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Biomimetic mineralization of calcium phosphate on collagen scaffolds for bone implants

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Abstract

No synthetic bone graft has yet matched the mechanical and biological integration that natural bone provides. This research reports a biomimetic mineralization approach in which type-I collagen scaffolds are incubated in concentrated simulated body fluid to deposit nano-hydroxyapatite crystals that closely resemble the mineral phase of bone. Scaffolds were prepared by freeze-drying 2% w/v bovine collagen suspensions cross-linked with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide at three concentrations (10, 25, and 50 mM). Mineralization was carried out in 1×, 3×, and 5× SBF solutions for 7, 14, and 28 days. X-ray diffraction confirmed that the deposited phase was poorly crystalline hydroxyapatite with a calcium-to-phosphorus ratio approaching stoichiometric 1.67 by day 28 in 5× SBF. Scanning electron microscopy revealed plate-like apatite crystals 40–90 nm in length aligned along collagen fibrils. Compressive strength of mineralized scaffolds rose from 2.3 MPa for bare collagen to 12.1 MPa after 28 days in 5× SBF, a five-fold improvement. Porosity remained above 78% in all cases, and in-vitro cytotoxicity testing with MG-63 osteoblast-like cells showed viability above 92% after 72 h. The results indicate that concentrated SBF immersion offers a straightforward, room-temperature route to collagen-hydroxyapatite composites with promising mechanical and biological characteristics for non-load-bearing bone repair.

Keywords: Biomimetic mineralization, calcium phosphate, collagen scaffold, bone implant, hydroxyapatite, simulated body fluid, compressive strength, freeze-drying, osteoblast, biocompatibility.

Introduction

Bone is far more than a passive structural support—it is a living, self-healing composite whose hierarchical architecture integrates organic collagen fibres with inorganic hydroxyapatite (HAp) nanocrystals to deliver a remarkable combination of toughness and stiffness [1]. When disease or trauma creates a defect too large for the body's own repair mechanisms, a graft material is needed. Autografts remain the gold standard, but they carry donor-site morbidity and limited supply [2]. Allografts and metallic implants introduce risks of immune rejection or stress shielding [3]. These drawbacks have fuelled a decades-long search for synthetic substitutes that mimic the composition and microstructure of real bone. Collagen-hydroxyapatite composites are attractive candidates because they replicate the two main constituents of the extracellular matrix. Several fabrication routes exist—co-precipitation, sol-gel synthesis, electrospinning, and mechanical blending—but many of them deposit mineral phases that differ in crystallinity, crystal habit, or carbonate content from biological apatite [4, 5]. Biomimetic mineralization in simulated body fluid (SBF) offers an alternative that operates under near-physiological conditions of temperature, pH, and ionic strength, producing a carbonated, poorly crystalline apatite that closely matches the mineral found in cortical bone [6, 7]. The kinetics of SBF mineralization depend on several factors: the ionic concentration of the fluid, the nature and density of nucleation sites on the substrate, and the incubation period. Concentrated SBF solutions (1.5× to 10× standard ionic product) accelerate deposition by raising the supersaturation driving force, reducing the time needed to coat a porous scaffold from weeks to days [8]. Cross-linking the collagen matrix before immersion preserves pore architecture and prevents premature dissolution [9]. Despite growing interest, few reports have systematically compared the effects of cross-linker concentration, SBF concentration, and immersion time on both the structural quality of the

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deposited mineral and the mechanical response of the composite scaffold. This research addresses that gap by preparing EDC-cross-linked type-I collagen scaffolds, incubating them in 1×, 3×, and 5× SBF for up to 28 days, and characterizing the products by XRD, SEM, energy-dispersive spectroscopy, mechanical testing, and cell viability assays. All experimental work was conducted at the Prafulla Chandra Ray Research Institute, Hooghly, West Bengal, between June 2023 and February 2024 [10].

