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Bioactive Glass Scaffolds Injected with Riboflavin-Loaded Cement

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Abstract

Critical-sized bone defects remain a major clinical challenge due to the limited intrinsic regenerative capacity of bone. Conventional treatments such as autografts and allografts are associated with significant limitations, including donor-site morbidity, limited tissue availability, risk of immune rejection, and potential disease transmission. Synthetic bone substitutes, while avoiding these issues, might have issues with that they may not degrade fully, may induce inflammation, may not react at an optimum rate, and more importantly are often associated with implant-related infections. Therefore, advanced biomaterial-based strategies are needed to enhance bone regeneration and prevent adverse reaction. In recent years, bone tissue engineering has emerged as a rapidly expanding field aimed at addressing these limitations through the integration of biomaterials science and additive manufacturing technologies. Three-dimensional printing, in particular, enables precise architectural control, allowing the fabrication of scaffolds that mimic the structural complexity of native bone. Among candidate materials, bioactive glasses have gained considerable attention due to their osteoconductive, osteoinductive, angiogenic, and biodegradable properties. Notably, 1393B20 bioactive glass overcomes some of the limitations of conventional silicate-based glasses, such as excessive crystallization during processing, while maintaining pro-osteogenic and pro-angiogenic potential.

The main objective of this thesis was the processing and characterization of a 3D-printed scaffold based on 1393B20 bioactive glass, incorporating a riboflavin-loaded cement aimed at enhancing mechanical stability and promoting bone regeneration. The scaffolds were designed with a central hollow channel intended to host the injectable cement. They were fabricated via robocasting and subsequently sintered to ensure structural consolidation while preserving the predefined porous architecture.

The injectable cement was formulated by combining 1393B20 bioactive glass powder with riboflavin and a chitosan solution, the latter employed as a biocompatible polymeric binder. This composite formulation was designed to improve cohesion within the scaffold structure while potentially introducing additional biological functionality through riboflavin incorporation. Following fabrication and sintering, the scaffolds were injected with the prepared cement to obtain a reinforced composite system.

To evaluate the influence of the riboflavin-loaded cement, mechanical compression tests, in vitro dissolution studies in TRIS buffer solution, and cytocompatibility assessments using Live/Dead assays were performed. The mechanical characterization showed that cement-loaded scaffolds did not exhibit significant differences compared to non-injected scaffolds. Although the compressive strength values were within the typical range of trabecular bone, further optimization may be necessary to enhance mechanical performance. It should be noted that the limited sample size may have influenced the statistical robustness of the results.

In vitro dissolution tests were conducted to investigate the degradation behavior and stability of the composite system through ICP-OES analysis.

Cell viability assays demonstrated cytocompatibility after 24 hours of culture, indicating that the materials did not induce acute cytotoxic effects. Nevertheless, extended biological evaluations are needed to assess long-term cellular response, proliferation, and osteogenic differentiation.

Overall, while the developed system shows promising preliminary results, further investigations are necessary to optimize material composition, improve mechanical properties, and validate its potential for future clinical applications in bone tissue engineering.

Keywords: 3D-printed scaffolds, bioactive glass, bone regeneration, cement, riboflavin, drug-delivery

Abstract in lingua italiana

I difetti ossei di dimensioni critiche rappresentano ancora una grande sfida clinica a causa della limitata capacità rigenerativa intrinseca del tessuto osseo. I trattamenti convenzionali, come gli autoinnesti e gli allotrapianti, sono associati a significative limitazioni, tra cui morbidità del sito donatore, disponibilità limitata di tessuto, rischio di rigetto immunitario e potenziale trasmissione di malattie. I sostituti ossei sintetici, pur evitando tali problematiche, possono presentare criticità quali degradazione incompleta, induzione di infiammazione, cinetiche di riassorbimento non ottimali e, soprattutto, un rischio di infezioni correlate all'impianto. Pertanto, sono necessarie strategie avanzate basate su biomateriali per migliorare la rigenerazione ossea e prevenire reazioni avverse.

Negli ultimi anni, l'ingegneria dei tessuti ossei è emersa come un campo in rapida espansione, volto ad affrontare tali limitazioni attraverso l'integrazione tra scienza dei biomateriali e tecnologie di manifattura additiva. La stampa tridimensionale, in particolare, consente un controllo architetturale preciso, permettendo la fabbricazione di scaffold in grado di mimare la complessità strutturale dell'osso nativo. Tra i materiali candidati, i vetri bioattivi hanno suscitato notevole interesse grazie alle loro proprietà osteoconduttive, osteoinduttive, angiogeniche e biodegradabili. In particolare, il vetro bioattivo 1393B20 supera alcune delle limitazioni dei vetri silicati convenzionali, come l'eccessiva cristallizzazione durante la lavorazione, mantenendo al contempo un potenziale pro-osteogenico e pro-angiogenico.

L'obiettivo principale di questa tesi è stata la produzione e la caratterizzazione di uno scaffold stampato in 3D a base di vetro bioattivo 1393B20, incorporando un cemento caricato con riboflavina finalizzato a migliorare la stabilità meccanica e a promuovere la rigenerazione ossea. Gli scaffold sono stati progettati con un canale centrale cavo destinato ad accogliere il cemento iniettabile. Sono stati fabbricati mediante robocasting e successivamente sinterizzati per garantire il consolidamento strutturale preservando l'architettura porosa predefinita.

Il cemento iniettabile è stato formulato combinando polvere di vetro bioattivo 1393B20 con riboflavina e una soluzione di chitosano, quest'ultima impiegata come legante polimerico

biocompatibile. Questa formulazione composita è stata progettata per migliorare la coesione all'interno della struttura dello scaffold, introducendo potenzialmente anche una funzionalità biologica aggiuntiva attraverso l'incorporazione della riboflavina. Dopo la fabbricazione e la sinterizzazione, gli scaffold sono stati iniettati con il cemento preparato al fine di ottenere un sistema composito rinforzato.

Per valutare l'influenza del cemento caricato con riboflavina, sono stati eseguiti test di compressione meccanica, studi di dissoluzione in vitro in soluzione TRIS e valutazioni di citocompatibilità mediante test Live/Dead. La caratterizzazione meccanica ha mostrato che gli scaffold caricati con cemento non presentavano differenze significative rispetto a quelli senza. Sebbene i valori di resistenza a compressione rientrassero nell'intervallo tipico dell'osso trabecolare, potrebbero essere necessarie ulteriori ottimizzazioni per migliorare le prestazioni meccaniche. Va sottolineato che il numero limitato di campioni potrebbe aver influenzato la robustezza statistica dei risultati.

I test di dissoluzione in vitro sono stati condotti per studiare il comportamento di degradazione e la stabilità del sistema composito mediante analisi ICP-OES.

Le analisi di citotossicità cellulare hanno dimostrato citocompatibilità dopo 24 ore di coltura, indicando che i materiali non inducevano effetti citotossici acuti. Tuttavia, sono necessarie valutazioni biologiche prolungate per analizzare la risposta cellulare a lungo termine, la proliferazione e la differenziazione osteogenica.

Nel complesso, sebbene il sistema sviluppato mostri risultati preliminari promettenti, sono necessarie ulteriori indagini per ottimizzare la composizione del materiale, migliorare le proprietà meccaniche e validarne il potenziale per future applicazioni cliniche nell'ambito dell'ingegneria dei tessuti ossei.

Parole chiave: scaffold 3D-stampati, vetro bioattivo, rigenerazione ossea, cemento, riboflavina, drug-delivery

Contents

Abstract	i
Abstract in lingua italiana	iii
Contents	v
1 Introduction and The State of The Art	1
1.1 Introduction	1
1.2 Bone	2
1.2.1 From macro-structure to nano-structure	3
1.2.2 Cells of the bone	5
1.2.3 Ossification and bone remodeling	7
1.2.4 Bone Defects and Bone Repair Process	8
1.3 Scaffolds for bone regeneration	11
1.4 Bioactive glasses	14
1.5 3D-printed Bioactive glass scaffolds	18
1.5.1 Additive manufacturing	18
1.6 Cements for bone regeneration and influence of riboflavin	21
1.6.1 Bone cements as drug delivery systems	22
1.6.2 Riboflavin	22
2 Materials and Methods	25
2.1 Preparation of 1393B20 glass	25
2.1.1 Glass Casting	25
2.1.2 Glass crushing	27
2.2 Preparation of the scaffolds	28
2.2.1 Preparation of the ink	29
2.2.2 The 3D-printing process	30
2.2.3 The sintering	30

2.2.4	Preparation of the Cement for the injection of Hollow Scaffolds . . .	31
2.3	Mechanical tests: compression tests	31
2.4	In vitro dissolution test	32
2.4.1	Preparation of the buffer solution: TRIS	32
2.4.2	The dissolution tests	32
2.4.3	ICP-OES	33
2.4.4	Mechanical tests: compression tests on dried scaffolds	33
2.5	Cytocompatibility Test	33
2.5.1	Preparation of the samples	33
2.5.2	Live and Dead assay	34
3	Results	37
3.1	Introduction	37
3.1.1	Mechanical tests	37
3.1.2	In vitro dissolution test	39
3.1.3	Mechanical tests before and after immersion	45
3.2	Cytocompatibility test: Live / Dead assay	46
4	Discussion	51
5	Conclusions and future developments	55
	Bibliography	57
	List of Figures	69
	List of Tables	71
	Acknowledgements	73

1 | Introduction and The State of The Art

1.1. Introduction

Since their discovery by L.L. Hench, bioactive glasses have emerged as promising materials for bone regeneration [1]. Indeed, bioactive glasses, dissolve in aqueous solution, releasing ions of therapeutic interests [2]. Not only the release of ions can trigger signaling pathways leading to cell recruitment, the significant calcium release, also, leads to the precipitation of hydroxyapatite (similar to the mineral phase of the bone) at the surface of the glass. Bioactive glass is, thus, classified as osteostimulative (Bioactive Class A) [3]. However, despite the tremendous clinical results obtained when using bioactive glasses, this material still suffers from major drawbacks, limiting their applications [4]. One drawback lies in their tendency to crystallize during heat treatments, such as sintering. Indeed, while silicate bioactive glasses have been usually clinically used in the form of granules [5], it is well known that the optimum shape would be a 3D scaffolds with large pores and a high interconnection [6]. To overcome such limitations borosilicate glasses, with reduced crystallization tendency have been developed and processed into 3D scaffolds [7]. A second drawback lies in the inability of bioceramics, in general, to act as drug delivery system. Indeed, bioceramics are known to degrade from their surface [8]. When the bone grafts are in the form of particles, bulk or scaffold, biomolecules and drugs can only be grafted at the surface of the ceramics. The drug release will henceforth start immediately post-implantation, and the release rate will be high, leading to uncontrolled drug/molecule release [9]. To overcome such challenges, in this thesis we propose to develop a bioactive glass scaffold with a hollow core. The hollow core will be loaded with a bioactive glass/chitosan cement acting as a drug reservoir. The cement will be loaded with riboflavin, a vitamin, taken as a model drug. Here, the mechanical properties of the designed materials will be studied in compression. The dissolution/degradation/bioactivity of the materials will be studied in simulated body fluid. The ability of the cements to release, in a controlled manner, the model drug will be assessed using spectroscopic

techniques.

