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Executive Summary of the Thesis

Bioactive Glass Scaffolds Injected with Riboflavin-Loaded Cement

Laurea Magistrale in Biomedical Engineering - Ingegneria Biomedica

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1. Introduction

Critical-sized bone defects represent a significant clinical challenge due to the limited intrinsic regenerative capacity of bone tissue. When the defect exceeds a critical dimension, spontaneous healing is impaired, often resulting in non-union and functional loss. Current clinical approaches, including autografts and allografts, remain the gold standard for reconstruction; however, they present important drawbacks. Autografts are limited by donor-site morbidity and restricted tissue availability, while allografts carry risks of immune rejection and potential disease transmission [1][2].

To overcome these limitations, synthetic bone substitutes have been widely investigated. Although they eliminate the need for donor tissue, their clinical performance is often compromised by incomplete degradation, suboptimal resorption rates, inflammatory responses, and, notably, implant-associated infections. These challenges highlight the need for advanced biomaterial-based strategies capable not only of supporting bone regeneration but also of modulating biological responses and minimizing adverse effects.

Tissue-engineered scaffolds provide a temporary 3D architecture for cell attachment, proliferation, differentiation, and extracellular matrix (ECM) deposition. To facilitate effective bone regeneration, these structures must satisfy several critical requirements: biocompatibility to support cellular activity without toxicity; mechanical integrity to match host tissue properties and withstand physiological loads; optimal porosity and pore size for nutrient transport and vascularization; and tailored surface topography to influence cell adhesion and osteogenic differentiation. Finally, the scaffold must exhibit bioactivity to actively stimulate bone ingrowth and integration with native tissue. [3].

Ceramic-based scaffolds, including hydroxyapatite, calcium phosphates, and bioactive glasses, are widely employed in bone tissue engineering due to their structural and biological properties. These materials feature a highly porous, interconnected architecture designed to mimic the in vivo features of native bone. This 3D network promotes physiological integration by facilitating cellular infiltration and nutrient exchange within the surrounding tissue.

The clinical utility of bioactive glasses is con-

strained by intrinsic brittleness and low fracture toughness, limiting their use in load-bearing applications. Furthermore, rapid dissolution kinetics can trigger localized alkaline pH shifts that, if not balanced with new tissue formation, compromise both structural stability and cellular viability.

While considered a gold standard, 45S5 bioactive glass faces specific challenges as a 3D scaffold due to its narrow sintering window. The proximity of the glass transition temperature to the crystallization onset often leads to devitrification, which reduces mechanical strength and slows hydroxyapatite conversion.

Borosilicate bioactive glasses, specifically the 1393B20 composition, offer better conversion rates to hydroxyapatite and enhanced thermal stability compared to traditional silicate glasses. These properties facilitate additive manufacturing without crystallization, enabling the fabrication of custom-shaped scaffolds for personalized bone tissue engineering. By incorporating both silica and boron oxide as network formers, these scaffolds provide a distinctive structural framework capable of time-controlled boron release, which is fundamental for supporting bone regeneration processes [4].

Additionally, bone cements are clinically essential for stabilizing implants and repairing irregular defects due to their ability to conform to complex geometries. However, their application is often restricted to non-load-bearing sites due to relatively low mechanical strength and rapid resorption rates, which can compromise structural stability. To mitigate these limitations, reinforcement strategies—such as the incorporation of bioactive glass—are frequently employed.

Calcium phosphate cements (CPCs) are particularly advantageous as versatile drug-delivery platforms. Their biphasic formulation (reactive powder and liquid) allows for the seamless integration of therapeutic agents, including antibiotics, growth factors, and osteogenic compounds, during preparation. This versatility enables the development of multifunctional scaffolds capable of providing both immediate physical filling and localized, long-term biochemical signaling for bone regeneration.

In this context, the present work focuses on the development and implementation of a 1393B20 bioactive glass scaffold–cement composite system designed to investigate its drug-release capabilities. Riboflavin was employed as a model compound to evaluate the release behavior of the system, with the broader aim of assessing its potential as a multifunctional platform for bone tissue engineering applications.

2. Materials and Methods

2.1. Preparation of scaffolds and cement

Three distinct scaffold configurations were developed: (i) cylindrical, (ii) hollow-core cylindrical, and (iii) hollow-core cylindrical injected with riboflavin-loaded cement. The cylindrical scaffolds were fabricated with a height of 3 mm and a diameter of 8 mm. For the hollow-core configurations, a central longitudinal channel was incorporated with an internal diameter of 3 mm. The scaffolds were fabricated via robocasting using an ink consisting of 1393B20 bioactive glass powder (particle size < 30 μm) and a 30 wt% Pluronic F-127 solution.The designs were generated using CAD software and printed using a Brinter®3D-printer. Following a 24-hour drying period at 37°C, the scaffolds underwent a sintering cycle at 625°C for one hour to achieve structural densification while preserving the designed porous architecture.

2.2. Compression Tests

Eight samples per scaffold type were compressed to failure using an Instron Electropuls E1000 (2 kN load cell) at a displacement rate of 0.5 mm/min. Force–displacement data were converted to stress–strain curves using the dimensions of the scaffolds. MATLAB was utilized to calculate fracture strength and Young's modulus.