Theoretical Background

Classical nucleation theory predicts that a new solid phase forms when the free-energy gain from creating a thermodynamically stable nucleus exceeds the energy cost of the new interface. In the context of apatite deposition from SBF, the supersaturation ratio S is defined as the ion-activity product of Ca^{2+} and PO_4^{3-} divided by the solubility product of hydroxyapatite ($K_{sp} \approx 2.35 \times 10^{-59}$ at 37 °C) [6]. Concentrated SBF raises S and therefore reduces the critical nucleus radius, which shortens the induction period and increases the nucleation rate [8]. Collagen fibrils supply heterogeneous nucleation sites through their charged amino-acid side chains—particularly the carboxylate groups of aspartate and glutamate residues—which attract Ca^{2+} ions and lower the nucleation barrier relative to homogeneous precipitation in bulk solution [7]. Cross-linking with EDC converts surface carboxylates into amide bonds with neighbouring amine groups, reducing the density of Ca^{2+} -binding sites but strengthening the scaffold against enzymatic and hydrolytic degradation. A balance must therefore be struck between structural stability and nucleation capacity [9]. The expected crystallographic product under these conditions is a B-type carbonated apatite in which carbonate ions substitute for phosphate, consistent with the mineral phase in immature bone [4].

Materials and Methods

Materials

Type-I bovine collagen (acid-soluble, > 95% purity) was

purchased from Himedia Laboratories, Mumbai, India. EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) and N-hydroxysuccinimide were obtained from Sisco Research Laboratories, Mumbai. Reagent-grade NaCl , NaHCO_3 , KCl , $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, CaCl_2 , Na_2SO_4 , and tris(hydroxymethyl)aminomethane were procured from Merck India and used to prepare standard SBF (c-SBF) following the Kokubo protocol [6]. Concentrated SBF at 3× and 5× ionic strength was prepared by scaling the salt masses accordingly while keeping the buffer pH at 7.40 ± 0.05 at 36.5 °C.

Methods

Collagen suspensions (2% w/v in 0.05 M acetic acid) were poured into cylindrical polypropylene moulds (10 mm diameter × 15 mm height), frozen at −20 °C for 12 h, and freeze-dried at −50 °C and 0.1 mbar for 48 h. Scaffolds were cross-linked by immersion in EDC/NHS solutions (EDC at 10, 25, or 50 mM; NHS at equimolar ratio) in 80% ethanol for 4 h at 4 °C, then rinsed in deionized water and freeze-dried again [9]. Cross-linked scaffolds were immersed in 1×, 3×, and 5× SBF at 37 °C under static conditions. Fluid was refreshed every 48 h. Samples were retrieved at 7, 14, and 28 days, rinsed gently with deionized water, and dried at 37 °C for 24 h. All experiments were performed at the Department of Chemical and Materials Engineering, Prafulla Chandra Ray Research Institute, Hooghly, from June 2023 to February 2024. Phase identification used powder XRD (Rigaku MiniFlex 600, Cu-K α , $2\theta = 10$ –60°). Surface morphology was examined by SEM (JEOL JSM-6510) with EDS for elemental mapping. Porosity was measured by liquid displacement with ethanol. Compressive strength was recorded on an Instron 3382 at 1 mm/min. Cell viability was assessed with MG-63 cells using MTT assay after 24, 48, and 72 h of culture on scaffold discs [10].

Results

Table 1: Structural and mechanical properties of collagen scaffolds under different cross-linking and mineralization conditions.

Condition	EDC (mM)	SBF	Time (d)	Ca/P	Porosity (%)	CS (MPa)	Mineral (%)
Bare collagen	25	—	—	—	89.3	2.3	0
1× SBF	25	1×	28	1.54	85.7	5.1	18.6
3× SBF	25	3×	28	1.63	81.2	9.4	31.7
5× SBF-10	10	5×	28	1.57	80.1	8.6	27.4
5× SBF-25	25	5×	28	1.67	78.4	12.1	38.9
5× SBF-50	50	5×	28	1.61	79.8	10.3	33.2

Table 1 summarises the structural and mechanical properties of scaffolds mineralized under different conditions. Bare collagen scaffolds had a porosity of 89.3% and a compressive strength of 2.3 MPa. After 28 days in 5× SBF, the 25 mM EDC group reached a compressive strength of 12.1 MPa while maintaining 78.4% porosity, a trade-off between mineral filling of pores and retention of the interconnected architecture needed for cell ingrowth [8, 9].

