhanced bone regeneration in rat cranial defects, which was linked to their quicker resorption compared to amorphous calcium phosphate.⁴⁷ Additionally, low crystalline nHAp can promote bioactivity due to its higher dispersibility in SBF.⁴⁸ Poorly crystalline calcium-deficient HA demonstrated better protein adsorption capacity and enhanced osteoblastic cell proliferation and gene expression.⁴⁹

Furthermore, a broad, slightly raised peak in the range of 10–25° was detected, indicating the amorphous nature of collagen and PLGA (see [Supplementary Figure S2](#)). Studies have shown that PLGA/collagen exhibits a broad peak, characteristic of a random polymer structure between 10° and 30°, indicative of its amorphous state.^{50,51} The XRD data also indicated broad, low-intensity peaks in the amorphous region (20–25°) at higher Sr/Zn concentrations (2.5% and 4%). These peaks may indicate an altered interaction between nHAp, collagen, and PLGA at elevated Sr/Zn concentrations, leading to changes in the composite structure and resulting in additional peaks within the amorphous region. The increased Sr/Zn concentrations may enhance the interactions between Sr/Zn-nHAp and the collagen/PLGA matrix,

potentially leading to more ordered structures or new phases at the interfaces. These structural changes could explain the additional peaks observed between 20° and 25° (2θ), possibly reflecting the formation of semi-crystalline phases or new structural arrangements influenced by Sr^{2+} and Zn^{2+} incorporation. Previous work has shown that collagen-nHAp-PLGA composites can form crystalline structures within the material as compared to PLGA alone, suggesting that collagen significantly influences the structural organization and can facilitate the formation of crystalline structures within the nHAp-PLGA composite.⁵²

Fourier-Transform Infrared Spectroscopy

The FTIR spectroscopy results for the different electrospun scaffolds are displayed in Figure 3. The spectroscopic analysis confirmed the presence of characteristic bands for nHAp, collagen, and PLGA, verifying successful bonding and composite formation. The nHAp exhibited distinct bands corresponding to the phosphate (PO_4^{3-}) groups, with peaks at 560 cm^{-1} and 1040 cm^{-1} , representing the ν_4 bending mode and ν_3 asymmetric stretching of phosphate ions, respectively.³⁶ Additionally, the hydroxyl (OH^-) groups were observed with a stretching mode at 3570 cm^{-1} and a vibrational mode at 630 cm^{-1} .^{53–55} A reduction in the peak intensity of the PO_4^{3-} and OH^- groups was observed as the percentage of Sr/Zn substitution increased. This decrease is likely due to changes in the lattice structure and crystallinity, as confirmed by the XRD analysis.^{56,57}

The characteristic band for collagen's amide I was observed at 1618 cm^{-1} , primarily attributed to the C=O stretching vibration of the peptide bonds in the collagen structure. According to previous studies, the amide I band typically appears within the range of $1600\text{--}1700\text{ cm}^{-1}$.^{58–60} The bands for collagen amides II and III were not prominent in the FTIR results, likely due to overlap with the strong bands attributed to PLGA CH_3 bending or nHAp phosphate stretching. Previous studies showed that in a PLGA/collagen-nHAp composite, the collagen amide II band can overlap with the carbonate group peak at 1631 cm^{-1} , while the C-O stretch from PLGA can overlap with the nHAp phosphate group at 1039 cm^{-1} .^{61,62} Additionally, the band at 1458 cm^{-1} (see Figure 3) is likely due to an overlap between the CH_2 wagging of glycine in collagen and the CH_3 bending in PLGA. Amide vibrational regions typically have absorption bands that are close in wavelength, which can result in a significant overlap between them.⁶³ Other characteristic bands of PLGA were observed at 1760 cm^{-1} , 1095 cm^{-1} , and 2940 cm^{-1} , corresponding to carbonyl stretching, C-O-C stretching, and