2.3. In vitro dissolution tests

Prior to immersion, TRIS buffer solution was equilibrated at 37°C. Scaffolds were placed in plastic tubes with TRIS buffer (10 mg 1393B20 glass per 1 mL TRIS), shielded with aluminum

foil to protect light-sensitive riboflavin, and incubated at 37°C with 100 RPM rotation. At six time points (0, 2, 4, 6, 24, and 48 h), samples were collected for pH measurement (at 37 ± 0.02°C) and UV-Vis analysis. Following removal and drying, 1 mL of immersion solution was diluted in 9 mL of 1M HNO_3 for ion concentration assessment, through ICP-OES.

2.4. Compression tests before and after immersion

Uniaxial compression tests were performed on all dry scaffolds ($n = 3$) at two time points: prior to immersion ($t = 0$) and after 48 hours of dissolution. The experimental setup and parameters remained identical to those previously described.

2.5. Cell tests

Cell viability was evaluated via Live/Dead assay using hADSCs (P1, 10k cells/mL). Initially, scaffolds and positive controls were seeded in 24-well plates with 2 mL alpha-MEM (5% HS, 1% P/S). After 24 h, 1 mL of medium was collected for future ICP analysis. To assess the impact of medium volume on proliferation, a second assay was performed using 6-well plates with 4 mL medium.
Following incubation, samples were rinsed with pre-warmed DPBS and treated with a staining solution (1.25 μL Calcein-AM and EthD-1 per 10 mL DPBS). After 30 minutes at RT, fluorescence images were captured across three areas per sample using an Olympus IX51 microscope and processed via ImageJ2.

3. Results

3.1. Mechanical tests

Uniaxial compression tests were performed ($n = 8$) on cylindrical, hollow, and cement-injected hollow scaffolds to evaluate fracture strength and Young's modulus. This analysis aimed to assess the influence of structural design and cement reinforcement on mechanical behavior.
Cylindrical scaffolds exhibited an average fracture strength of 7.40 ± 2.87 MPa, while hollow scaffolds reached 8.92 ± 3.16 MPa, indicating that geometric modification did not compromise mechanical integrity. Hollow scaffolds injected with cement showed the highest strength at 12.23 ± 2.57 MPa. However, despite this higher mean, the large standard deviation precludes the conclusion of a significant reinforcing effect from the cement.
Young's modulus values were 137.4 ± 62.4 MPa for cylindrical, 154.0 ± 46.2 MPa for hollow, and 170.0 ± 49.4 MPa for cement-injected hollow scaffolds. Similar to the fracture strength results, no significant differences in Young's modulus were observed between groups.

3.2. In vitro dissoution tests

In vitro dissolution tests ($n = 3$ per scaffold type) were conducted to evaluate riboflavin release and degradation. Samples were collected at 2, 4, 6, 24, and 48 hours for UV–Vis spectrophotometric analysis and pH measurement at 37°C. All data were processed in MATLAB, with results reported as mean values ± standard deviation.

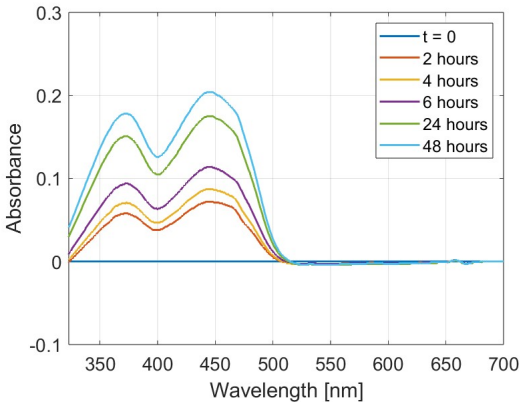

Figure 1: UV-Vis spectra of the TRIS solution after immersion of scaffold with. cement loaded with riboflavin for up to 48 hours of immersion

Absorption spectra exhibited two characteristic riboflavin bands at 372 nm and 444 nm, with intensities increasing over 48 hours without morphological changes to the peaks. Riboflavin release was initially rapid for 6 hours before stabilizing, showing a linear correlation with the square root of time ($R^2 = 0.9616$). Concentration was determined via a standard curve ($y = 7.1212x + 0.0002$; $R^2 = 0.9982$) based on the 372 nm absorbance peak. The consistent increase in intensity confirms a time-dependent release of riboflavin into the TRIS buffer.

3.3. ICP-OES results

ICP–OES was performed to quantify ionic release (Si, B, Ca, Mg, Na, K, P) from 1393B20 bioactive glass scaffolds during immersion in TRIS buffer. Regardless of scaffold geometry or cement presence, concentration for all ions increased over time, with B, Mg, Na, K, and P exhibiting similar release profiles across all groups. Notably, despite the presence of cement, Si and Ca concentrations were lower in cement-loaded samples than in glass-only scaffolds. While all solutions showed a time-dependent pH increase, the cement-loaded scaffolds consistently exhibited lower pH values, correlating with the observed ion release trends.