1.2. Bone

The human body possesses a highly complex structure that enables movement and mechanical stability, primarily ensured by the musculoskeletal system [10]. The musculoskeletal system represents the main structural framework responsible for body movement, posture, and support. It is composed of bones, muscles, tendons, ligaments, joints, cartilage, and other connective tissues, including collagen and elastic fibers, which work synergistically to allow locomotion and load bearing [10].

In particular, the human skeleton consists of 206 bones and forms the rigid component of the musculoskeletal system. These bones are composed of bone tissue, a specialized mineralized connective tissue characterized by its ability to provide structural support, protect vital organs, and contribute to metabolic functions such as mineral homeostasis [11].

Bone is a living and dynamic organ that plays multiple essential roles in the human body. Beyond providing structural support and protection for vital organs, bone enables movement in coordination with the musculoskeletal system. Additionally, bone is the primary site of hematopoiesis: the production of red blood cells, white blood cells, and platelets within the bone marrow [11, 12]. Its multifunctional nature, combining mechanical, protective, and metabolic roles, highlights the complexity and importance of bone tissue in overall human physiology.

Bone tissue exhibits a hierarchical organization, encompassing multiple structural levels that contribute to its mechanical strength and biological function. At the macroscale, bone can be classified into cortical (compact) and trabecular (spongy) bone, which differ in density, porosity, and mechanical properties. At the microscale, the structural unit is the osteon, or Haversian system, which consists of concentric lamellae surrounding a central Haversian canal. The sub-microscale includes the lamellae and the network of Haversian canals, while the nanoscale is characterized by collagen fibers interspersed with mineralized hydroxyapatite crystals. Finally, at the sub-nanoscale, individual collagen molecules and bone mineral crystals can be observed, forming the fundamental building blocks of bone tissue [13]. This hierarchical arrangement is crucial for providing bone with its unique combination of strength, toughness, and biological functionality.

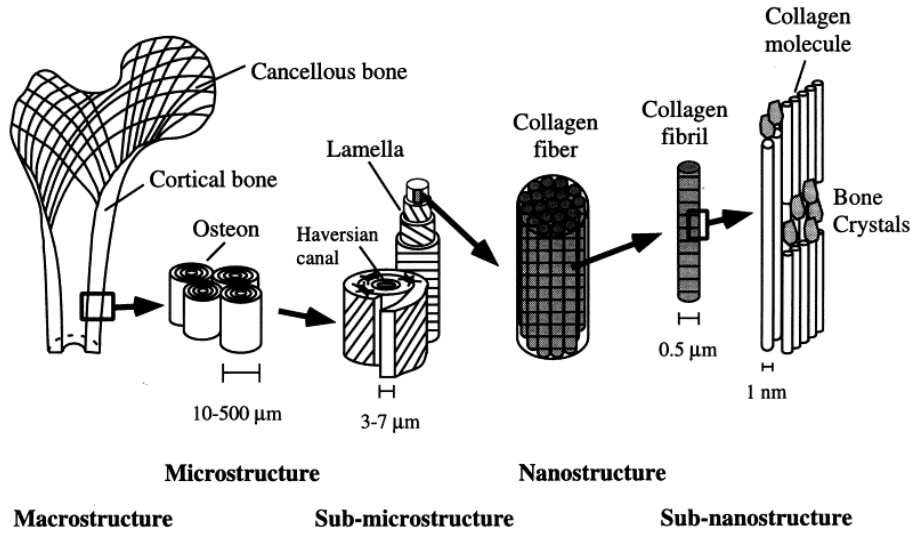


Figure 1.1: Hierarchical system of the bone [13].

1.2.1. From macro-structure to nano-structure

Cortical bone

Cortical bone, also known as compact bone, constitutes the majority of the human skeleton and forms its dense outer layer. It provides structural support, protection for internal organs, and resistance to mechanical loads such as compression and torsion. Cortical bone is predominantly located in the diaphysis of long bones and forms a thin layer in the epiphysis [14, 15].

The basic structural unit of cortical bone is the osteon. Each osteon is traversed by a central Haversian canal containing blood vessels, which ensure vascularization of the surrounding bone tissue. Adjacent osteons, oriented parallel to one another, are interconnected through Volkmann canals, facilitating communication and nutrient transport between Haversian systems. Surrounding the Haversian canals, concentric layers of extracellular matrix, called lamellae, form the cylindrical structure of the osteon. Each mature osteon typically comprises approximately of 4-8 layer of lamellae [15].

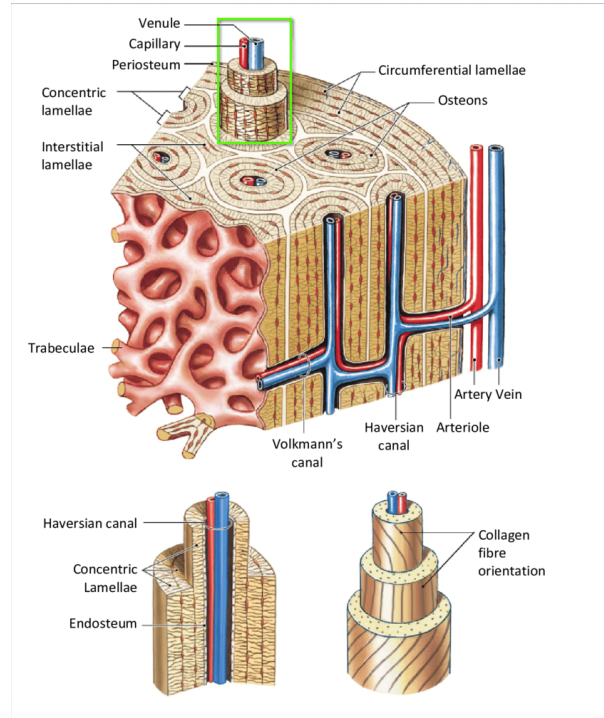


Figure 1.2: Organization in osteons of the cortical bone [16].

The blood vessels within the Haversian canals are responsible for supplying nutrients to bone cells, particularly osteocytes embedded within the lacunae of the lamellae. Osteons are generally aligned parallel to the long axis of the bone, contributing to directional mechanical strength. The highly organized structure of cortical bone underlies its mechanical properties, with reported value of Young's modulus of around 17 GPa [17].

Trabecular bone

Trabecular bone, also referred to as cancellous bone, is composed of a three-dimensional network of trabeculae that provide structural support and contribute to the distribution of mechanical loads within the skeleton [18]. It is predominantly located in the epiphyses of long bones and within the interior of short and flat bones [19]. The trabeculae are oriented along axes corresponding to the principal mechanical stresses experienced by the bone. This organization results in an anisotropic structure that adapts to the type and direction of applied loads, allowing trabecular bone to optimize its strength-to-weight ratio while maintaining a lightweight architecture [20].

Compared to cortical bone, trabecular bone exhibits higher porosity and lower density, which translates into reduced mechanical stiffness. Its Young's modulus is approximately 1 GPa, significantly lower than that of cortical bone, reflecting its function as a load-distributing, energy-absorbing component of the skeletal system [21].

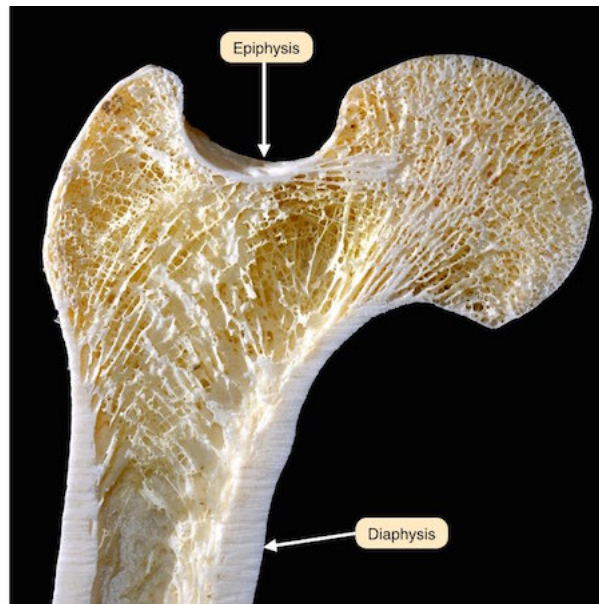


Figure 1.3: Cortical and Trabecular Bone [22].

1.2.2. Cells of the bone

The bone tissue is composed of three types of cells: osteoblasts, osteoclasts and osteocytes. These cells work synergistically to build, destroy and maintain bone tissue in a continuous cycle. Osteoblasts deposit new bone matrix, osteoclasts resorb old bone and osteocytes detect mechanical stress to regulate strength [23].

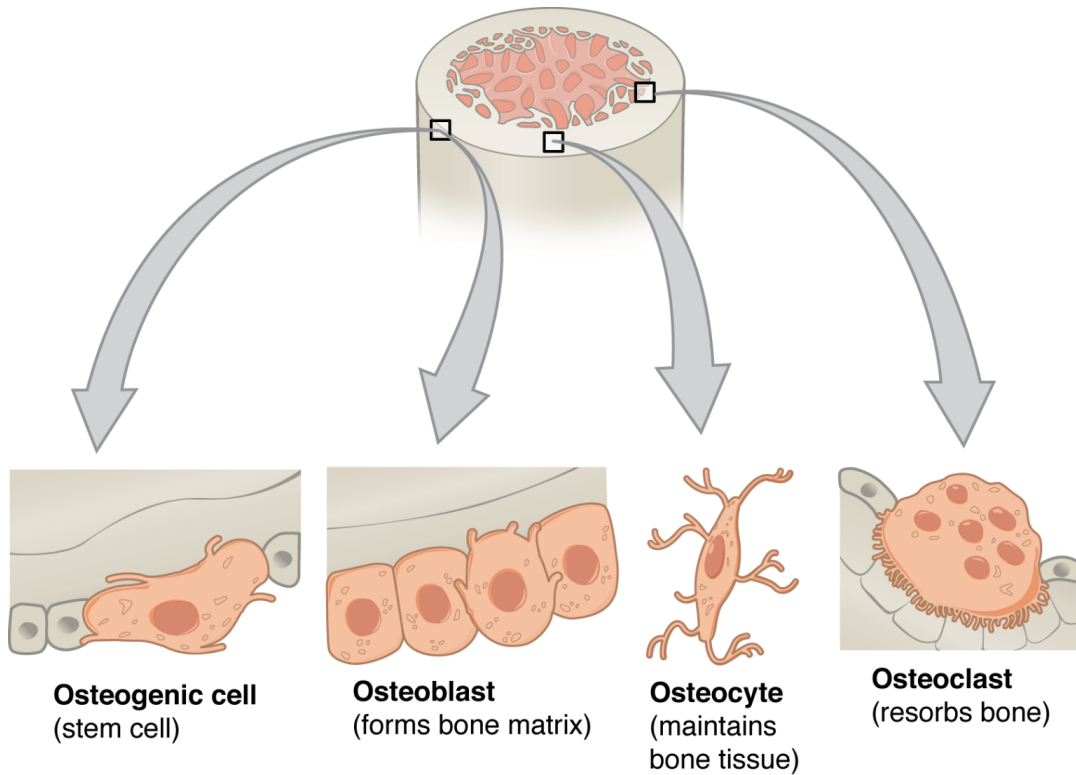


Figure 1.4: Bone Cells: Osteogenic cell, Osteoblast, Osteocyte, Osteoclast [24].