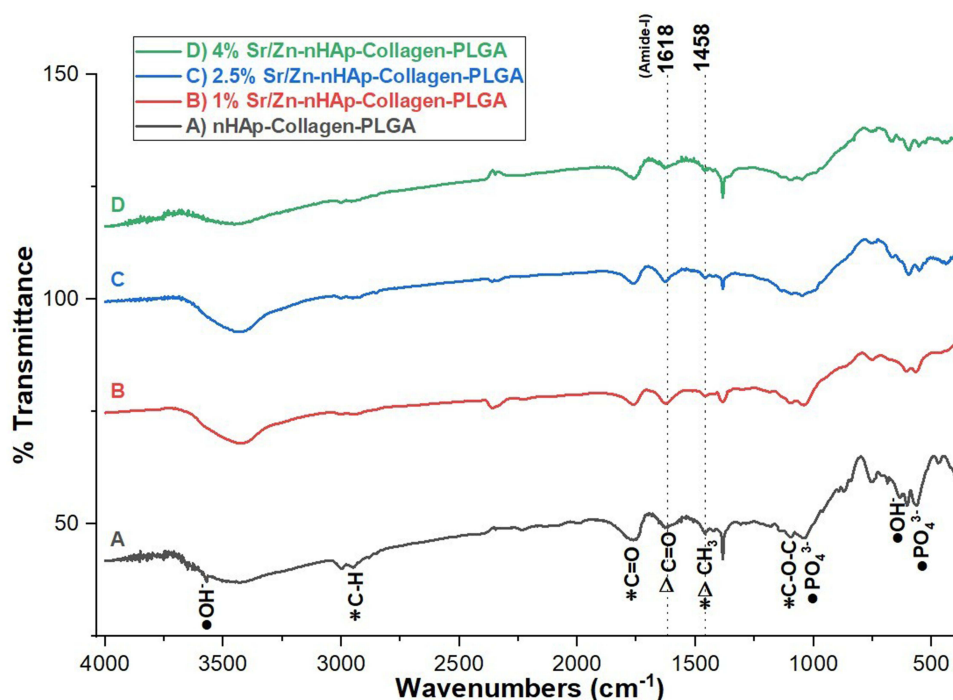


Figure 3 FTIR spectroscopic analysis of different scaffolds. Functional groups correspond to the following components: nHAp (•), PLGA (*), and collagen (Δ).

C-H stretching vibrations, respectively.⁶⁴ The FTIR analysis confirmed the successful integration of Sr/Zn-nHAp-collagen-PLGA scaffolds, indicating modifications in the lattice structure. FTIR analysis suggests a chemical interaction between nHAp, collagen, and PLGA, as there was a shift in OH and C=O regions as we increased the dopant concentration, which could suggest potential interaction through hydrogen or covalent bonding.^{65,66} The combination of these materials and the observed spectral features suggests that the scaffolds have significant potential to enhance both bioactivity and biocompatibility in BTE.

Electrospun Scaffolds Mechanical Properties

Understanding the mechanical behavior of biomaterials is crucial for assessing their potential applications. Figure 4 represents the results of the mechanical properties, namely Young's modulus (E) and hardness (H) of various electrospun fibers using the nanoindentation technique. The E values for nHAp-collagen-PLGA and 1%, 2.5%, 4% Sr/Zn-nHAp-collagen-PLGA were 3.37 ± 0.69 , 7.46 ± 0.95 , 9.0 ± 1.6 , and 9.91 ± 1.7 GPa, respectively. Moreover, the H values were 0.13 ± 0.02 , 0.12 ± 0.05 , 0.21 ± 0.09 , and 0.3 ± 0.08 GPa. The results showed that scaffolds doped with Sr^{2+} and Zn^{2+} exhibited a significant increase in mechanical properties compared to non-doped scaffolds. This suggests that the incorporation of Sr^{2+} and Zn^{2+} significantly enhanced the mechanical properties of various scaffolds, with a doping percentage of 4% showing the highest E and H values ($P = 0.005$ for E, $P = 0.017$ for H) compared to non-doped scaffolds.

The improvement in mechanical performance with Sr/Zn doping can be attributed to the partial substitution of Sr^{2+} and Zn^{2+} for Ca^{2+} within the hydroxyapatite lattice, which induces lattice distortion and enhances crystallinity. These changes improve load transfer between the ceramic phase and the polymer matrix. In addition, ionic interactions between the doped nHAp particles and collagen/PLGA macromolecules may promote cross-linking within the composite, thereby contributing to the increased stiffness and hardness observed in the 4% Sr/Zn-doped scaffolds. The same finding was confirmed by Sprio et al,⁶⁸ where multi-doped hydroxyapatite exhibited Young's modulus and hardness values 8% higher than those of singly doped scaffolds when assessed using nanoindentation. This implies that incorporating multiple dopants in materials can enhance their mechanical properties, resulting in increased hardness, stiffness, and structural uniformity compared to materials doped with a single element. Nanoindentation studies have reported a wide range of average Young's moduli for human cancellous bone, spanning between 1.3–22.3 GPa.²⁰

Mechanical properties are critical in bone scaffolds, where the stiffness is expected to mimic that of native bone ECM. This similarity helps regulate cell attachment and migration, as cells can sense the ECM's stiffness and generate signals for attachment within the scaffold.⁶⁹ Scaffold, consisting of 4% Sr/Zn-nHAp-collagen-PLGA, exhibited

