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Mechanistically Guided Development of a Bioactive Chitosan/Magnesium-Doped Hydroxyapatite Nanocomposite Scaffold for Osteogenic and Antibacterial Bone Regeneration

Proposed Research Based on the Preliminary Findings of an Electrospun CS/Mg-HAp Scaffold Co-Loaded with Icariin, Lithium Chloride and Naringin

1. Research Aim

The primary aim of the proposed study is to develop and systematically validate a multifunctional bioactive nanocomposite scaffold for bone tissue regeneration by combining a chitosan (CS) polymer matrix with magnesium-doped hydroxyapatite (Mg-HAp) and selected osteogenic bioactive agents. The study will specifically investigate how scaffold composition, architecture, controlled release and molecular signaling interact to regulate osteogenic differentiation and antibacterial activity.

The proposed work will build on preliminary evidence showing that an electrospun CS/Mg-HAp scaffold co-loaded with icariin, lithium chloride (LiCl), and naringin can provide sustained bioactive release, antibacterial activity, enhanced osteogenic marker expression and improved bone regeneration. The new study will move beyond proof-of-concept by introducing appropriate intermediate controls and mechanistic experiments to determine the contributions of individual components and their interactions.

2. Scientific Relevance and Background

Bone regeneration is a complex biological process involving coordinated cellular proliferation, osteogenic differentiation, extracellular matrix deposition, vascularization and remodeling. Conventional bone grafts and passive implant materials primarily provide structural support, but often lack the ability to actively regulate the cellular and molecular events required for efficient regeneration. Consequently, current bone tissue engineering research increasingly focuses on bioactive scaffolds capable of providing both an appropriate three-dimensional microenvironment and controlled biological stimulation.

Chitosan is a promising natural polymer for scaffold fabrication because of its biocompatibility, biodegradability, hydrophilicity, and favorable interaction with cells. Hydroxyapatite, in turn, provides an osteoconductive inorganic phase that resembles the mineral component of native bone. The incorporation of magnesium into hydroxyapatite is particularly attractive because magnesium is a physiologically relevant ion associated with bone metabolism and can influence osteoblast activity and mineralization.

The signaling-oriented concept of the proposed scaffold is further supported by the use of bioactive agents capable of modulating osteogenic pathways. Icariin, LiCl, and naringin represent complementary bioactive components with reported effects on osteogenic differentiation and cellular signaling. Their localized incorporation into a porous scaffold may provide prolonged stimulation while reducing the limitations associated with systemic administration.

The central mechanistic focus will be the Wnt/β-catenin pathway, together with downstream osteogenic markers including RUNX2, ALP, and osteocalcin. The preliminary study demonstrated increased expression of Wnt/β-catenin-related markers and nuclear β-catenin localization, but a causal relationship was not established. Therefore, pathway inhibition will be incorporated into the proposed design.

3. Research Gap

• The individual contributions of icariin, LiCl, and naringin have not been adequately separated from the effects of the complete formulation.

• The biological advantage of Mg-HAp over conventional HAp requires direct comparative assessment.

• The causal role of Wnt/β-catenin signaling requires pharmacological inhibition and protein-level validation.

• Agar diffusion alone is insufficient for comprehensive antibacterial characterization; quantitative MIC, MBC, and biofilm assays are required.

• The relationship between scaffold architecture, degradation, drug release, and biological activity requires an integrated analysis.

• A stronger experimental design is required to establish whether the observed effects result from additive or synergistic interactions among scaffold components.

4. Scientific Hypothesis

We hypothesize that a CS/Mg-HAp nanocomposite scaffold co-loaded with icariin, LiCl, and naringin will provide a favorable three-dimensional microenvironment, controlled local release, and enhanced osteogenic and antibacterial activities compared with non-loaded and single-agent controls. We further hypothesize that the osteogenic response will be mediated, at least in part, through the activation of the Wnt/β-catenin pathway.

5. Specific Objectives

1. To optimize the composition and fabrication parameters of CS/Mg-HAp nanocomposite scaffolds.

2. To compare undoped HAp and Mg-HAp formulations and confirm Mg incorporation using complementary physicochemical techniques.

3. To characterize scaffold morphology, porosity, pore interconnectivity, surface properties, swelling, degradation, and mechanical behavior.

4. To determine the loading efficiency and release kinetics of icariin, LiCl, and naringin.

5. To quantitatively evaluate antibacterial activity against *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922.

6. To evaluate cytocompatibility, apoptosis, adhesion, proliferation and osteogenic differentiation using Saos-2 cells and, where feasible, a mesenchymal stem cell model.

