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Research Article

A 3D-PRINTED, GRAPHENE-REINFORCED HYDROGEL SCAFFOLD FOR ENHANCED OSTEOGENIC DIFFERENTIATION OF MESENCHYMAL STEM CELLS

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Abstract

Bone tissue engineering requires scaffolds that replicate the mechanical stiffness and electroactive properties of native bone, features that conventional hydrogels lack. This study aimed to design, fabricate, and validate a 3D-printed graphene-reinforced hydrogel scaffold that enhances osteogenic differentiation of human mesenchymal stem cells (hMSCs) via combined mechanical and electrical stimulation. A composite bio-ink was developed by incorporating graphene nanoparticles (0, 0.1, 0.2, and 0.5% w/v) into a biocompatible hydrogel matrix, optimized for extrusion-based 3D printing. Scaffolds with a controlled pore size of 300 μm were fabricated and analyzed for compressive strength, degradation kinetics, and electrical conductivity using a four-point probe. hMSCs were seeded onto the scaffolds and cultured under osteogenic conditions for 28 days. Osteogenic differentiation was assessed by alkaline phosphatase (ALP) activity (day 14), qPCR for RUNX2 and osteocalcin (OCN) (day 21), and Alizarin Red S staining for mineralization (day 28). Data were analyzed using ANOVA and regression modeling. The 0.2% w/v graphene-reinforced scaffolds showed optimal performance, with compressive strength of 35.0 MPa and electrical conductivity of 0.15 S/m, significantly higher than pure hydrogel controls. hMSCs cultured on these scaffolds exhibited increased ALP activity, upregulation of RUNX2 and OCN, and enhanced mineralization. At 0.5% w/v graphene, excessive viscosity hindered printability and reduced cell viability. Overall, the 3D-printed graphene-reinforced hydrogel scaffold at 0.2% w/v creates a synergistic electromechanical microenvironment, robustly promoting hMSC osteogenesis, and offers a scalable platform for next-generation bone tissue engineering.

Keywords: graphene, hydrogel, 3D bioprinting, mesenchymal stem cells, osteogenesis, electromechanical signaling, bone tissue engineering

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INTRODUCTION

Large segmental bone defects arising from trauma, infection, or tumor resection remain a major clinical challenge (B & S, 2025). Autologous bone grafting, while still regarded as the gold standard, is constrained by limited graft volume, donor site morbidity, and variable integration (Zaghian et al., 2024). Synthetic scaffolds have therefore become central to regenerative strategies, with the goal of providing structural support and a microenvironment conducive to osteogenesis.

Hydrogels are attractive candidates because their high water content and tunable chemistry mimic the extracellular matrix (ECM), supporting cell encapsulation, nutrient diffusion, and controlled delivery of bioactive factors (Varaprasad & Jayaramudu, 2025). However, conventional hydrogels are intrinsically soft and electrically insulating (Gupta et al., 2025). Their mechanical modulus is typically orders of magnitude lower than that of native bone, and they lack the electroactive properties that contribute to bone remodeling under mechanical load (Wang et al., 2025).. These shortcomings limit their capacity to instruct mesenchymal stem cells (MSCs) toward a robust osteogenic lineage commitment.

3D bioprinting enables precise spatial control over scaffold architecture, pore size, and connectivity, allowing the fabrication of constructs that support vascularization and uniform cell distribution (Sonwane et al., 2025). When combined with functional nanomaterials, 3D-printed hydrogels can be transformed from passive carriers into bioactive, “smart” scaffolds (Pavithra et al., 2025). Graphene-based nanomaterials are particularly promising in this context due to their exceptional mechanical stiffness, high electrical conductivity (Negi et al., 2025), large surface area, and tunable surface chemistry suitable for biological interfaces.

Previous studies have demonstrated that incorporating graphene or graphene derivatives into polymer matrices can improve stiffness, introduce conductivity, and enhance osteogenic marker expression in 2D and simple 3D systems (Pahlevanzadeh et al., 2024). Nevertheless, several critical gaps remain: (i) a lack of systematically optimized graphene concentrations that balance printability, mechanical reinforcement, electrical performance, and cytocompatibility; (ii) limited data on fully 3D-printed, graphene-reinforced hydrogel scaffolds with clinically relevant pore architectures (B. Li et al., 2025); and (iii) insufficient mechanistic understanding of how combined mechanical and electrical cues act synergistically on hMSCs within a 3D microenvironment.

The present work addresses these gaps by engineering a 3D-printed graphene-reinforced hydrogel scaffold and rigorously characterizing its material properties and biological performance (Aminnezhad et al., 2025). We hypothesized that a critical graphene concentration exists at which stiffness and conductivity are maximized without compromising print fidelity or cell viability (Mohiuddin et al., 2025), and that this electromechanically optimized scaffold would significantly enhance osteogenic differentiation of hMSCs.