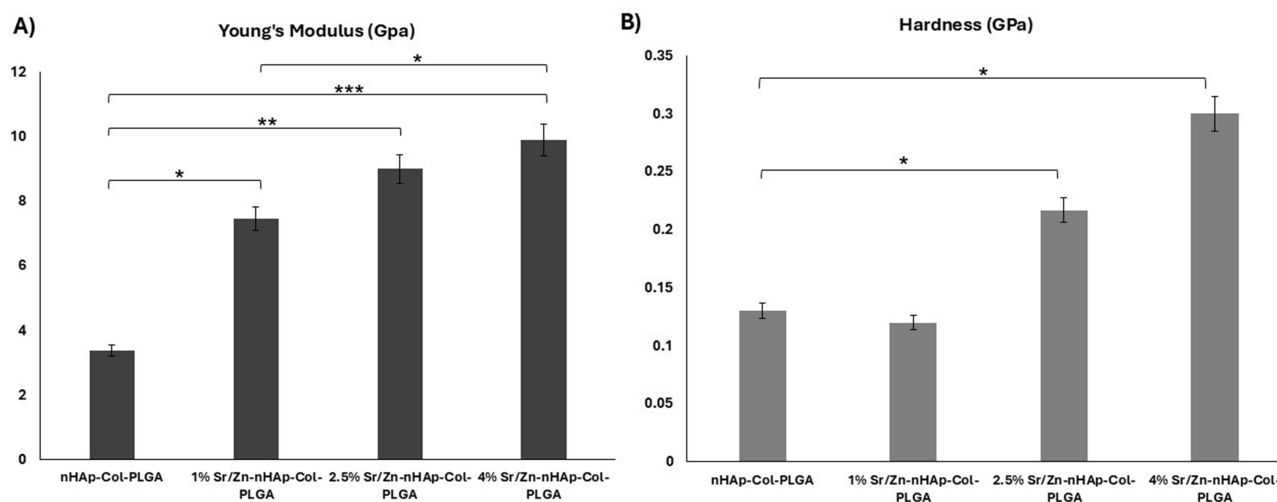


Figure 4 Young's modulus (A) and hardness (B) for different electrospun scaffolds. Statistical analysis was performed using one-way ANOVA with Bonferroni's post-hoc test, $P < 0.05$; asterisks indicate significant differences (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$). Reproduced with permission from Hassan, M. and Mohsin, S., IEEE, 2024.⁶⁷

a Young's modulus of 9.91 ± 1.7 GPa, suggesting its suitability for bone regeneration applications. Our previous scaffold fabricated using super-critical CO_2 (ScCO_2) showed that higher doping percentages (4% Sr/Zn-nHAp-PLGA) demonstrated reduced mechanical properties, most likely due to poor dispersion.³⁶ However, in electrospun scaffolds, the 4% Sr/Zn-nHAp-collagen-PLGA scaffolds exhibited the highest mechanical properties, indicating improved dispersion between scaffold components.

Electrospun Scaffold Morphology

Nanofibers Diameters and Porosity

The nanofibrous mats produced from 4% Sr/Zn-nHAp-collagen-PLGA were visualized using SEM, which revealed a smooth, uniform, and continuous nanofiber network without bead formation, indicating a homogeneous structure. The average fiber diameter measured was 331.6 ± 96.11 nm ($0.3 \mu\text{m}$), and the scaffold was porous with an average pore size of $2.9 \pm 0.76 \mu\text{m}$ (Figure 5A). Histograms of the average fiber diameters and pore size obtained from SEM images are shown in Figure 5B and C.

Many studies have shown that smaller fiber diameters (~ 300 – 350 nm) can enhance cell proliferation, maintain cellular phenotype, promote osteogenic differentiation, and modulate immune activity more effectively than larger fibers or flat surfaces.^{70–72} However, the scaffold pore size is shown to be below the typical pore size required for bone tissue