7. To quantify Wnt, β-catenin, RUNX2, ALP, and OCN at the gene and protein levels.

8. To verify the involvement of Wnt/β-catenin signaling using an appropriate pathway inhibitor such as DKK1 or XAV939.

9. To determine the regenerative efficacy of the optimized scaffold in a critical-size calvarial bone defect model.

10. To integrate material, microbiological, cellular, molecular, and *in vivo* findings to identify the most promising formulation for future translational studies.

6. Materials and Methods

6.1 Scaffold Formulation and Fabrication

Chitosan will be used as the polymeric matrix and hydroxyapatite or magnesium-doped hydroxyapatite as the inorganic phase. The previously investigated CS/Mg-HAp ratio will serve as the starting formulation, followed by optimization where necessary. Icariin, LiCl, and naringin will be incorporated individually and in combination. Electrospinning parameters will be optimized to obtain a reproducible morphology and a highly interconnected porous architecture.

6.2 Experimental Groups

Group	Formulation	Purpose	Principal Comparison
G1	Defect/ Untreated control	Negative control	Baseline
G2	CS	Polymer control	Effect of inorganic phase
G3	CS/HAp	HAp control	Effect of Mg substitution
G4	CS/Mg-HAp	Base nanocomposite	Effect of bioactive agents
G5	CS/Mg-HAp + Icariin	Single-agent	Icariin contribution
G6	CS/Mg-HAp + LiCl	Single-agent	LiCl contribution
G7	CS/Mg-HAp + Naringin	Single-agent	Naringin contribution
G8	CS/Mg-HAp + Icariin + LiCl + Naringin	Complete formulation	Combined effect

6.3 Physicochemical and Structural Characterization

Scanning electron microscopy (SEM) will be used to evaluate fiber morphology, pore architecture, and particle distribution. Fourier-transform infrared spectroscopy (FTIR) will assess functional groups and chemical interactions. X-ray diffraction (XRD) will characterize crystalline phases and changes associated with Mg substitution. Energy-dispersive X-ray spectroscopy (EDS) will determine the elemental composition and Mg distribution, while transmission electron microscopy (TEM) will be used where appropriate to assess nanoparticle morphology and dispersion. Porosity, pore size distribution, swelling, degradation, wettability, and mechanical properties will also be quantified.

6.4 Bioactive Loading and Release

The loading efficiency of icariin and naringin will be quantified using a validated chromatographic method, while LiCl release will be determined using an appropriate quantitative ion analysis method. Cumulative release profiles will be evaluated over the predefined experimental period and fitted to zero-order, first-order, Higuchi, and Korsmeyer–Peppas models. Model selection will consider the goodness of fit and appropriate statistical criteria.

6.5 Antibacterial Assessment

Antibacterial activity will be evaluated against *S. aureus* ATCC 25923 and *E. coli* ATCC 25922. Agar well diffusion will be retained as an initial screening method, but quantitative broth microdilution will additionally be performed to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Time-kill kinetics and biofilm inhibition/eradication assays will provide complementary quantitative evidence.

6.6 In Vitro *Cytocompatibility and Osteogenic Differentiation*

Saos-2 cells will be used to maintain continuity with the preliminary work. Where resources permit, a mesenchymal stem cell model will be incorporated to improve translational relevance. Cell viability will be assessed using a metabolic assay together with Annexin V/PI flow cytometry. Cell adhesion, morphology, and proliferation will be evaluated by microscopy and quantitative assays. Osteogenic differentiation will be assessed using ALP activity, mineralization, and the expression of RUNX2, ALP, OCN, and related osteogenic markers.

6.7 Molecular Mechanism of Wnt/β-catenin Signaling

Wnt and β-catenin expression and β-catenin subcellular localization will be investigated by quantitative PCR, immunofluorescence, and, where available, Western blotting. To establish pathway dependence, cells treated with the complete scaffold will be exposed to a validated Wnt/β-catenin inhibitor, such as DKK1 or XAV939, following preliminary dose-response and cytotoxicity testing. Inhibitor-only controls will be included. Changes in RUNX2, ALP, and OCN responses after pathway inhibition will be interpreted as evidence supporting or weakening the causal role of Wnt/β-catenin signaling.

6.8 In Vivo *Evaluation*

Following prospective ethical approval and an *a priori* power analysis, an established critical-size calvarial defect model will be used. Animals will be randomized to appropriate experimental groups, with blinding applied to outcome assessments where feasible. Micro-computed tomography will be used to quantify the bone volume fraction and related structural parameters. Histological and histomorphometric analyses will assess new bone formation, maturation, vascularization, and scaffold degradation. All animal procedures will follow institutional animal welfare requirements and ARRIVE-aligned reporting.

7. Statistical Analysis

Data will be analyzed using statistical methods appropriate to the experimental design. Multifactorial experiments will use factorial or two-way ANOVA to evaluate main effects and interactions between components, followed by appropriate *post hoc* comparisons. Longitudinal release and degradation data will be analyzed using repeated-measures or mixed-effects approaches where appropriate. Effect sizes, 95% confidence intervals, and adjusted *p*-values will be reported where applicable. An *a priori* power analysis will determine the required *in vivo* sample size. Statistical significance will be defined as $p < 0.05$.