Osteoblasts

Osteoblasts are the primary bone-forming cells responsible for the synthesis and mineralization of the bone matrix. They exhibit cuboidal or cylindrical morphologies and secrete osteoid, an organic extracellular matrix rich in proteins, including type I collagen. Upon mineralization, the osteoid becomes the hardened bone tissue, contributing to skeletal growth and regeneration [25].

Osteoblasts are essential not only for bone development but also for the continuous remodeling of bone throughout life, maintaining skeletal integrity and responding to mechanical and metabolic demands. These cells originate from progenitor cells located in the periosteum and the stromal compartment of the bone marrow, where they differentiate under the influence of specific growth factors and signaling pathways [25].

Osteoclasts

Osteoclasts are specialized, terminally differentiated cells responsible for bone resorption, in contrast to osteoblasts which mediate bone formation. They are large, multinucleated, dome-shaped cells that attach to the bone surface and create resorption pits by secreting acids and proteolytic enzymes, thereby degrading both the mineral and organic

components of the bone matrix [26], [27]. Bone remodeling is a dynamic process in which osteoclast-mediated resorption is closely coupled to osteoblast-mediated formation, allowing for the renewal of bone tissue and adaptation to mechanical and metabolic demands [28]. Osteoclasts also play a key role in bone development and the regulation of calcium and phosphorus homeostasis [29].

Osteocytes

Osteocytes are the most abundant cells within bone tissue and are derived from osteoblasts that become embedded in the mineralized matrix. These cells reside in small cavities called lacunae and extend cytoplasmic processes through canaliculi, forming an extensive communication network throughout the bone [23].

Osteocytes serve a fundamental role in bone remodeling by acting as mechanosensors. They detect mechanical stress and strain and translate these stimuli into biochemical signals that regulate the activity of osteoblasts and osteoclasts, thereby controlling bone formation and resorption. Additionally, osteocytes contribute to the regulation of calcium and phosphorus homeostasis through the production of signaling molecules [30].

Through these functions, osteocytes are essential for maintaining bone mass, structural integrity, and overall skeletal strength, ensuring that the bone can adapt to mechanical demands throughout life [30].

1.2.3. Ossification and bone remodeling

Ossification

Ossification is the biological process by which bone tissue is formed, beginning from mesenchymal connective tissue and initiated during the embryonic stage of development [31]. This process involves the differentiation of mesenchymal cells into bone-forming cells and the subsequent deposition of mineralized extracellular matrix to create mature bone tissue.

There are two primary modes of ossification: intramembranous ossification, also known as direct ossification, and endochondral ossification, or indirect ossification. In both processes, the original mesenchymal tissue is ultimately replaced by bone; however, the mechanisms differ. In intramembranous ossification, mesenchymal tissue is directly transformed into bone without an intermediate cartilage stage. In contrast, endochondral ossification proceeds through a cartilage template that is gradually replaced by mineralized bone.

Bones formed through intramembranous ossification are typically short or flat bones, and are therefore also referred to as membranous bones. The process begins with the con-

densation of mesenchymal cells, followed by their differentiation into osteoblasts, which secrete osteoid. This osteoid subsequently mineralizes, forming trabecular structures that are later remodeled into mature bone, often with a compact outer layer [31].

Endochondral ossification primarily gives rise to long bones, which function as structural supports for the body. In this process, mesenchymal cells first differentiate into chondrocytes to form a hyaline cartilage model of the future bone. The cartilage undergoes proliferation, hypertrophy, and calcification. Subsequently, blood vessels invade the calcified cartilage, bringing osteoprogenitor cells that differentiate into osteoblasts and deposit bone matrix.

Bone remodeling

Bone remodeling is a dynamic and lifelong process through which mature bone tissue is continuously replaced by newly formed bone, ensuring structural integrity, repair of micro-damages, and regulation of systemic calcium homeostasis [32]. This tightly regulated cycle is mediated by the coordinated actions of osteoclasts and osteoblasts and is strongly influenced by hormonal signaling and mechanical stimuli.

Bone resorption is carried out by osteoclasts, which adhere to the bone surface and secrete acids and proteolytic enzymes capable of dissolving both the mineral phase and the collagenous organic matrix. This degradative activity is essential for maintaining skeletal health, enabling repair, and allowing bone to adapt to mechanical and metabolic demands [29].

Importantly, bone remodeling is not performed by isolated cells acting independently, but rather by organized cellular assemblies known as basic multicellular units (BMUs)[33]. BMUs operate on the periosteal and endosteal surfaces, as well as within trabecular and cortical bone, replacing old bone with newly formed tissue in discrete remodeling packets. Each BMU follows a well-defined and sequential process referred to as the activation–resorption–formation sequence. During activation, osteoclast precursors are recruited and differentiate; during resorption, mature osteoclasts remove the existing bone matrix; and during formation, osteoblasts deposit new osteoid that subsequently mineralizes, restoring the structural integrity of the tissue [34].

1.2.4. Bone Defects and Bone Repair Process

Bone defects are damaged areas in bone tissue caused by tumors, trauma or diseases. Usually, micro-sized damages and defects can be self-treated by the bone remodeling process. However, severe cases of bone defect require external interventions: bone grafts,

tissue engineering techniques for effective regeneration. The size of the defect is very important as it influences the spontaneous bone repair process [35].

Bone Repair Process

Fracture healing occurs in 4 overlapping stages: the inflammatory phase, soft callus formation, hard callus formation and the bone remodeling [32].

- Inflammatory response

The inflammatory phase begins immediately after the fracture. Blood vessels are disrupted, causing bleeding and formation of a fibrin-rich haematoma. The hematoma plays a crucial role by releasing growth factors such as PDGF, TGF- β and VEGF. The growth factors stimulate angiogenesis, recruit inflammatory cells and allow tissue repair. Macrophages remove necrotic tissue and coordinate the transition to the repair phase. This stage lasts from 3 to 4 days, and longer based on the gravity of the lesion. The hematoma is reabsorbed by the end of the first week unless there are infections in the lesion site, excessive motion happened or extensive necrosis took place in the surrounding soft tissues [32].

- Soft Callus (cartilage) formation

Within a few days the hematoma starts to be replaced by granulation tissue which gradually becomes fibrous connective tissue. Mesenchymal stem cells from the periosteum, endosteum and bone marrow proliferate and differentiate into chondrocytes under the influence of TGF- β and BMPs. This leads to the formation of a soft callus across the fracture site. The soft callus is the one providing increasing mechanical stability, while still remaining flexible. These phase occurs during the first three weeks [32].

- Hard Callus formation (endochondral ossification)

As the Vascularization gets better and the stability increases, the soft callus starts mineralizing through the endochondral ossification, leading to the formation of a hard callus. Chondrocytes regulate calcification, the cartilage gets resorbed, vascularization is terminating and new trabecular bone is deposited. At this stage an increase in strength and rigidity occurs, although the structure is still immature [32].

- Bone Remodelling

The final stage reshapes the bone to restore the original architecture and the mechanical properties. Osteoclasts resorb excess bone and at the same time osteoblasts deposit new bone according to mechanical stress, following Wolff's law [32].

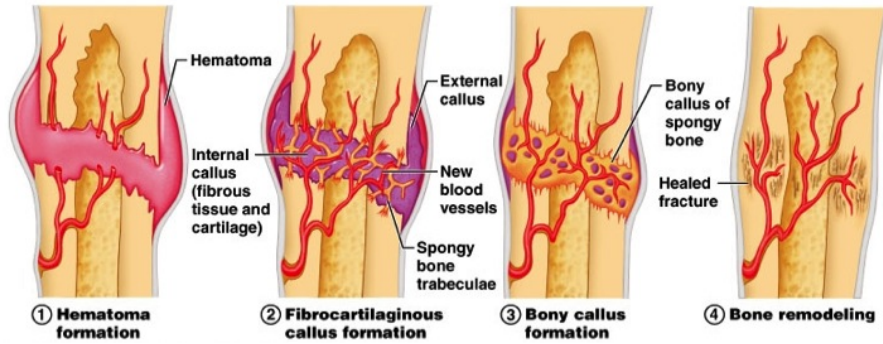


Figure 1.5: Bone Repair Process [36].

Bone defects

A bone defect is defined as the absence of bone tissue resulting from trauma, tumor, bacterial infection and diseases such as osteoporosis or arthritis [37]. Spontaneous bone repair does not occur in cases where the defect exceeds a critical size, as the regenerative capacity of the tissue is insufficient to bridge the defect without clinical intervention [35].

Treatments

The conventional clinical approaches currently used for the treatment of critical-size lesions are:

- **Distraction osteogenesis**

Distraction osteogenesis is a surgical technique that promotes bone growth by slowly and gradually separating two surgically osteomized bone segments using a specialized device (distractor). Based on the tension–stress principle introduced by Ilizarov, controlled mechanical traction induces the formation of a fibrous callus within the gap, which progressively undergoes intramembranous ossification. This method allows bone lengthening, deformity correction, and reconstruction of craniofacial and long-bone defects without the need for grafting. However, it requires prolonged treatment periods and strict patient compliance [38].

- **Autograft**

A bone autograft is a surgical procedure involving the transplantation of bone tissue from one anatomical site to another within the same patient to repair defects, promote healing, or enhance fusion. It is considered the gold standard for bone defect repair because it provides osteogenic cells, osteoinductive growth factors, and an osteoconductive matrix. Additionally, the risk of immune rejection is eliminated. Nevertheless, autografting is limited by donor site morbidity, restricted tissue avail-

ability, and increased surgical time [39], [40].

- **Allograft**

Bone allografts consist of decellularized and sterilized bone tissue harvested from human donors. They primarily serve as osteoconductive scaffolds, supporting new bone ingrowth in procedures such as spinal fusion and joint reconstruction. Although allografts eliminate donor site morbidity, they lack viable osteogenic cells and present potential risks of immune response and disease transmission [40]. A more specific bone allograft is bone marrow allograft more accurately referred to as autologous hematopoietic stem cell transplantation, involves the collection and reinfusion of a patient's own stem cells following high-dose chemotherapy or radiation therapy. While primarily used in the treatment of hematological malignancies, this procedure is not typically considered a standard approach for structural bone defect repair. Its relevance to orthopedic regeneration lies mainly in the osteogenic potential of mesenchymal stem cells derived from bone marrow aspirates [41]

- **Synthetic bone substitutes**

Synthetic bone substitutes are engineered biomaterials designed to mimic the composition and structure of native bone. Common materials include calcium phosphates, hydroxyapatite, and bioactive glasses. These substitutes provide osteoconductive support and offer advantages such as unlimited availability and reduced infection risk. Biomaterials used as bone substitutes may not completely degrade [42], can trigger inflammatory responses [43], and may not resorb at an optimal rate for proper bone regeneration [42].