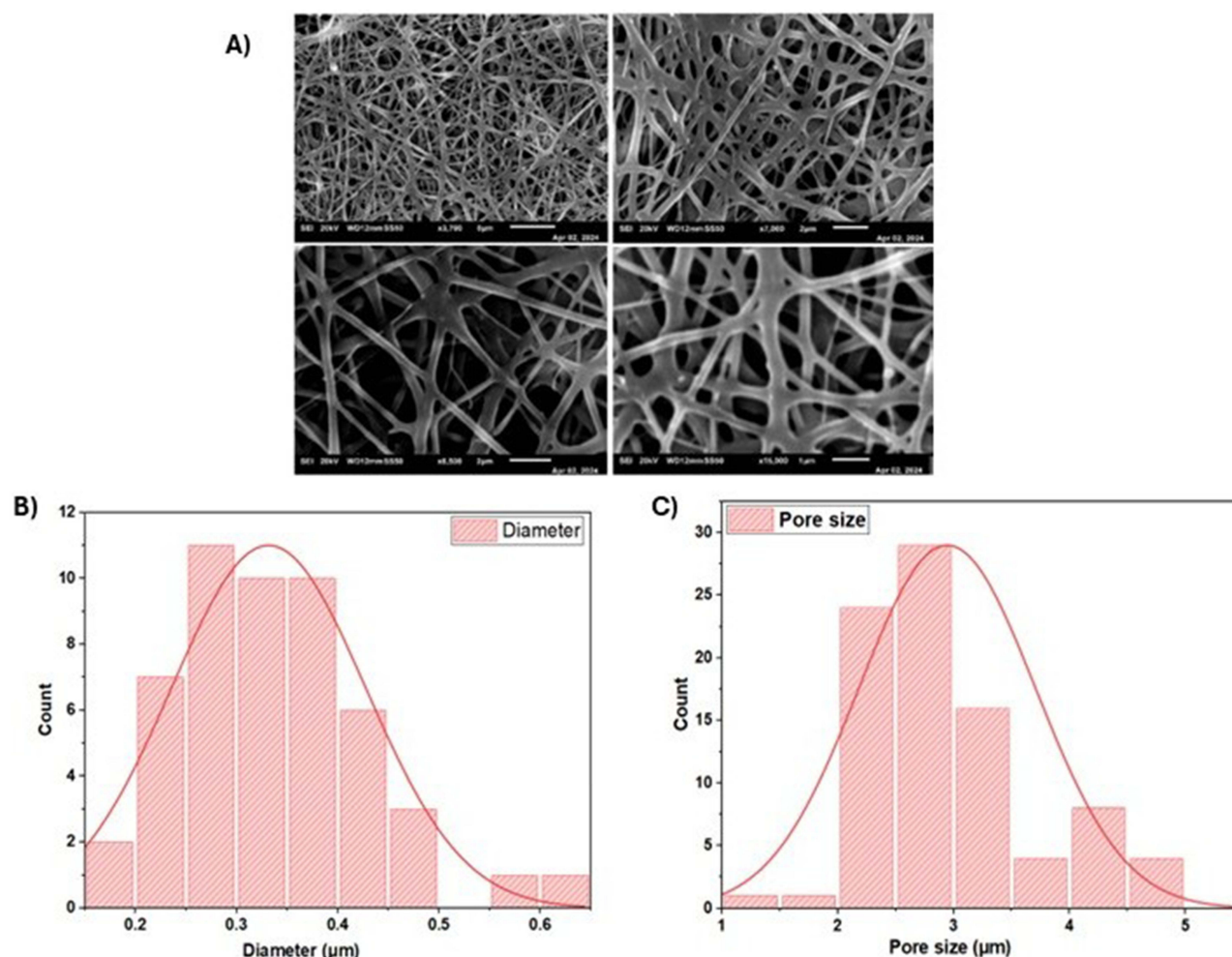


Figure 5 SEM images of 4% Sr/Zn-nHAp-collagen-PLGA scaffolds showing the nanofibrous structure (top) (A), along with histograms of average fiber diameters (B) and pore size distribution (C) obtained from SEM analysis (bottom). SEM images reproduced with permission from Hassan, M. and Mohsin, S., IEEE, 2024.⁶⁷

ingrowth. Generally, the size of osteoblasts ranges from 10–50 μm and requires a pore size of 100–200 μm for cell proliferation and infiltration.⁷³ Such a limitation is well-known for electrospun fiber mats.^{74,75}

The inherently tiny pore size is a direct consequence of the electrospinning process, as ultrafine fibers deposit densely in a random, sheet-like layer, resulting in high overall porosity but extremely small pore size (typically <10 μm).⁷⁶ Despite these pore-size limitations, electrospun nanofiber scaffolds remain highly relevant in BTE. Their fibrous architecture mimics the ECM, providing a high surface-area-to-volume ratio and biomimetic topography that promote cell adhesion and osteogenic differentiation on the scaffold surface.⁷⁷ Recent studies have shown that a multilayered scaffold (stacked) configuration creates interlamellar pores and increased vertical porosity, which can enhance cell infiltration beyond what 2D electrospun mats typically allow.⁷⁸ For instance, nanofiber mats are often combined with or layered onto coarse, 3D-printed or foam scaffolds to provide both the ECM-like nanoscale cues and the larger pore channels needed for vascularized tissue ingrowth.⁷⁷ Additionally, multilayered scaffolds of PLGA and collagen were able to support MC3T3-E cell adhesion and spread.⁷⁹ Similarly, in our study, the stacked electrospun layers formed a three-dimensional architecture that introduced interlayer spacing to enhance overall scaffold thickness and porosity, which may contribute to improved oxygen diffusion, nutrient transport, and potential cell infiltration despite the small intrinsic pore size of the individual fiber layers.