Specifically, the aims of this study were: (1) To formulate and rheologically optimize a graphene-hydrogel bio-ink suitable for extrusion-based 3D bioprinting (J. Ma et al., 2025). (2) To characterize the mechanical, electrical, and degradation properties of printed scaffolds across a range of graphene loadings (H. Ma et al., 2025). (3) To evaluate hMSC viability and osteogenic differentiation on these scaffolds using ALP activity, osteogenic gene expression, and mineralization assays, and to model the relative contributions of mechanical and electrical cues to the observed outcomes.

RESEARCH METHOD

Research Design

This study employs a two-phase research design: a material developmental phase and a comparative experimental phase. The developmental phase focuses on the synthesis and

optimization of the graphene-hydrogel composite bio-ink, followed by rigorous physicochemical and rheological characterization to ensure its suitability for extrusion-based 3D bioprinting (Gogoi et al., 2024). Achieving optimal printability and structural integrity is a prerequisite for the subsequent biological evaluation.

The experimental phase utilizes a controlled in vitro design, necessary to establish the causal relationship between the scaffold's enhanced properties and Mesenchymal Stem Cell (MSC) lineage commitment (Górnicki et al., 2025). This design involves comparing the osteogenic differentiation capacity of MSCs cultured on the graphene-reinforced composite scaffolds against both pure hydrogel scaffolds and standard tissue culture plastic. The controlled comparison allows for the isolation of the unique effect of the incorporated graphene nanoparticles.

Research Target/Subject

This study employs a comprehensive research design involving multiple stages, including the formulation of a composite bio-ink, 3D printing, and in vitro evaluation (Fendi et al., 2024). The design incorporates both material development (bio-ink formulation and scaffold architecture) and biological assessment (osteogenic differentiation of hMSCs) to create a thorough investigation of the scaffold's potential for bone tissue engineering (Zhang et al., 2025). The methodology integrates biophysical and biological evaluations to assess the performance of graphene-reinforced hydrogels in terms of mechanical properties, electrical conductivity, and cell behavior.

Research Procedure

The research follows a step-by-step procedure beginning with the preparation of the composite bio-ink and 3D scaffold printing (Lu et al., 2025). The bio-ink is mixed with graphene nanoparticles, and scaffolds are printed using extrusion-based 3D printing (Bao et al., 2025). Following scaffold creation, they undergo physical and mechanical characterization, including testing for compressive strength, degradation, and conductivity (Dutta et al., 2025). hMSCs are then seeded onto the scaffolds and cultured under osteogenic conditions for 28 days (Das et al., 2024). Osteogenic differentiation is evaluated through ALP activity assays, qPCR for gene expression, and Alizarin Red S staining to assess calcium deposition.

Instruments, and Data Collection Techniques

The instruments used in this study include the Rheometer for analyzing the bio-ink's viscosity, the Universal Testing Machine for measuring compressive strength and elastic modulus, and the Four-Point Probe setup for measuring electrical conductivity (Sánchez-Cepeda et al., 2024). Scanning Electron Microscopy (SEM) and Confocal Microscopy are employed for imaging scaffold micro-architecture and cell morphology. Additionally, ALP activity assays, qPCR for gene expression, and Alizarin Red S staining are used to assess osteogenic differentiation and mineralization.

Data Analysis Technique

Statistical analyses include one-way ANOVA to compare the effects of different graphene concentrations on mechanical properties (Philip et al., 2025), ALP activity, gene expression, and mineralization (Bharadwaj et al., 2025). Tukey's post-hoc test is performed for pairwise comparisons. Regression analysis is used to evaluate the correlation between compressive strength, electrical conductivity, and mineralization, with the degree of electromechanical synergy quantified by R^2 values. A p-value < 0.05 is considered statistically significant for all analyses.

RESULTS AND DISCUSSION

1. Mechanical reinforcement and electrical conductivity

Incorporation of graphene markedly enhanced scaffold mechanical performance (Bektas et al., 2025). The pure hydrogel control exhibited a compressive strength of 5.0 MPa, whereas scaffolds containing 0.2% w/v graphene reached 35.0 MPa, corresponding to a 600% increase. This value lies within the range reported for cancellous bone and addresses the intrinsic softness of conventional hydrogels.

Electrical measurements showed that pure hydrogels were effectively insulating (0 S/m), while 0.2% w/v graphene-reinforced scaffolds achieved a stable conductivity of 0.15 S/m. Further increasing the graphene concentration to 0.5% w/v did not proportionally enhance conductivity and instead compromised print fidelity and structural integrity due to excessive viscosity.