1.3. Scaffolds for bone regeneration

To overcome the limitations of allograft and autografts, that are still today the gold standard in bone repair, biomaterials tissue engineered devices have been developed.

Tissue-engineered scaffolds are designed to provide a temporary three-dimensional structure that supports cell attachment, proliferation, differentiation, and extracellular matrix deposition. For effective bone regeneration, scaffolds must satisfy several critical requirements [44], [45]:

- **Biocompatibility and non-toxicity**

The material must not elicit adverse inflammatory or immune responses and should support cellular viability and function.

- **Mechanical properties**

Appropriate stiffness and compressive strength are critical, especially in load-bearing applications. In particular, tuning the scaffold's elastic modulus to closely resemble that of the surrounding native bone is fundamental, as mechanical stimuli play a key role in directing stem cell fate through mechanotransduction mechanisms. If the device is intended for load-bearing applications, its mechanical properties should ideally match those of cortical bone, as this tissue provides the primary structural support and resistance to bending and compression. In contrast, for non-load-bearing applications, mechanical properties comparable to cancellous (trabecular) bone are generally sufficient. Moreover, even in load-bearing scenarios, targeting the mechanical properties of cancellous bone may often be adequate in practice. This is because surgeons typically adopt a conservative approach and frequently use additional fixation systems (e.g., plates and screws) to stabilize the fracture site, thereby reducing the mechanical demand on the graft and minimizing the risk of graft failure [46].

- **Porosity and pore size**

Interconnected porosity is fundamental for nutrient diffusion, vascularization, and cell migration. Pore size directly influences cell infiltration and osteogenic differentiation. Generally, pore sizes above 100–300 μm are considered favorable for bone tissue ingrowth and vascular formation [47].

- **Surface topography**

Surface roughness and micro-/nano-scale architectural features modulate protein adsorption and integrin-mediated cell adhesion. These parameters significantly influence cellular morphology, proliferation, and lineage commitment.

- **Load-bearing and mechanical stimulation**

The ability to withstand cyclic compressive loading is particularly relevant in bone tissue engineering. Mechanical stimulation enhances osteogenic differentiation, promotes matrix mineralization, and improves the overall functional integration of the scaffold.

Overall, the rational design of bone scaffolds requires the integration of biological compatibility, structural architecture, and mechanical performance in order to replicate, as closely as possible, the *in vivo* bone microenvironment [44], [48].

The materials employed in the fabrication of scaffolds for bone regeneration are primarily classified into natural and synthetic polymers. These two categories are often combined in order to balance biological performance with adequate mechanical strength [1]. The integration of different material classes allows the development of scaffolds that simulta-

neously promote cellular activity and provide structural support.

Natural polymers are derived from renewable sources, including animal and plant-based materials, and are widely appreciated for their high biocompatibility. Among the most commonly used natural polymers are proteins and polysaccharides. For example, collagen, which represents the main organic component of the bone extracellular matrix (ECM), is extensively utilized due to its intrinsic biological relevance [49], [50]. Polysaccharides such as chitosan and hyaluronic acid are also employed, as they provide favorable conditions for cellular processes and tissue integration [51], [52].

In contrast, synthetic polymers are mainly selected for their predictable mechanical properties and tunable degradation rates [53]. Their physicochemical characteristics can be carefully controlled during synthesis, enabling the design of scaffolds with defined performance profiles [54]. Synthetic polymers may be biodegradable, such as polylactic acid (PLA) and poly(lactic-co-glycolic acid) (PLGA), or non-biodegradable, such as polyethylene glycol (PEG) [51]. Notably, through copolymerization strategies, certain non-biodegradable polymers can be modified to exhibit biodegradable behavior, further expanding their applicability in bone tissue engineering. However, the use of polymeric materials for the regeneration of load-bearing bones can be challenging for their low mechanical strength [55].

In addition to natural and synthetic polymers, bone tissue engineering also makes use of ceramic materials. These components can also be incorporated into polymer–ceramic composite systems to further enhance both the biological response and the structural integrity of the construct. Such materials are particularly valued for their osteoconductive properties and for their ability to mimic the inorganic phase of native bone tissue, thereby contributing to improved interaction with the host environment [1], [56], [57].

Ceramic-based bone scaffolds, including hydroxyapatite, calcium phosphates, bioactive glasses, and bioactive silicates, are widely employed in bone tissue engineering due to their structural and biological properties. These materials are characterized by a highly porous architecture with interconnected microstructures, specifically designed to mimic the in vivo features of native bone tissue. This porous and interconnected network resembles the natural organization of bone, thereby promoting physiological integration with the surrounding tissue [48], [3].

One of the main advantages of ceramic-based scaffolds lies in their intrinsic bioactivity. Their composition enables direct interaction with the biological environment, supporting processes that are essential for tissue repair. In addition, their structural design provides mechanical support during the healing phase, contributing to the stabilization of the

defect site while regeneration occurs [48].

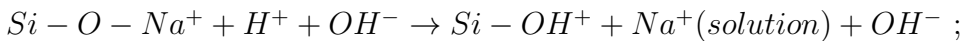
Furthermore, these scaffolds are capable of supporting key cellular activities, including cell proliferation, migration, and differentiation. By providing a suitable three-dimensional environment, they facilitate cellular colonization and tissue formation within the construct. Some ceramic-based scaffolds can also serve as vehicles for the controlled release of growth factors, thereby contributing to enhanced bone regeneration through localized and sustained delivery [48], [58].

1.4. Bioactive glasses

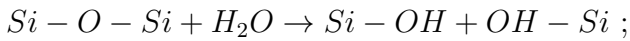
Professor Larry L. Hench first developed Bioglass 45S5 in the late 1960s [57]. Unlike inert biomaterials, which remain biologically passive after implantation, 45S5 is able to form a strong and stable bond with bone tissue through the formation of a hydroxycarbonate-apatite (HCA) layer on its surface, which is chemically similar to the mineral phase of natural bone [57], [59].

The mechanism of dissolution and bone bonding of a bioactive glass, as proposed by L. L. Hench, is a multi-stage process that occurs at the glass–body fluid interface and involves the following sequential steps [1], [60]:

1. A rapid ion exchange occurs between alkali and alkaline-earth cations from the glass (for example Na^+ , Ca^{2+}) and H^+ ions from the surrounding body fluid (or SBF). This process leads to the formation of silanol groups and the release of modifier cations into solution:



2. The breaking of $Si - O - Si$ bonds due to hydrolysis results in the dissolution of soluble silica (effectively silicic acid) into the surrounding medium. This reaction leaves behind silanol ($Si - OH$) groups at the glass–solution interface:



3. The $Si - OH$ groups undergo condensation and repolymerisation reactions, leading to the formation of a silica-rich, cation-depleted layer on the glass surface;
4. Subsequently, Ca^{2+} and $[PO_4]^{3-}$ groups migrate toward the surface through the SiO_2 -rich layer, forming a $CaO - P_2O_5$ -rich film on top of the silica-rich layer. This amorphous $CaO - P_2O_5$ -rich film continues to grow through the incorporation of soluble calcium and phosphate species from the surrounding solution or body fluid;

5. Finally, the amorphous $CaO - P_2O_5$ film undergoes crystallization through the incorporation of OH^- and $[CO_3]^{2-}$ anions from solution, resulting in the formation of a hydroxycarbonate-apatite (HCA) layer.

After the crystallization of the HCA layer, Hench proposed that a series of biologically mediated events follow, ultimately leading to complete bone bonding and regeneration [59]:

6. Adsorption of proteins and growth factors onto the reaction layers formed during the first stages of bioactivity. This adsorption process begins already during the initial surface reactions and plays a crucial role in determining the biological properties of the HA layer, including its ability to support cell attachment and signaling;
7. Activation and action of macrophages at the implantation site. These immune cells contribute to the early inflammatory response and participate in the regulation of tissue healing and remodeling;
8. Attachment of stem cells to the surface of the implant. The HCA layer provides a favorable biochemical and topographical environment for cell adhesion;
9. Proliferation and differentiation of the attached stem cells into mature osteoblasts under the influence of local biochemical signals and growth factors;
10. Mature osteoblasts begin producing additional growth factors that further stimulate cellular attachment, mitosis, and the synthesis of extracellular matrix proteins, leading to the formation of new bone matrix;
11. Following matrix deposition, the extracellular matrix undergoes mineralization, typically within approximately 10–12 days, resulting in the formation of mature bone tissue.

After the discovery of Bioglass, numerous bioactive glasses have been developed for BTE applications [61], [62].

The main types of bioactive glasses that have been developed are:

Silicate-based bioactive glasses

The silicate bioactive glass 45S5, commercially known as Bioglass® 45S5, has been the most extensively investigated composition for biomedical applications since the discovery of its bone-bonding ability [63]. It is based on a three-dimensional SiO_2 network, in which silicon is tetrahedrally coordinated with oxygen [64]. Its bioactivity derives from its specific composition: compared with conventional silicate glasses, 45S5 contains a lower

SiO_2 content and higher concentrations of network modifiers such as Na_2O and CaO , as well as a high CaO/P_2O_5 ratio. These features reduce chemical durability and enhance ionic release, promoting rapid surface reactivity in physiological environments [61].

The bonding of 45S5 to bone is attributed to the formation of a carbonate-substituted hydroxyapatite-like (HCA) layer on its surface upon contact with body fluids [59]. Because this layer closely resembles the mineral phase of bone, it enables strong interfacial bonding with living tissue. After HCA formation, biological events including growth factor adsorption, osteoprogenitor cell attachment, proliferation, and differentiation occur at the implant surface. Mature osteoblasts then produce extracellular matrix, which mineralizes to form a nanocrystalline bone-like structure while the glass progressively degrades [59], [63].

The biocompatibility of 45S5 is well documented [65], [66], [67]. Upon implantation, it undergoes controlled degradation, releasing ions such as Na^+ , Ca^{2+} , and soluble silicon species (primarily $Si(OH)_4$). In vivo studies have shown that released silicon is excreted in soluble form without evidence of toxicity [68].

Despite being considered the gold standard among bioactive glasses, 45S5 presents limitations when used as a three-dimensional scaffold [69][70]. Its narrow sintering window - due to the small temperature range between the glass transition temperature (T_g) and crystallization onset - makes viscous flow sintering challenging [71]. As a result, scaffolds often undergo devitrification [72] [73], forming combeite ($Na_2O - 2CaO - 3SiO_2$), which reduces mechanical strength and slows conversion to an HA-like phase. Moreover, the relatively slow and sometimes incomplete degradation of 45S5 can hinder synchronization between scaffold resorption and new bone formation, potentially leaving residual SiO_2 -rich material in vivo [6], [74].