Dissolution and Stability of Electrospun Scaffolds

Figure 6A shows the pH measurements of different electrospun scaffolds after incubation in SBF. The pH for different scaffolds A: nHAp-collagen-PLGA, B: 1% Sr/Zn-nHAp-collagen-PLGA, C: 2.5% Sr/Zn-nHAp-collagen-PLGA, and D: 4% Sr/Zn-nHAp-collagen-PLGA started at a physiological pH of 7.4 at day 0. A slight steady increase in the pH was observed across all scaffolds in days 1–7, which indicates PLGA dissolution and ion release. PLGA breaks down through the hydrolysis of its ester bonds, resulting in the release of lactic acid and glycolic acid.⁸⁰ Scaffold D exhibited the highest pH by day 7, from 7.4 to 7.61, corresponding to the highest content of Sr and Zn ions (4%). By day 35, the pH further increases, with scaffold D also having the highest pH (8.04), followed closely by scaffolds B and C. The rise in pH could indicate the release of ions from the scaffolds into the SBF, which may contribute to the alkaline environment. All scaffolds exhibited higher pH, indicating an ongoing dissolution process; however, the increase was minimal, suggesting that the dissolution occurs at a slow rate, as appeared in the weight loss study. Mao et al have shown that HA can neutralize the acidity of PLGA by reacting with the hydrogen dissociated from lactic and glycolic acid, resulting in a stabilized pH of 7.1.⁸¹ Also, the addition of HA to PLGA can control the pH during dissolution.⁸² Another study showed that Sr-doped Zn-Ca-P coatings maintained the pH and exhibited a negligible rise upon immersion in SBF.⁸³ Moreover, Zreiqat et al showed that adding Zn and Sr to a calcium-silicon system maintained a controlled pH between 7 and 8, reducing the dissolution rate and improving scaffold chemical stability. In contrast, undoped CaSiO_3 scaffolds showed higher, potentially toxic pH levels. The addition of Sr and Zn improved the chemical stability, biocompatibility, and bioactivity of the scaffolds.⁸⁴ The results suggest that the incorporation of Sr/Zn doped nHAp into PLGA matrix

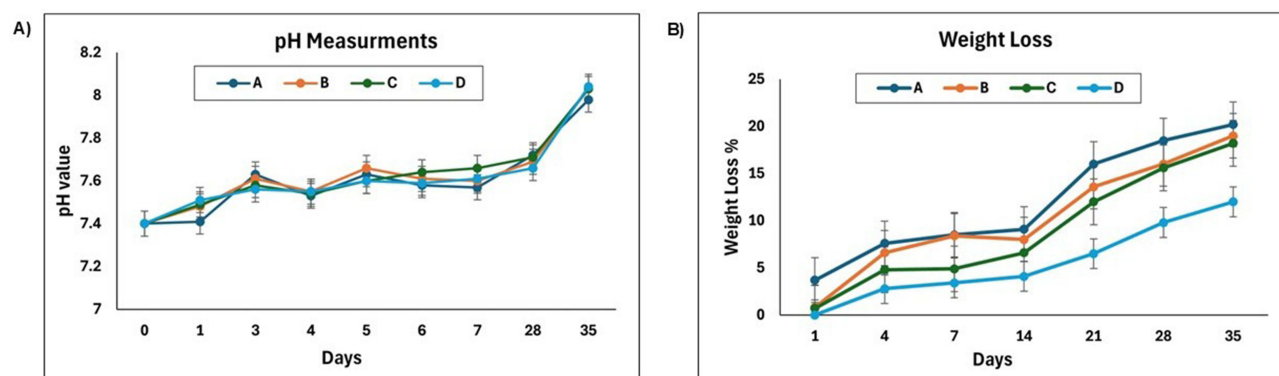


Figure 6 pH values (A) and weight loss (B) of different scaffolds after immersion in SBF: (A) nHAp-collagen-PLGA, (B) 1% Sr/Zn-nHAp-collagen-PLGA, (C) 2.5% Sr/Zn-nHAp-collagen-PLGA, (D) 4% Sr/Zn-nHAp-collagen-PLGA.

neutralizes the pH as dissolution progresses. Higher concentrations of Sr/Zn tend to increase pH levels as more ions will be released, resulting in neutralization effects which can enhance the scaffold's biological activity, improve its stability and make it more suitable for long-term implantation.