8. Expected Results

- A reproducible CS/Mg-HAp scaffold with controlled porosity, an interconnected architecture, and suitable mechanical behavior.
- Experimental confirmation of Mg incorporation and a clear distinction between HAp and Mg-HAp formulations.
- Controlled and reproducible release of the incorporated bioactive agents.
- Quantitative antibacterial activity, including measurable MIC/MBC and biofilm effects.
- High cytocompatibility with enhanced cell adhesion and proliferation.
- Greater osteogenic differentiation in the combined bioactive formulation than in single-agent or non-loaded controls.
- Increased Wnt/β-catenin signaling accompanied by elevated RUNX2, ALP and OCN expression.
- Attenuation of the osteogenic response following pathway inhibition, providing mechanistic evidence for Wnt/β-catenin involvement.
- Enhanced new bone formation *in vivo* compared with appropriate controls.

9. Scientific Novelty

The novelty of the proposed study lies in the transition from a multifunctional scaffold proof-of-concept toward a controlled mechanistic investigation. Rather than evaluating only the complete scaffold against a defect-only control, the proposed design separates the effects of the polymer, ceramic phase, Mg substitution, and individual bioactive agents. The combination of factorial comparisons, quantitative antibacterial testing, and pathway-inhibition experiments will allow the biological contributions of each component and their interactions to be evaluated more rigorously.

The study will also connect scaffold architecture and degradation with controlled bioactive release and cellular signaling. This integrated design is intended to generate evidence that is more informative for international biomaterials and tissue engineering journals and more useful for subsequent translational development.

10. Expected Scientific and Clinical Significance

The proposed research is expected to contribute to the development of multifunctional biomaterials that combine structural support, localized bioactive delivery, antibacterial protection, and osteogenic stimulation. Such a platform may be particularly relevant to non-load-bearing craniofacial and maxillofacial bone defects, where local regeneration and infection control are important considerations. The study will also identify which components of the formulation are biologically essential, thereby reducing unnecessary complexity in future scaffold optimization.

11. Limitations and Risk Management

• Electrospun scaffolds may have limited mechanical capacity for load-bearing applications; therefore, initial translational claims will focus on non-load-bearing defects.

• Bioactive agents may interact with the polymer/ceramic matrix and alter their release; analytical validation and mass-balance assessments will be used.

• Pathway inhibitors may produce off-target or cytotoxic effects; dose-response and inhibitor-only controls will be included.

• *In vitro* degradation and release may not fully reproduce *in vivo* behavior; both datasets will therefore be interpreted within their experimental context.

• Animal sample size will be determined by a power analysis rather than convenience, and ethical principles including reduction, refinement, and humane endpoints, will be followed.

12. Proposed Work Plan

Stage	Main Activities	Estimated Period
I	Formulation optimization and scaffold fabrication	Months 1–2
II	Structural, physicochemical, and mechanical characterization	Months 2–4
III	Drug loading/release and antibacterial evaluation	Months 4–6
IV	Cell viability, osteogenic differentiation, and mechanism studies	Months 5–9
V	*In vivo* bone regeneration, micro-CT and histology	Months 8–12
VI	Statistical integration and manuscript preparation	Months 12–14

13. Relationship to Preliminary Findings

The proposal is based on preliminary work in which an electrospun CS/Mg-HAp nanocomposite co-loaded with icariin, LiCl, and naringin demonstrated sustained release, antibacterial activity, cytocompatibility, the upregulation of Wnt/β-catenin-related osteogenic markers, and improved bone formation in a rat calvarial defect model. The preliminary study also identified important experimental limitations, particularly the absence of intermediate control groups and the need for quantitative microbiological testing and pathway inhibition. The proposed project is therefore designed as a logically connected next-stage investigation rather than a simple repetition of the preliminary study.

14. Keywords

Bone tissue engineering; nanocomposite scaffold; chitosan; magnesium-doped hydroxyapatite; electrospinning; icariin; lithium chloride; naringin; Wnt/β-catenin signaling; osteogenesis; antibacterial activity; 3D biomaterials; bone regeneration.

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Механістично керована розробка біоактивного нанокомпозитного каркаса на основі хітозану та магнієвмісного гідроксіапатиту для остеогенної та антибактеріальної регенерації кісткової тканини

Пропоноване дослідження на основі попередніх результатів щодо каркаса з хітозану та гідроксиапатиту, легованого магнієм (CS/Mg-HAp), отриманого методом електроформування та одночасно насиченого ікаріїном, хлоридом літію й нарингіном

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