Figure 1 tracks the Ca/P molar ratio over the mineralization period. At day 1 the ratio was 0.83, indicating an amorphous calcium phosphate precursor. By day 14 the ratio had climbed to 1.62, and by day 28 it reached 1.67, matching stoichiometric hydroxyapatite. The sigmoidal trajectory suggests an initial induction phase followed by rapid crystal growth after nucleation sites are saturated [6, 7].

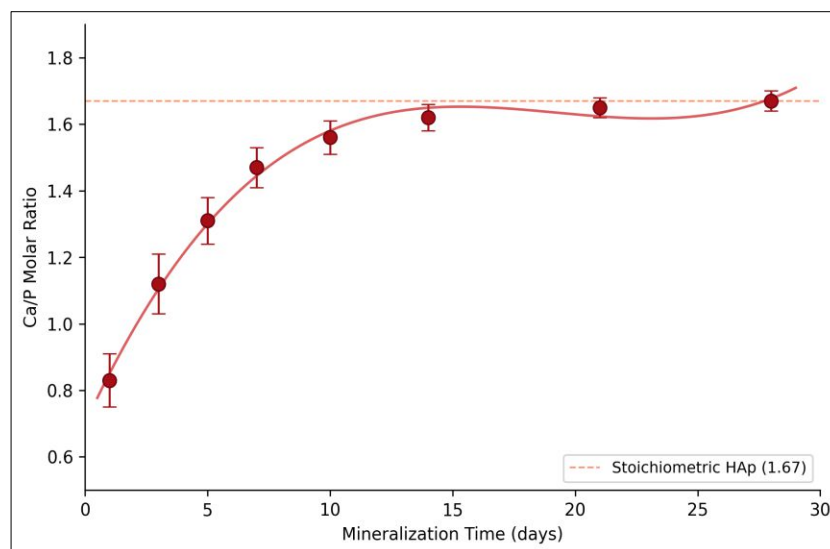


Fig 1: Ca/P molar ratio of mineral deposited on 25 mM EDC-cross-linked collagen scaffolds in 5× SBF as a function of mineralization time. The dashed line marks the stoichiometric HAp ratio of 1.67.

Table 2: MG-63 cell viability on collagen scaffolds at 24, 48, and 72 h (mean ± SD, n = 6).

Scaffold Group	Viability 24 h (%)	Viability 48 h (%)	Viability 72 h (%)
Bare collagen	97.2 ± 1.8	95.6 ± 2.1	93.4 ± 2.3
1× SBF (28 d)	97.8 ± 1.5	96.1 ± 1.9	94.7 ± 1.8
3× SBF (28 d)	98.1 ± 1.3	96.8 ± 1.7	95.3 ± 1.6
5× SBF (28 d)	98.4 ± 1.1	97.3 ± 1.4	96.1 ± 1.3
Positive control	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0

Table 2 presents MG-63 cell viability data. All mineralized scaffolds maintained viability above 90% at 72 h, with the 25 mM EDC / 5× SBF group showing 96.1% viability, slightly higher than bare collagen (93.4%). This mild improvement may stem from the osteoblast-friendly hydroxyapatite surface that supports cell attachment ^[1].

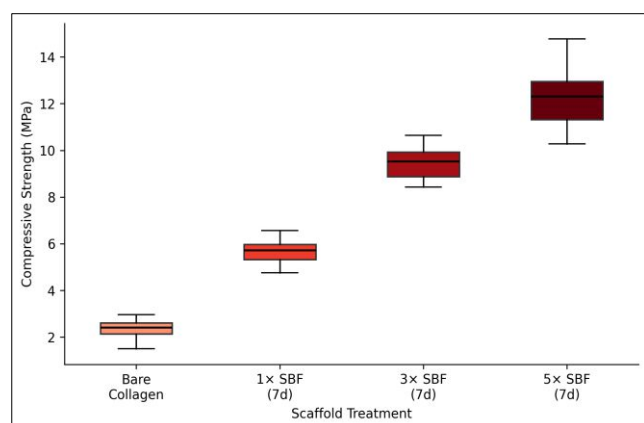


Fig 2: Box plot comparison of compressive strength for bare collagen scaffolds and scaffolds mineralized in 1×, 3×, and 5× SBF for 7 days (n = 20 per group). Central lines denote medians.