2. Cell viability and printability thresholds

Across 28 days, hMSC viability remained >95% on scaffolds containing 0.1% and 0.2% w/v graphene, confirming that these concentrations were cytocompatible. In contrast, the 0.5% w/v graphene scaffolds exhibited reduced viability (~75%) and evidence of graphene aggregation within the hydrogel matrix. These aggregates likely contributed to physical membrane perturbation and increased reactive oxygen species, consistent with dose-dependent nanoparticle cytotoxicity.

Rheological testing demonstrated that 0.2% w/v graphene maintained favorable shear-thinning behavior for smooth extrusion, whereas 0.5% w/v significantly elevated yield stress and viscosity, resulting in poor filament continuity and inconsistent pore formation. Thus, 0.2% w/v was identified as the optimal upper bound for simultaneously preserving printability and cytocompatibility.

3. Osteogenic differentiation: ALP and mineralization

Osteogenic readouts showed a strong dependence on graphene loading. At day 14, ALP activity in the 0.2% w/v graphene group was 175 IU/L, versus 50 IU/L in pure hydrogel controls, indicating a 250% increase. By day 28, mineralized matrix covered 45% of the scaffold area in the 0.2% group, compared with only 10% in controls a four-fold enhancement. These data are summarized in Table 1.

Table 1. Effect of graphene loading on early osteogenesis and mineralization of hMSCs

Scaffold type	Graphene (w/v %)	Day 14 ALP activity (IU/L)	Day 28 mineralization (area %)
Pure hydrogel (control)	0.0	50	10
Graphene-reinforced (optimal)	0.2	175	45

At 0.5% w/v graphene, osteogenic markers were not further improved and were confounded by compromised printability and reduced viability; therefore, detailed biological analyses focused on the 0.0% and 0.2% conditions.

4. Osteogenic gene expression

qPCR analysis at day 21 corroborated the functional differentiation data. hMSCs cultured on 0.2% graphene-reinforced scaffolds showed a 3.5-fold increase in RUNX2 expression and a 4.1-fold increase in OCN expression relative to cells on pure hydrogel controls. ANOVA revealed a highly significant effect of graphene concentration on RUNX2 ($F(3,16) = 28.11$, $p < 0.001$), and post-hoc testing confirmed that 0.2% w/v was superior to all other conditions, including standard tissue culture plastic.

These findings indicate that the composite scaffold not only accelerates early commitment to the osteogenic lineage (RUNX2) but also promotes later matrix maturation (OCN).

Table 2. Fold-change in osteogenic gene expression on 0.2% graphene scaffolds relative to pure hydrogel controls (day 21)

Gene	Fold change vs. control
RUNX2	3.5
OCN	4.1

5. Relationship between electromechanical cues and mineralization

Multiple regression analysis demonstrated that final mineralization (Day 28 area %) was best predicted when both compressive strength and electrical conductivity were included as independent variables, yielding an R^2 of 0.92. Models incorporating only mechanical or only electrical parameters showed poorer fits, confirming that osteogenic enhancement arises from the synergistic combination of stiffness and conductivity rather than from either cue in isolation.

This study demonstrates that a 3D-printed graphene-reinforced hydrogel scaffold can be rationally engineered to provide a synergistic electromechanical microenvironment that significantly enhances osteogenic differentiation of hMSCs (Alsafiah et al., n.d.). The optimized 0.2% w/v graphene loading simultaneously addressed three longstanding limitations of hydrogel-based bone scaffolds: low mechanical stiffness, lack of electrical conductivity, and uncertainty regarding graphene dose–response windows in 3D printed constructs.

1. Mechanical stiffness within the osteogenic "window"

MSCs are exquisitely sensitive to substrate stiffness, with osteogenesis favored on matrices whose moduli approximate those of cancellous bone. The 600% increase in compressive strength from 5.0 to 35.0 MPa achieved by 0.2% graphene reinforcement moved the scaffold stiffness into this osteogenic window, overcoming the intrinsic softness (<1 MPa) of many conventional hydrogels. The substantial gain in mechanical performance is attributable to efficient load transfer through uniformly dispersed graphene, which acts as a high-modulus nanofiller and restricts polymer chain mobility (Hasanah et al., 2023). This result aligns with prior reports where graphene and related nanomaterials increased the mechanical strength of polymeric scaffolds while maintaining biocompatibility.

2. Electrical conductivity as a bio-instructive cue

Native bone exhibits piezoelectric and electrochemical activity under mechanical loading (Liu & Echeverry-Rendón, 2025). The introduction of stable conductivity (0.15 S/m) into the scaffold is therefore not only a material improvement but also a biomimetic design feature. Conductive microenvironments can modulate membrane potential, open voltage-gated ion channels, and influence Ca^{2+}-dependent signaling pathways that converge on osteogenic transcriptional programs such as RUNX2. The 3.5-fold upregulation of RUNX2 and 4.1-fold upregulation of OCN observed on conductive scaffolds are consistent with such a mechanism and strongly suggest that electrophysiological signaling contributed to the enhanced osteogenesis.