3.4. Compression tests before and after immersion

Mechanical performance was evaluated via compression tests ($n = 3$) before and after 48 h of immersion and drying to assess the impact of degradation.

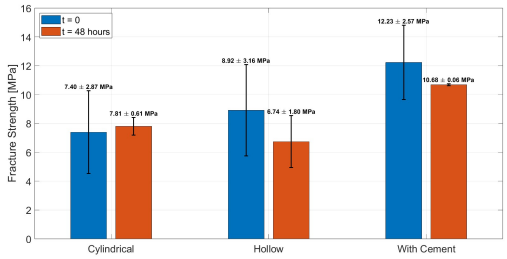

Figure 2: Fracture strength - before and after immersion in TRIS, for all three types of scaffolds

Results showed that immersion did not significantly affect the fracture strength across any scaffold type. While cement-loaded scaffolds exhibited a slight increase in strength, the change remained statistically insignificant.

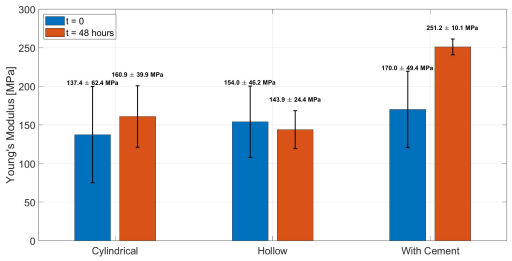

Figure 3: Young's modulus - Before and After immersion in TRIS, for all three types of scaffolds

Conversely, the Young's modulus of glass-only scaffolds remained stable post-immersion, whereas cement-loaded samples showed a slight increase following the 48 h immersion period.

4. Discussion

Compression tests revealed Young's modulus and fracture strength values (7.40 ± 2.87 to 12.23 ± 2.57 MPa) that, while lower than cortical bone, align with the ranges reported for cancellous bone. These properties are likely dictated by strut-level defects rather than overall geometry [5]. The expected reinforcement from the cement was not observed, potentially due to its post-processing viscoelastic nature.

Upon immersion in TRIS, mechanical properties remained largely stable, except for a slight increase in the Young's modulus of cement-loaded samples. This change is attributed to cement hydration, which transitions the material from a viscoelastic to a stiffer, brittle state.

While mass loss was higher for cement-loaded samples, ICP analysis showed that release profiles for most ions remained identical to glass-only scaffolds. The lower concentrations of Si and Ca in cement-loaded samples suggest these ions are trapped within the chitosan matrix via electrostatic interactions or cross-linking [6, 7]. Thus, the excess mass loss in these samples is primarily attributed to chitosan degradation rather than accelerated glass dissolution.

pH levels increased over time for all samples due to Na^+/K^+ and H^+ ion exchange. However, cement-loaded scaffolds showed a lower pH increase compared to glass-only controls. This is

explained by the acidic by-products of chitosan degradation and the trapping of alkaline ions within the polymer matrix [8].

Riboflavin presence did not significantly impact the degradation of the glass or chitosan. Release was initially rapid but slowed over time, following a linear correlation with the square root of time, which indicates a diffusion-controlled mechanism. The stability of the riboflavin was confirmed by the lack of changes in the shape of the absorption band (ratio between band at 372 nm and the one at 444 nm) at all time point of investigation.

Biocompatibility was assessed using hADSCs (P1). Initial tests in 24-well plates (2 mL medium) resulted in significant mortality due to high local ion concentrations exceeding toxicity thresholds. Increasing the volume to 4 mL in 6-well plates mitigated this effect, confirming the scaffolds are not inherently cytotoxic.

Fluorescence microscopy in the 6-well setup revealed high densities of viable, elongated cells, indicating successful adhesion and a favorable environment for proliferation. The results confirm that the 1393B20 glass and riboflavin (regardless of photodegradation) support basic biological requirements for bone tissue engineering.

While these 1-day results are promising, future studies at 3 and 7 days are required to fully evaluate long-term proliferation and differentiation potential.

5. Conclusions

The primary objective of this study was to develop and characterize 3D-printed 1393B20 bioactive glass scaffolds integrated with a riboflavin-loaded chitosan cement for bone regeneration. The scaffolds were successfully fabricated via robocasting and sintering, maintaining a porous architecture with a longitudinal channel for cement injection.

Mechanical analysis revealed that cement integration did not significantly alter compressive strength, which remained within the range of trabecular bone. In vitro dissolution tests confirmed the structural stability of the composite system over 48 hours and demonstrated the successful, diffusion-controlled release of riboflavin. Live/Dead assays verified cytocompatibility, showing that neither the cement nor light exposure induced cell death under optimized medium volumes.

Limitations include the need for mechanical optimization, the restriction to short-term (24 h) biological evaluations, and the lack of comprehensive statistical analysis due to sample size.

Overall, this work establishes a promising platform for bioactive, drug-releasing scaffolds in tissue engineering. Future research should focus on enhancing mechanical properties to approach cortical bone levels, optimizing cement formulations to mitigate volume-dependent cytotoxicity, and investigating the release kinetics of various drugs at different molecular weights.

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