Degradation of 45S5 also alters the local biological environment by increasing ionic concentrations and pH, particularly during early stages of dissolution [8]. The biological consequences of these changes are complex and not always predictable from in vitro models [75].

To overcome processing limitations, alternative silicate compositions have been developed, including 13-93 glass [76]. Derived from 45S5 but containing higher SiO_2 and additional modifiers such as K_2O and MgO , 13-93 exhibits a wider sintering window, allowing fabrication of porous scaffolds with improved structural integrity and reduced crystallization. While in vitro studies show comparable osteoblastic responses between 45S5 and 13-93 [56], the latter degrades more slowly and converts to an HA-like material at a reduced rate [1].

Borate-based bioactive glasses

Recent research has shown that bioactivity is not exclusive to silicate-based glasses such as Bioglass 45S5 or 13-93, but can also be achieved in borate-based systems. Owing to their lower chemical durability, borate bioactive glasses degrade more rapidly and tend to convert more completely into a hydroxyapatite (HA)-like phase compared with traditional silicate compositions [77]. Although the overall mechanism of HA formation follows a sequence similar to that described for 45S5, borate glasses do not form a silica-rich (SiO_2) layer during conversion, reflecting fundamental differences in their glass network structure [78].

The higher dissolution rate of borate glasses allows modulation of scaffold resorption through compositional design [1]. Partial substitution of SiO_2 with B_2O_3 yields borosilicate glasses, while complete replacement produces fully borate-based systems. Through this strategy, degradation kinetics can be tuned over a wide range, potentially enabling better synchronization between scaffold resorption and new bone formation [79].

Biologically, borate bioactive glasses have been shown to support cell proliferation, osteogenic differentiation, and tissue infiltration in vivo [80][81]. They have also been investigated as platforms for controlled drug delivery, particularly in the treatment of bone infections [82]. However, concerns have been raised regarding the potential cytotoxicity associated with the release of boron as borate ions (BO_3)³⁻ [83]. In static in vitro conditions, certain borate compositions exhibited cytotoxic effects, whereas under dynamic culture conditions such effects were significantly reduced [78].

An additional advantage of borate-based glasses lies in their compositional flexibility, which enables the incorporation of trace elements known to enhance bone regeneration, such as Zn, Cu, F, Mn and Sr [84][85][86][87]. As the material degrades in vivo, these ions can be released at controlled rates, contributing to osteogenesis and angiogenesis [87]. The combination of tunable degradation, processing versatility, and therapeutic ion delivery makes borate-containing bioactive glasses promising candidates for bone regeneration applications [1].

One concern with borate and borosilicate bioactive glasses lies in their potential toxic nature, in vitro, due to rapid release of boron in large concentration [83]. Furthermore, the rapid dissolution of borate glasses, as well as borosilicate glasses with large boron content, leads to significant rise in pH [88][7].

Borosilicate bioactive glasses present several advantages compared to more traditional and commercially available silicate-based compositions. Among these advantages are an

increased conversion rate into hydroxyapatite and improved thermal properties, which enable more complex processing through heat treatment [88]. From a compositional standpoint, borosilicate bioactive glasses contain both silica and boron oxide as network formers, contributing to their distinctive structural and functional characteristics [7].

Borosilicate scaffolds have demonstrated a higher potential to support bone regeneration and hydroxyapatite formation in dynamic in vivo fluid environments. These findings have positioned borosilicate glasses, particularly the 1393B20 composition, within the category of highly promising bioactive materials. Their relevance is attributed to several key features, including the time-controlled release of boron, the possibility of being processed by additive manufacturing techniques without undergoing crystallization, and their ability to promote bone regeneration [7].

Consequently, 3D-printed borosilicate scaffolds appear particularly promising for applications in bone tissue engineering and personalized medicine. Their capacity to be custom-shaped allows precise adaptation to specific defect sites, enhancing their clinical potential. Further developments and material modifications could be investigated to endow these scaffolds with additional properties tailored to specific tissue localizations and clinical requirements [7].

1.5. 3D-printed Bioactive glass scaffolds

1.5.1. Additive manufacturing

Additive manufacturing is a fabrication technique used in biomedical applications in which the materials is deposited layer-by-layer in order to obtain any desired shape starting from a CAD model [89]. It enables highly customized, patient-specific solutions in biomedical engineering, including implants, prosthetics, and surgical models created from medical imaging data [90].

In particular, three-dimensional (3D) printed scaffolds have gained increasing attention in tissue engineering due to their ability to provide a highly controllable and reproducible architecture. Unlike conventional fabrication methods, 3D printing allows precise tuning of scaffold geometry, pore size, porosity, and interconnectivity, which are key parameters for cell infiltration, nutrient diffusion, and tissue regeneration [91].

3D-printed scaffolds are widely used in bone tissue regeneration because they allow precise control over the structural and biological features required for new bone formation [92]. In bone repair, the scaffold acts as a temporary template that supports cell attachment,

proliferation, and differentiation, while gradually being replaced by newly formed tissue [93]. As already mentioned, the main advantages of 3D printing are the ability to design scaffolds with controlled pore size, porosity, and interconnectivity. These parameters are crucial in bone applications because: the pore size and the porosity influence cell penetration, proliferation and differentiation, as well as to promote angiogenesis and bone ingrowth (usually a range of 100-700 μm for pore size and 60-70% of porosity are considered to be a good compromise) [47] [94]; interconnectivity is essential when it comes to both cell migration and a good nutrient-exchange, as well as vascularization [94].

As already mentioned, in order to have a good scaffold for bone regeneration, the scaffolds should exhibit some important characteristics such as balancing biocompatibility, mechanical strength, and controlled degradation in scaffold materials [92].

Different biomaterials are used for 3D-printed scaffolds for bone regeneration:

- **Polymers**

Polymers can be divided into two main categories: natural and synthetic. Natural polymers generally exhibit good biocompatibility and provide cell-recognition signals; however, they present several limitations, including susceptibility to microbial contamination, potential immune reactions, uncontrollable degradation rates, and poor mechanical strength, which restrict their application in hard tissue regeneration [95].

Synthetic polymers, on the other hand, are favored for their predictable mechanical properties and tunable degradation rates. They can be engineered to achieve specific characteristics and typically degrade gradually in the body without leaving toxic residues or byproducts. Their mechanical behavior and degradation kinetics can be tailored by adjusting parameters such as molecular weight, polymerization methods, and processing techniques, allowing for standardized mass production [95]. Nevertheless, synthetic polymers still exhibit limited mechanical strength compared to native bone, and their structure does not fully replicate the hierarchical organization of human bone tissue.

- **Metals**

Metallic biomaterials mainly include stainless steel, cobalt-based alloys, titanium, and titanium alloys. These materials provide excellent mechanical strength and good biocompatibility. However, metallic scaffolds may release ions that can induce local toxicity, thus often requiring surface modifications to improve their biological performance. In addition, metals are generally non-degradable, whereas biodegradability is a desirable feature in bone regeneration applications, where the scaffold

should gradually be replaced by newly formed tissue [96].

- **Bioceramics**

Bioceramic materials exhibit high bioactivity and can promote bone tissue regeneration as well as neovascularization. These materials act as three-dimensional matrices that mimic the mineral phase of bone, promoting osteoconduction and supporting new tissue formation. Ceramic-based synthetic bone scaffolds, such as HA, TCP, bioactive glasses, beta-tricalcium phosphate, bioactive silicates, are typically designed with porous architectures characterized by interconnected macro and microstructures that resemble the mechanical and structural properties of natural bone [96], [48], [97].

Among the different classes of materials used for bone tissue engineering, ceramics, and in particular bioactive glasses, have demonstrated significant potential as scaffold materials. Their main advantage lies in their intrinsic bioactivity, which enables direct interaction with the physiological environment after implantation [97].

When exposed to body fluids, bioactive glasses undergo a series of surface reactions, including ionic exchange, silica network dissolution, and the formation of a silica-rich layer. This process is followed by the precipitation of a calcium–phosphate layer, which progressively crystallizes into a hydroxyapatite-like layer on the scaffold surface [59]. This layer is chemically and structurally similar to the mineral phase of natural bone, allowing strong interfacial bonding between the scaffold and the surrounding tissue and thereby promoting osteointegration [98].

In addition to their osteoconductive properties, bioactive glasses can release biologically active ions (such as Si, Ca, P, and in some formulations Mg, Sr, or Zn), which can stimulate cellular responses [99]. These ionic dissolution products have been shown to enhance osteoblast proliferation and differentiation, promote angiogenesis, and modulate gene expression related to bone formation. Therefore, bioactive glass scaffolds not only provide structural support but also actively contribute to the regenerative process [100].

However, despite these advantages, bioactive glasses also present some limitations. Their intrinsic brittleness and low fracture toughness restrict their use in load-bearing applications [101]. Moreover, their degradation rate may be difficult to precisely control and must be carefully balanced with the rate of new tissue formation. Rapid dissolution can lead to local pH changes, potentially affecting cell viability if not controlled [102].

1.6. Cements for bone regeneration and influence of riboflavin

Bone cement is widely used in orthopedics and dentistry as a bone-filling material for the stabilization of implants and the repair of irregular bone defects. Its clinical relevance lies in its ability to conform to complex defect geometries and provide immediate mechanical support. Among the currently available materials, polymethylmethacrylate (PMMA) remains one of the most commonly used bone cements [103]. However, PMMA is non-degradable, which limits its long-term biological performance [103], [104].

Bone cements are self-setting bone substitute materials, which are generally composed of powder and liquid phases that mixed together turn into a paste, easily injectable and moldable [105]. Furthermore, cements such as calcium phosphates and calcium sulfates have been proven to be osteoconductive [106], [58].

Calcium Phosphate Cements (CPCs)

Calcium phosphate cement (CPC) is widely used for the repair of bone defects due to its close chemical similarity to the mineral phase of natural bone [107], [108]. This compositional resemblance provides excellent biocompatibility and bioactivity, making CPCs particularly attractive for orthopedic and dental applications. CPCs offer several advantageous properties, including self-setting ability, injectability, moldability, osteoconductivity, resorbability [105], and potential for controlled drug delivery [109]. For these reasons, they are considered among the most promising and convenient injectable bone fillers for minimally invasive treatment of bone defects. Currently, CPCs are defined as a combination of one or more calcium phosphate powders that, upon mixing with a liquid phase, form a paste capable of self-setting and hardening in situ at the defect site to generate a scaffold structure [110], [107].

Despite the advantages, CPCs present several critical limitations. One of the major drawbacks is their poor mechanical strength [111]; although they exhibit acceptable compressive strength for certain indications, their tensile and flexural strengths are low, restricting their use primarily to non-load-bearing or moderate-load-bearing applications [112]. In addition, CPCs are inherently brittle and possess low fracture toughness, making them susceptible to catastrophic failure under mechanical stress [110].

Calcium Sulfate (CS) Bone Cements

Calcium sulfate (CS) bone cements represent another class of resorbable bone fillers commonly used in clinical practice. These materials are highly biocompatible, bioresorbable, and relatively cost-effective, making them suitable for filling non-load-bearing bone defects and for use as drug delivery vehicles [106]. CS cements exhibit favorable handling and setting properties, as well as good osteoconductivity, which supports bone tissue ingrowth during the healing process [113].