Figure 6B represents the dissolution and weight loss percentage of different scaffolds in SBF. Scaffold A exhibited the highest dissolution rate throughout the study and lost about 20.2% of its weight by day 35, indicating rapid dissolution compared to Sr/Zn doped nHAp-collagen-PLGA scaffolds. Scaffolds B and C showed a moderate increase in weight loss, reaching 19% and 18.2% respectively, by day 35. Scaffold D with the highest Sr/Zn concentration (4%) consistently displayed the slowest degradation, losing 12% of its weight by day 35. Dissolution study results demonstrated that adding Sr/Zn ions into the scaffold reduces the dissolution rate, with scaffold D exhibiting the highest stability over time, which was also proved by the results of mechanical testing. Previous studies have also shown that doped nHAp exhibited low dissolution and increased stability.^{85–87} This makes the scaffold suitable for extended implantation and provides great stability under physiological conditions.⁸⁸

Electrospun Scaffolds Bioactivity in vitro

Electrospun 4% Sr/Zn-nHAp-collagen-PLGA scaffolds exhibited significant bioactivity after a 2-week immersion in SBF (**Figure 7**). SEM images revealed the formation of a needle-like, rosette-shaped crystal. Fibers and pores were also observed, as indicated by red and white arrows in SEM images. The organized arrangement of needle-shaped crystals suggests active mineralization, which is crucial for promoting a positive interaction between the scaffold and the bone tissue environment.⁸⁹ The presence of these needle-shaped nanocrystals indicates a well-organized mineralization process, resembling the natural composition of bone minerals, which are mainly made of calcium phosphate. The needle-

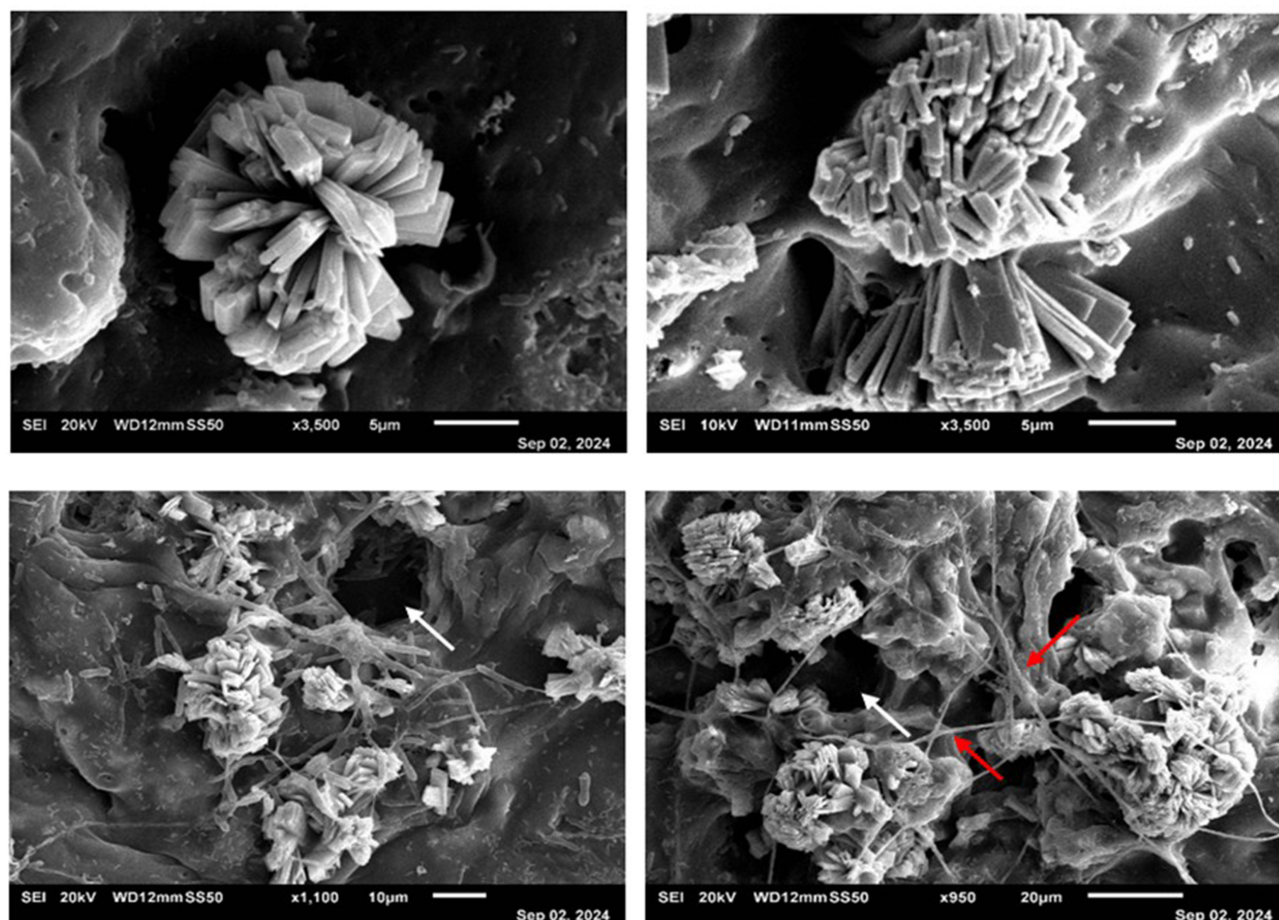


Figure 7 Formation of apatite crystals on 4% Sr/Zn-nHAp-collagen-PLGA scaffolds after 14 days of incubation in SBF. Fibers are indicated in red arrows and pores in white arrows.