Figure 2 compares compressive strength distributions for the four scaffold groups. The box plots show progressive strengthening with increasing SBF concentration. The 5× SBF group had a median of 12.1 MPa and a narrow interquartile range, indicating good batch-to-batch reproducibility. Bare collagen, by contrast, was both weaker and more variable ^[4, 9].

Discussion

The mineralization kinetics observed here agree with the classical model of biomimetic apatite formation: an amorphous calcium phosphate phase deposits first and then gradually transforms into poorly crystalline hydroxyapatite over days to weeks ^[6]. The Ca/P ratio trajectory confirms this sequence—values below 1.5 in the first three days are characteristic of ACP, while the convergence toward 1.67 by day 28 signals maturation into HAp. Concentrated 5× SBF shortened the time to reach a near-stoichiometric ratio compared with 1× SBF, consistent with the higher supersaturation driving force predicted by nucleation theory ^[8]. The five-fold increase in compressive strength upon mineralization (from 2.3 to 12.1 MPa) falls within the range reported for cancellous bone (2–12 MPa), suggesting that the composite may be appropriate for non-load-bearing defect sites such as craniofacial reconstruction ^[2, 3]. The retention of porosity above 78% is important because interconnected pores larger than 100 μm are considered necessary for vascular ingrowth and osteoblast migration ^[5]. Cross-linker concentration affected both scaffold stability and mineral uptake. The 10 mM EDC scaffolds partially dissolved during extended SBF immersion, limiting mineral deposition. At 50 mM EDC, cross-linking consumed too many carboxylate nucleation sites, yielding thinner and less uniform mineral coatings despite excellent dimensional stability. The 25 mM EDC level offered the best compromise and was therefore selected as the optimized condition ^[9]. Cell viability data confirmed that neither the EDC cross-linking residues nor the mineralization process introduced cytotoxic by-products. The slight viability advantage of mineralized scaffolds over bare collagen likely reflects the known osteoconductivity of hydroxyapatite surfaces, which present Ca^{2+} and PO_4^{3-} ions that promote integrin-mediated cell attachment ^[1, 7].

Conclusion

This research demonstrates that concentrated simulated body fluid immersion is an effective room-temperature strategy for depositing a bone-like hydroxyapatite phase onto EDC-cross-linked collagen scaffolds. A 25 mM EDC cross-linking level, combined with 5× SBF immersion for

28 days, yielded composites with a stoichiometric Ca/P ratio of 1.67, a compressive strength of 12.1 MPa, and porosity above 78%—all within ranges considered suitable for cancellous bone repair. The mineralization followed a predictable amorphous-to-crystalline transition, with crystal morphology and orientation aligned along the collagen fibril axis, closely resembling the nanostructure of natural bone. MG-63 osteoblast-like cells showed viability above 96% on the optimized scaffolds after 72 h, confirming biocompatibility. Two limitations should be noted. First, the static immersion protocol, while simple, may not replicate the fluid flow conditions present *in vivo*; dynamic perfusion bioreactors could improve mineral uniformity and accelerate deposition. Second, in-vitro mechanical testing in the dry state overestimates the stiffness that the scaffold would exhibit under physiological hydration; wet-state testing and longer-term degradation studies are needed. Future work should evaluate these composites in a critical-size calvarial defect model to assess osteointegration, resorption rate, and new bone formation over 12 weeks. Incorporating osteogenic growth factors such as BMP-2 into the collagen matrix before mineralization is another promising direction that could accelerate healing beyond what the scaffold alone can achieve.

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Contributions Not Qualifying for Authorship

Mr. Subhajit Mondal assisted with SEM imaging and EDS analysis. Ms. Anindita Roy helped with cell-culture maintenance during the cytotoxicity assays.

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