However, calcium sulfate cements also present notable drawbacks. One of the primary limitations is their rapid degradation rate in vivo, which may result in premature loss of mechanical support before sufficient new bone has formed [110]. In addition, CS cements possess relatively low mechanical strength, further restricting their application to non-load-bearing sites. The fast resorption can also create transient voids within the defect area, potentially compromising structural stability.

To overcome these limitations, various reinforcement strategies have been explored, including the incorporation of additives such as bioactive glass, magnesium, or biodegradable polymers [114], [115]. These modifications aim to improve mechanical performance, modulate degradation kinetics, and enhance overall clinical efficacy.

1.6.1. Bone cements as drug delivery systems

As already mentioned, several bone cements have been investigated as local drug delivery systems for bone regeneration applications [109], [116].

Calcium phosphate cements (CPCs) are especially attractive as drug delivery platforms due to the intrinsic nature of their formulation. Since CPCs consist of a reactive powder phase mixed with a liquid phase, therapeutic agents can be incorporated into either component during material preparation. This versatility allows incorporation of a wide range of bioactive molecules, including antibiotics, anti-inflammatory agents, growth factors, and osteogenic compounds [116].

1.6.2. Riboflavin

In this work, riboflavin (vitamin B_2) was considered because of its strong photosensitivity and its ability to undergo photocleavage when exposed to light [117].

Riboflavin absorbs mainly in the UV and blue light range (around 260–470 nm) [117]. When it is irradiated, it becomes photoexcited and can react with surrounding molecules. In this excited state, it acts as a photosensitizer, leading to the formation of reactive

oxygen species (ROS), such as singlet oxygen and other radical species [118], [119].

Under blue or UV light exposure, riboflavin can break down into photoproducts like lumichrome and lumiflavin [117]. These degradation products are still photoactive and contribute to the overall biological effect. The ROS generated during this process are responsible for the observed antibacterial activity [119].

One of the photoproducts, lumiflavin, is not just an inactive byproduct. Lumiflavin is still a photoactive flavin derivative, and in some cases it can be even more photoreactive than riboflavin itself [120]. Compared to riboflavin, lumiflavin is more hydrophobic which means it can interact more easily with bacterial membranes, insert into lipid bilayers, localize closer to membrane structures. Since ROS have a very short diffusion distance [118], being close to the membrane makes the oxidative damage more effective.

Lumichrome also acts as a photosensitizer, although generally it is considered slightly less reactive than lumiflavin. Its antibacterial effect is mainly linked to light-induced ROS generation [120].

In this thesis, riboflavin was incorporated into a cement based on 1393B20 bioactive glass, which was then injected inside a 3D-printed 1393B20 glass scaffold. The release of riboflavin within this system was investigated. The choice of riboflavin was based on its photocleavable nature and its well-known antibacterial properties. More specifically, riboflavin was selected because it can undergo light-induced degradation under UV or blue light, generating photoactive byproducts and reactive oxygen species, which contribute to its antimicrobial effect.

This work focuses on evaluating how the addition of riboflavin influences the properties and behavior of the 1393B20 glass cement and scaffold.

The following section describes in detail the materials used and the experimental methods adopted to prepare and characterize the developed system.

2 | Materials and Methods

2.1. Preparation of 1393B20 glass

2.1.1. Glass Casting

Analytical grade (Na_2CO_3 , $\text{NH}_4\text{H}_2\text{PO}_4$, CaCO_3 , MgO , H_3BO_3 (Sigma Aldrich, St Louis, MO, USA), K_2CO_3 (Alfa Aesar, Haverhill, MA, USA) and Belgian quartz sand were weighted and mixed, before being placed inside a platinum crucible.

Table 2.1: Reagents and raw material weights.

No.	Reagents	Mw	Weight (g) of raw materials
1	CaCO_3	100.086	31.640
2	H_3BO_3	61.935	19.349
3	MgO	40.31	4.439
4	$(\text{NH}_4)\text{H}_2\text{PO}_4$	115.029	5.594
5	K_2CO_3	138.20	15.618
6	Na_2CO_3	105.99	9.097
7	SiO_2	60.08	37.542

The crucible was then placed inside the furnace and the furnace was turned on to reach 800°C in 50 minutes. Once the temperature was reached, this temperature was kept constant for 15 minutes to prevent foaming and evaporation of carbonates. Then, the furnace temperature was increased up to 1450°C in 50 minutes and, once reached, this temperature was kept constant for 30 minutes. The whole melting process lasted 2 hours and 25 minutes.

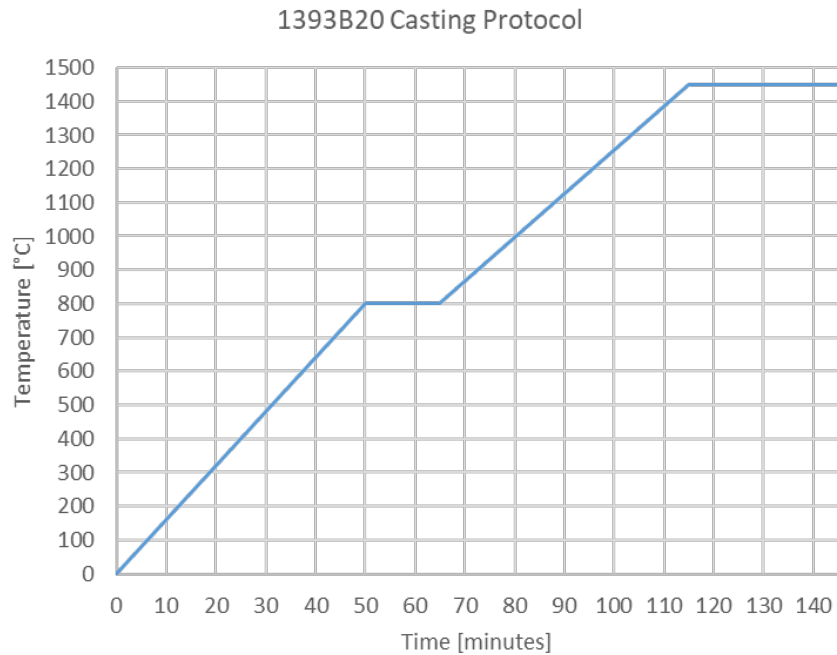


Figure 2.1: 1393B20 Glass melting protocol.

Once the melting was done, the casting process took place, so the melted glass was taken out and poured inside a cylindrical mold, where it stayed 1-2 minutes before being transferred inside the annealing oven.

The annealing process, in view of removing internal stress, lasted 8 hours at 500°C. The furnace was then let to decrease in temperature, using the furnace inertia to prevent thermal stress and the glass was taken out once the temperature of the furnace was below 40°C.

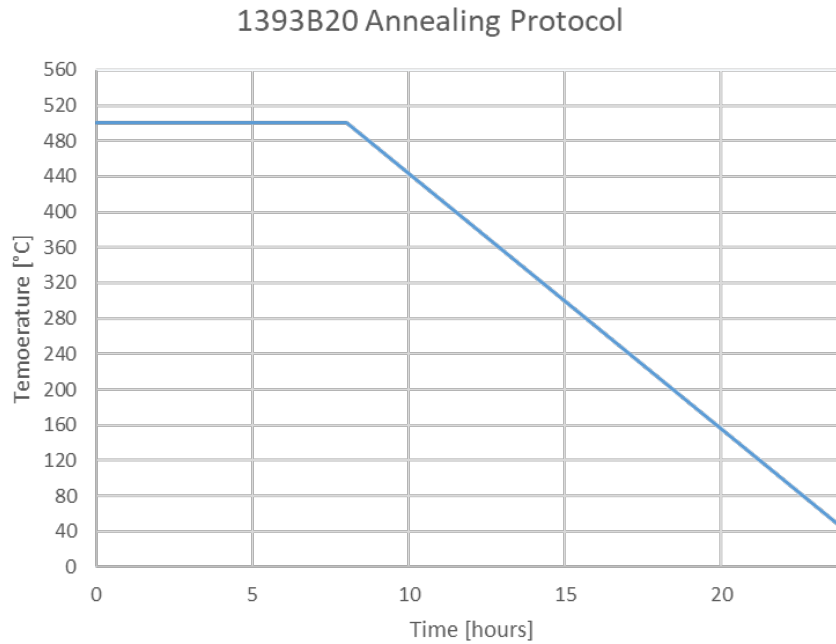


Figure 2.2: Annealing protocol.

2.1.2. Glass crushing

The bulk glass was initially crushed using a hammer and subsequently subjected to ball milling in order to reduce the material to a fine powder. The resulting 1393B20 glass powder obtained from both the hammer crushing and ball milling steps was then sieved to achieve the target particle size, defined as particles with a diameter smaller than 38 μm .



Figure 2.3: Different size 1393B20 glass powder before sieving.

The production of a sufficient quantity of powder with a particle size below 38 μm is

a time-consuming process and requires multiple repetitions of the mentioned steps, including crushing, sieving, and collection, to progressively isolate the finer fraction of the material.



Figure 2.4: 1393B20 Glass powder with size $<38\text{ }\mu\text{m}$.

The final powder was stored in a desiccator to maintain controlled humidity conditions and to prevent moisture uptake by the glass. The glass powder was used within two months following the crushing process in order to preserve its physicochemical properties.

2.2. Preparation of the scaffolds

Three different types of scaffolds were prepared for this study: solid cylindrical scaffolds, hollow cylindrical scaffolds, and hollow cylindrical scaffolds in which a cement composed of 1393B20 glass powder, chitosan solution, and riboflavin was injected. The preparation of multiple scaffold geometries was intended to enable comparative evaluation of their structural and functional characteristics.



Figure 2.5: Cylindrical and hollow scaffolds before sintering.

All scaffolds were fabricated by robocasting. The printing ink consisted of 1393B20 glass powder with a particle size smaller than $38\ \mu\text{m}$, dispersed in a pluronic solution at a concentration of 30 wt-%. The use of fine glass powder and a high-viscosity pluronic carrier allowed the production of a printable ink suitable for controlled extrusion and shape fidelity during the robocasting process.

2.2.1. Preparation of the ink

Based on the required number of scaffolds, the total ink volume was determined prior to preparation. However, the effective number of printable scaffolds may vary due to unforeseen factors such as the presence of air bubbles within the ink, ambient humidity conditions, and fluctuations in the pressure applied by the 3D printer during extrusion. The ink was prepared using a fixed composition of 70 wt-% pluronic and 30 wt-% glass powder. The masses of pluronic and glass powder required for a given ink volume were calculated according to the following equations:

$$mass(pluronic) = density \cdot 0.7 \cdot inkVolume$$

$$mass(glass) = densityGlass \cdot 0.3 \cdot inkVolume$$

where *inkVolume* represents the desired total volume of ink and corresponds to the same value used for the calculation of both components. After weighing the required amount of 1393B20 glass powder and adding the calculated quantity of pluronic, the composite ink was mixed using a vortex mixer. Each mixing cycle consisted of 20 seconds of vortexing followed by 30 seconds of rest in an ice bath to prevent premature gelation of the pluronic. This mixing cycle was repeated ten times to ensure adequate dispersion and homogeneity

of the glass powder within the polymeric matrix. Upon completion of the mixing process, the ink was transferred into a printing cartridge using a syringe and was then ready for the robocasting procedure.