like morphology suggests the nucleation and growth of apatite crystals in a hierarchical arrangement, mimicking the structure of natural bone.⁹⁰ Additionally, rosette-shaped hydroxyapatite crystals were observed in the electrospun samples due to rapid solvent evaporation and high supersaturation, which promote fast, multidirectional nucleation and aggregation of crystals around collagen fibrils. Previous studies have demonstrated that a fibrous structure can encourage the formation of needle-like apatite crystals, which promote crystal enlargement and enhance the mechanical strength of the scaffold.⁹¹

Numerous studies have shown that Zn-doped HA promotes the formation of apatite crystals in SBF, indicating enhanced bone activity.^{92–94} Similarly, Sr-doped HA has been found to improve bioactivity and facilitate apatite formation, as it nucleates more easily compared to pure HA.^{95–98} In summary, bone mineralization is closely linked to bone stiffness, as higher mineral content leads to increased stiffness.⁹⁹ This correlation aligns with the mechanical testing results, which suggest that the simultaneous doping of Sr and Zn, combined with the fibrous scaffold structure, synergistically promoted apatite crystal growth and improved the scaffold's mechanical strength.

Ions Release Kinetics

The ion release profiles of the various scaffolds are shown in Figure 8. The Ca^{2+} release profile varied across scaffold compositions. The nHAp-Col-PLGA scaffold exhibited a low initial release, peaking by Day 7, followed by a decline and plateau. The 2.5% Sr/Zn-nHAp-Col-PLGA scaffold showed high initial release, with a rapid decrease by Day 7 and continued reduction until Day 35. The 1% Sr/Zn-nHAp-Col-PLGA scaffold demonstrated a more sustained release, from Day 1 to Day 21, before decreasing. The 4% Sr/Zn-nHAp-Col-PLGA scaffold exhibited an initial peak followed by a sharp decline and gradual decrease. The initial release in the 4% scaffolds can promote early bioactivity, potentially supporting long-term calcium availability for bone mineralization.¹⁰⁰ Regarding the Zn^{2+} release profile, which varied with doping concentration, the 4% Sr/Zn-nHAp-Col-PLGA scaffold exhibited the highest initial release, followed by subsequent controlled reduction until Day 35. In comparison, the 2.5% and 1% scaffolds exhibited lower initial releases with similar declining patterns. This initial release in 4% Sr/Zn scaffolds may provide effective antibacterial activity