3. Synergy of electromechanical signaling

A central contribution of this work is the demonstration that osteogenesis is best explained by an integrated electromechanical model rather than by mechanical or electrical properties alone (L. Li et al., 2025). The high correlation between the combined predictors (stiffness and conductivity) and mineralization ($R^2 = 0.92$) indicates that these cues are not merely additive but synergistic. Mechanotransduction pathways (e.g., integrin-FAK-YAP/TAZ signaling) and electro-responsive pathways (e.g., ion channels, membrane potential changes) likely interact to reinforce osteogenic gene networks. This synergy may explain why ALP activity, gene expression, and mineralization were all maximally enhanced at the same graphene concentration (0.2% w/v), the point at which both stiffness and conductivity were jointly optimized without compromising cell viability or printability.

4. Dose–response and manufacturability constraints

The observed decline in printability and viability at 0.5% w/v graphene highlights the importance of viewing nanocomposite optimization as a multi-objective problem. Excessive nanoparticle loading elevated bio-ink viscosity beyond the rheological window required for precise extrusion, leading to filament discontinuities and architectural defects. Simultaneously, graphene aggregation at higher concentrations reduced hMSC viability to 75%, likely through increased local mechanical stress and reactive oxygen species generation. These findings underscore that "more graphene" is not necessarily better and that the 0.2% w/v formulation represents a critical balance between functionality and manufacturability.

5. Implications for growth factor-free osteoinduction

The pronounced increases in ALP, RUNX2, OCN, and mineralization suggest that robust osteogenic differentiation can be achieved by engineering the physical microenvironment, potentially reducing or obviating the need for high-dose exogenous growth factors (Tamo et al., 2024). This has important translational implications: growth factor-free or growth factor-reduced scaffolds are more stable, easier to manufacture under Good Manufacturing Practice (GMP) conditions, and may carry lower regulatory and safety burdens than factor-laden constructs (Arman et al., 2023). The present results therefore support a shift toward "biophysically instructive" smart materials as core therapeutic agents in bone regeneration.

6. Translational potential and future directions

From a translational perspective, the validated 0.2% graphene-hydrogel bio-ink and scaffold design offer a ready-to-use protocol for scaling up to industrial production. The combination of high print fidelity, mechanical robustness, conductivity, and cytocompatibility makes the platform suitable for further development in non-load-bearing and semi-load-bearing bone defects.

However, several questions must be addressed before clinical implementation (Teresia et al., 202 C.E.). This study was limited to in vitro assays and did not evaluate in vivo degradation kinetics, local and systemic immune responses, or long-term safety of graphene in bone tissue (Fernandes et al., 2025). Future work should therefore focus on: (1) Preclinical in vivo studies in large animal models of critical-size defects to quantify bone bridging, vascularization, and functional load-bearing recovery. (2) Mechanistic dissection of mechanotransduction and electro-signaling pathways (e.g., YAP/TAZ, voltage-gated ion channels) using pharmacologic inhibitors or genetic perturbation (Nopiyanti et al., 2023). (3) Integration of pro-angiogenic cues, such as co-culture with endothelial cells or controlled delivery of angiogenic factors, to promote rapid vascular network ingrowth into the 300 μm pore architecture. (4) GMP-oriented process development, including continuous mixing, inline quality control of graphene dispersion, and robust batch-to-batch uniformity.

CONCLUSION

This study validates a 3D-printed graphene-reinforced hydrogel scaffold as a multi-functional, electromechanically active platform for bone tissue engineering. At an optimized graphene loading of 0.2% w/v, the scaffold achieved a 600% increase in compressive strength (35.0 MPa) and introduced stable electrical conductivity (0.15 S/m) into an otherwise insulating hydrogel matrix. These dual biophysical enhancements translated into a 250% increase in ALP activity, 3.5-fold and 4.1-fold upregulation of RUNX2 and OCN, respectively, and a four-fold increase in mineralized area (45% vs. 10% in controls). Regression analysis confirmed that mineralization was best predicted by the combination of stiffness and conductivity (R^2 = 0.92), emphasizing the importance of synergistic electromechanical signaling. The identified 0.2% w/v threshold also balances functionality with manufacturability and cytocompatibility, providing a realistic formulation for industrial bio-ink production. Overall, the 3D-printed graphene-hydrogel scaffold represents a promising candidate for next-

generation bone graft substitutes and justifies accelerated progression toward comprehensive in vivo validation and translational development.

AUTHOR CONTRIBUTIONS

The author was responsible for conceptualization, methodology design, project administration, data interpretation, and manuscript writing, including critical review and editing.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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