2.2.2. The 3D-printing process

A 3D printer (*Brinter*) was used to fabricate both the solid cylindrical and hollow cylindrical scaffolds. The printing process was carried out using predefined CAD models, and the robocasting technique was applied to enable layer-by-layer deposition of the glass-based ink. The extrusion pressure was manually adjusted during printing based on the observed printability of the ink, in order to ensure continuous filament extrusion and adequate shape fidelity. Printing parameters such as nozzle diameter (0.25 mm), printing speed (8 mm/a), layer height (0.27 mm), and applied pressure ($2000 - 2300\text{ mbar}$) were optimized experimentally and kept constant for all samples.

Following the printing process, the scaffolds were carefully removed from the printing platform and placed in an oven at 37°C . The samples were left to dry overnight to allow solvent evaporation and structural stabilization prior to the subsequent sintering process.

2.2.3. The sintering

The printed scaffolds were sintered following a controlled thermal protocol. The samples were placed inside a furnace at room temperature (RT), and the temperature was increased to 625°C over a period of one hour. This temperature was maintained for one hour to allow sintering of the glass structure. Subsequently, the furnace was allowed to cool naturally to room temperature overnight. The scaffolds were removed from the furnace once the temperature dropped below $30\text{-}40^\circ\text{C}$.

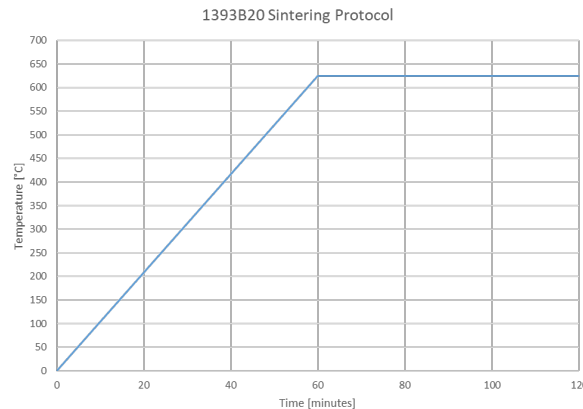


Figure 2.6: 1393B20 Glass scaffold sintering protocol.

2.2.4. Preparation of the Cement for the injection of Hollow Scaffolds

For the preparation of the cement, a solution of chitosan was first prepared. 100 mL of distilled water was put in a beaker and stirred at 500 rpm with a magnetic stirrer. To this, 20 g of citric acid was added and mixed until the solution became clear. 2 g of chitosan was added then added. The whole solution was left on the hot plate with the magnetic stirrer for two hours until it became homogeneous.

The cement was prepared starting with the chitosan solution prepared as said before, the 1393B20 glass powder and Riboflavin. Solid to liquid ratio was 1g/mL was optimized in the research group in previous studies (unpublished). The riboflavin was adjusted to ensure that if all the riboflavin is released during an immersion test, a 0.04 mg/mL of TRIS ratio would be maintained. Based on the final concentration needed, 8.7 mg of Riboflavin and 991.28 mg of glass powder was used. First 1393B20 glass powder was added in a glass jar, then Riboflavin was added and to complete the cement the chitosan was added. Everything was mixed with a spoon as fast as possible and stirred to avoid clumps. The cement paste was then introduced inside a syringe using a spoon. The cylindrical hollow scaffolds were then immediately injected with the cement and let to dry overnight.

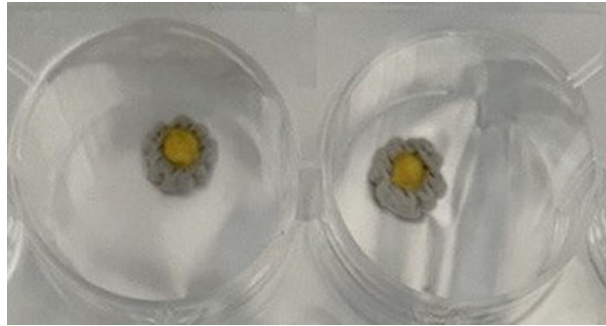


Figure 2.7: Cement injected scaffolds.

2.3. Mechanical tests: compression tests

8 samples per scaffold type have been used. A cell load of 2kN and a displacement speed of 0.5 mm/min were used to execute the compression tests with Instron Electropuls E1000. All scaffolds were crushed and values of the force and displacement were registered. The data were then used to plot the stress-strain curve Stress and Strain, using the dimensions of the scaffolds, measured with a caliper. All data were processed on MatLab to calculate both the fracture strength and the Young's modulus.

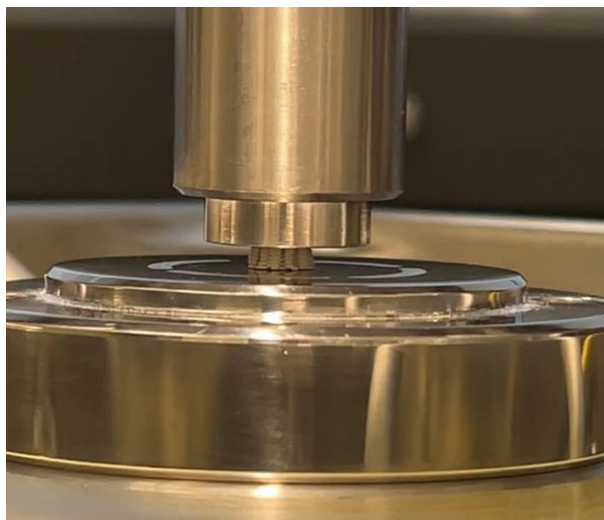


Figure 2.8: Compression test.

2.4. In vitro dissolution test

The 3D-printed scaffolds were subjected to immersion tests in TRIS buffer solution for up to 48 hours in order to investigate ion release from the glass and riboflavin release from the cement-containing scaffolds.

2.4.1. Preparation of the buffer solution: TRIS

The TRIS buffer solution was prepared prior to the immersion tests. A 0.05 M TRIS buffer solution was obtained using Trizma base Ultra Pure (1.66 g) and Trizma HCl (5.72 g), were dissolved in 700 mL ultrapure water in a plastic beaker and stirred using a magnetic stirrer until complete dissolution. After dissolution, the solution was placed in an incubator at 37°C for 3 hours to allow temperature equilibration. The pH of the buffer was measured at 37°C and adjusted to 7.4 ± 0.02 .

2.4.2. The dissolution tests

Prior to the start of the immersion tests, the TRIS buffer solution was equilibrated at 37°C.

A ratio of 1 mL of TRIS solution per 10 mg of 1393B20 glass was used. The scaffolds were placed inside plastic tubes, and the corresponding volume of TRIS solution was added. The tubes containing the scaffolds with cement were covered with aluminum foil to prevent light exposure, as it is known that the riboflavin is light sensitive [117]. All samples were incubated at 37°C in an incubator rotating at 100 RPM.

At each predetermined time point, the samples were collected, and pH measurements and UV-Vis spectrophotometric analyses were performed. Six time points were considered: 0, 2, 4, 6, 24, and 48 hours. This experimental timeline was selected based on literature data and previous studies. At each time point, the pH of the immersion solution was measured at $37 \pm 0.02^\circ\text{C}$. The samples were removed and dried. 1mL of immersion solution was collected and diluted in 9mL of 1M ultrapure HNO_3 for further assess the ion concentration in the solution.

2.4.3. ICP-OES

Prior to performing the ICP-OES (Inductively Coupled Plasma Optical Emission Spectroscopy) analysis, calibration standards were prepared. The standard solutions included all the elements of interest, namely Boron (B), Calcium (Ca), Potassium (K), Magnesium (Mg), Sodium (Na), Phosphorus (P) and Silicon (Si). Four different calibration concentrations were prepared for each element: 4, 10, 20 and 40 mg/L (ppm). To prepare the standards all elements were diluted in Nitric Acid 1 M.

The wavelengths taken into consideration for the ions were: 249.772 nm for Boron, 422.673 nm for Calcium, 769.897 nm for Potassium, 285.213 nm for Magnesium, 589.592 nm ppm for Sodium, 253.561 nm for Phosphorus and 288.158 nm for Silicon. The wavelengths were chosen based on the accuracy of the calibration curves from the standards.

All collected data was processed through MatLab.

2.4.4. Mechanical tests: compression tests on dried scaffolds

Compression tests were performed on all dry scaffolds at two time points: prior to immersion (time 0) and after 48 hours of dissolution testing. 3 samples per time point were used.

The mechanical tests were conducted under uniaxial compression using the same experimental setup described previously.

2.5. Cytocompatibility Test

2.5.1. Preparation of the samples

In order to study the cytotoxicity of the scaffolds injected with cement (with and without riboflavin), cell tests were conducted. To prepare the samples, the 1393B20 scaffolds were first sterilized soaking the scaffolds in EtOH 70% for 10 minutes. Once the 10 minutes

passed, the scaffolds were left under the laminar hood to completely dry. The cements with and without the Riboflavin were prepared under the laminar hood and injected into the scaffolds once they were dry. The cement with Riboflavin was prepared in absence of light. The scaffolds were then left overnight in order for the cement to dry.

To assess potential cytotoxic effects, two control groups were included in the study, for cell tests in the dark and through light exposure. Cells were exposed to light in the absence of scaffolds to evaluate whether light exposure itself could affect cell viability. Scaffolds containing cement without riboflavin were used to determine whether the cement matrix had any cytotoxic effects on the cells. Scaffolds containing riboflavin but maintained in the dark were included to assess whether riboflavin could be inherently cytotoxic independent of light activation. This experimental design allowed the discrimination of potential toxicity arising from the scaffold material, the incorporated riboflavin, or light exposure. Light exposure was a critical parameter, as riboflavin is photosensitive and its biological activity is triggered upon illumination, leading to activation of the components within the cement [117].

The hADSCs were defrosted and cultured in a T-75 polystyrene flask (Cellstar® 75 cm² Flask, Greiner bio-one, Frickenhausen, Germany), which contained a culture medium (CM) composed of MEM Alpha Medium (MEM Alpha Medium (1X), Minimum Essential Medium, Gibco, Thermo Fisher) without nucleosides and enriched with 5% Human Serum (HS; EU Eligible, Type AB Male, Serana, Pessin, Germany) and 1% antibiotics, Penicillin/Streptomycin (P/S) (Gibco, Thermo Fisher, Life Technology Corporation, Grand Island, New York, USA). The cells were cultured in an incubator (Thermo Scientific formastri-cycle i160 CO₂) at 37 °C with controlled atmosphere parameters (air with 5% CO₂, controlled humidity) until they reached 80% confluence. Before every experiment, the cells were detached from the flask using 3 mL of trypsin (TryPLE™ Select (1X), Gibco, Thermo Fisher) and resuspended in culture medium.