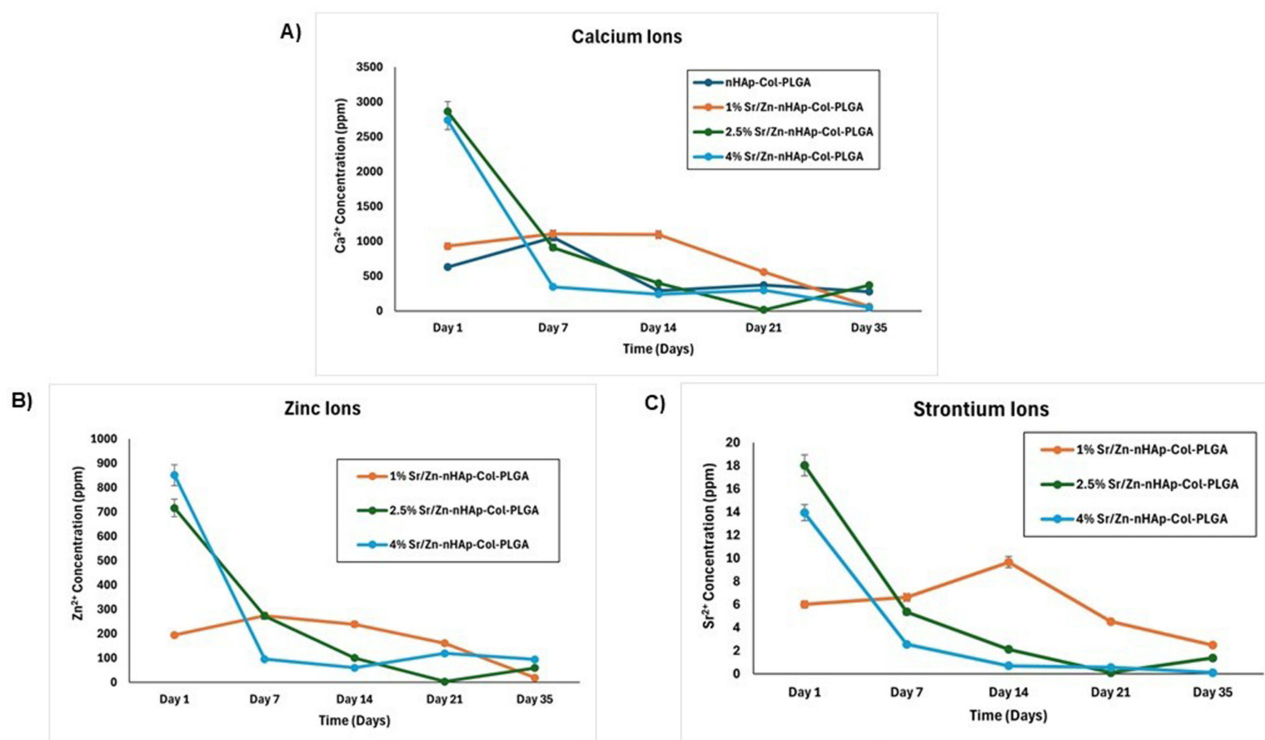


Figure 8 The release profile of Calcium (A), zinc (B), and strontium (C) ions from the scaffolds after immersion in SBF.

during the critical early implantation phase, supporting the scaffold's potential for infection control while maintaining a favorable environment for osteogenic activity.¹⁰¹

The release of Sr^{2+} followed a concentration-dependent pattern. The 2.5% Sr/Zn-nHAp-Col-PLGA scaffold exhibited the highest initial release, followed by a rapid decline by Day 7. The 4% Sr/Zn-nHAp-Col-PLGA scaffold showed a similarly elevated initial release followed by a sharp decrease within the first week. In contrast, the 1% Sr/Zn-nHAp-Col-PLGA scaffold had a lower initial release, peaking around Day 14 before gradually declining. The 4% Sr/Zn-nHAp scaffold's high initial release, paired with a controlled reduction, may effectively support early osteogenic stimulation while minimizing the risk of excessive ion exposure.^{102,103} The ion release profiles demonstrate the influence of scaffold composition, ion doping, and material degradation. In our study, we restricted Sr/Zn doping to $\leq 4\%$, as our earlier work showed 4% achieved good cytocompatibility and mechanical balance, while higher levels caused poor dispersion.^{23,36} Reports also indicated that $\sim 10\%$ Zn can induce cytotoxicity, whereas $\sim 5\%$ is generally safe.¹⁰⁴ The 4% Sr/Zn-nHAp-Col-PLGA scaffold, with its pronounced initial release, demonstrates strong potential for antibacterial and osteoinductive properties during the early implantation phase.

Limitations and Future Directions

A limitation of the present study is that we did not directly assess antibacterial activity or osteogenic potential of the electrospun Sr/Zn-nHAp-collagen-PLGA scaffolds. Although our earlier work has demonstrated these effects in related scaffold systems, future studies are needed to validate them specifically in this electrospun platform. In addition, the effects of the scaffold materials on cell adhesion, proliferation, and osteogenic differentiation were not investigated. These cellular-level evaluations are critical to fully establish the scaffold's potential for bone regeneration and will be addressed in future work.

Further work should also explore higher Sr/Zn doping concentrations with optimized processing, along with careful monitoring of ion release and cytocompatibility, to enhance scaffold performance. Tensile testing was also not carried out in this study, although it would have complemented nanoindentation by assessing bulk mechanical behavior and overall scaffold strength. Future work should include tensile testing to provide a more comprehensive evaluation, particularly for load-bearing applications. Finally, long-term in vivo studies are required to evaluate the scaffold's interactions within living systems, optimize ion release kinetics, and assess performance in complex bone defect models to advance clinical translation.

Conclusion

In this study, we successfully fabricated and characterized multicomponent scaffolds for potential application in BTE. The integration of Sr/Zn-nHAp-collagen-PLGA resulted in a biomimetic structure that closely mimics the natural bone ECM, offering an optimal balance of bioactivity, mechanical strength, and structural characteristics. Among the formulations tested, the 4% Sr/Zn-nHAp-Collagen-PLGA scaffold demonstrated the most promising performance. SEM and spectral analysis confirmed its uniform fiber morphology and structural integrity. Additionally, this scaffold demonstrated enhanced mechanical stability and superior bioactivity. The scaffold's pore size and mechanical properties closely resembled those of cancellous bone, indicating its potential to support bone regeneration in critical-size defects. Furthermore, the formation of needle-like apatite crystals in SBF highlighted the scaffold's bioactivity and its capacity to promote mineral deposition, essential for osteointegration and bone healing.

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Disclosure

The authors report no conflicts of interest in this work.

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