2.5.2. Live and Dead assay

Cell viability around the scaffolds was evaluated using a Live/Dead assay. hADSCs at passage P1 were suspended in culture medium (alpha-MEM with 5% HS and 1% P/S) at a density of 10k *cells/mL* and seeded into 24-well plates (ThermoFisher Scientific, Waltham, MA, USA) with 2 mL of cell suspension per well, together with the scaffolds. Three scaffold samples per condition were tested for 24 hours. Three wells without scaffolds were included as positive controls.

After 24 hours, 1 mL of CM from each well was collected and stored at – 20°C for future

ICP analysis.

Given the results from the first Live and Dead assay, an additional cell analysis was performed to verify how cell proliferation changed with a higher volume of medium. In particular 6-well plates (ThermoFisher Scientific, Waltham, MA, USA) were used with 4 mL of MEM Alpha Medium.

The scaffolds and control wells were then rinsed with pre-warmed (37°C) Dulbecco's Phosphate Buffered Saline (DPBS, Gibco, Life Technologies) and treated with 3 mL of staining solution per well. The staining solution was prepared by adding 1.25 μ L of each reagent from the Live/Dead Cell Double Staining Kit (Sigma-Aldrich, 04511) to 10 mL of DPBS at 37°C. Live cells were stained green with Calcein-AM, while dead cells were stained red with Ethidium Homodimer-1 (EthD-1). Samples were incubated with the staining solution for 30 minutes at 37°C and then rinsed again with DPBS.

Fluorescence images were captured from three areas per sample using an Olympus IX51 microscope (Tokyo, Japan) and processed with ImageJ2 1.54 (National Institutes of Health, USA).

3 | Results

3.1. Introduction

This chapter presents the experimental results obtained for the developed scaffolds intended for bone regeneration. The objectives of this work were 1) to assess changes in mechanical properties of the scaffolds with and without a hollow core, 2) to assess the impact of the cements on the mechanical properties as well as the cements/glass dissolution profile and 3) to assess the release of riboflavin, a model drug, into the surrounding medium upon immersion. Finally, a cytocompatibility test was performed using human adipose stem cells

To achieve this, a comprehensive experimental plan was conducted including mechanical characterization, dissolution studies, and in vitro cell assays. Mechanical testing was performed to determine the mechanical properties and suitability of the scaffolds for bone tissue engineering applications. Dissolution experiments were carried out to investigate the Riboflavin release kinetics under physiological-like conditions, while biological assays were conducted to evaluate cell viability and scaffold cytocompatibility.

The results are presented following the chronological sequence in which the experiments were performed. This organization allows a clear understanding of the progressive evaluation of scaffold performance, from physicochemical characterization to biological validation.

3.1.1. Mechanical tests

Uniaxial compression tests were performed to evaluate the mechanical performance of the different scaffold configurations. A total of $n = 8$ samples per group were tested, including: (i) cylindrical scaffolds, (ii) hollow scaffolds, and (iii) hollow scaffolds injected with cement. The aim of this analysis was to determine the fracture strength and Young's modulus of each scaffold type, and to assess the influence of structural design and cement reinforcement on mechanical behavior.

Figure 3.1 exhibits an example of a stress-strain curve obtained. The figure indicates the linear region taken as the elastic domain, as well as the maximum stress before fracture.

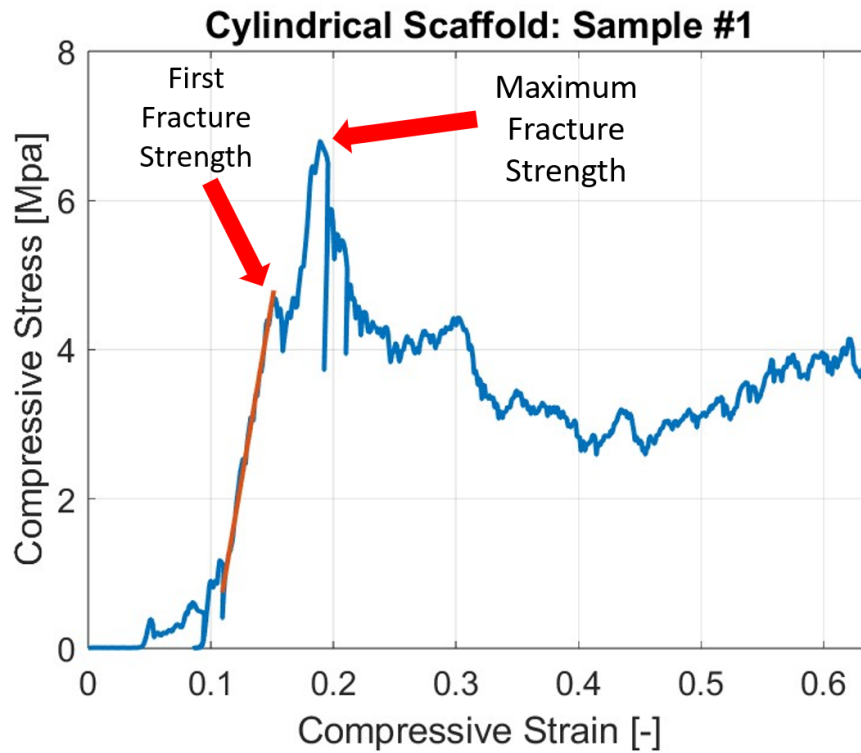


Figure 3.1: Stress-strain curve obtained from mechanical testing of a cylindrical scaffolds, taken as an example, in compression

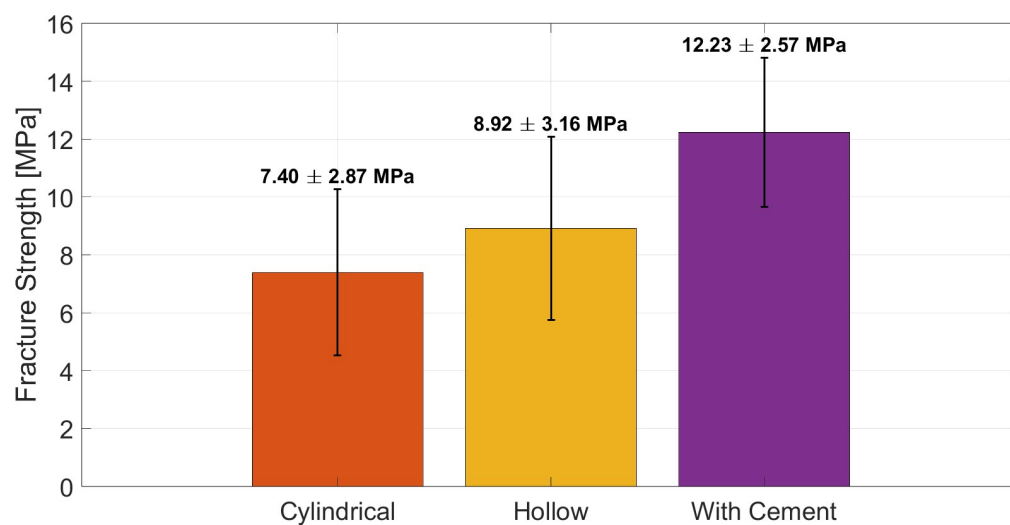


Figure 3.2: Fracture Strength of the scaffolds of investigation (means $\pm$ SD)

As shown in Figure 3.2, the average fracture strength of the cylindrical scaffolds was 7.40 ± 2.87 MPa. Hollow scaffolds exhibited a fracture strength of 8.92 ± 3.16 MPa, indicating that the geometric modification did not compromise the mechanical properties. The highest fracture strength was observed in hollow scaffolds injected with cement, reaching 12.23 ± 2.57 MPa. However, while the value seems slightly higher than those reported for scaffolds without cements, the large accuracy of measurements does not allow to speculate a reinforcing effect of the cements.

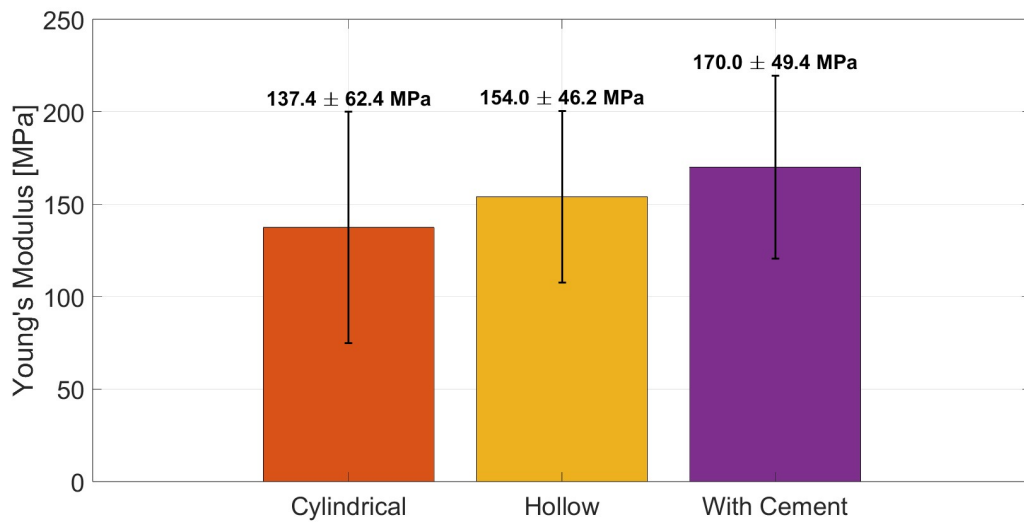


Figure 3.3: Young's modulus of the scaffolds of investigation (means±SD)

The cylindrical scaffold's Young's modulus is 137.4 ± 62.4 MPa, the hollow scaffold's Young's Modulus is 154.0 ± 46.2 MPa and the Young's Modulus of the hollow scaffold with cement is 170.0 ± 49.4 MPa. As for the maximum stress, no significant changes are reported.

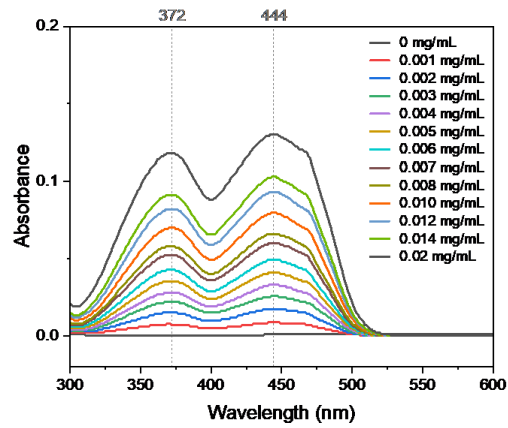
3.1.2. In vitro dissolution test

In vitro dissolution tests were conducted to evaluate Riboflavin release and scaffold degradation over time. A total of $n = 3$ samples per scaffold type were analyzed at each time point.

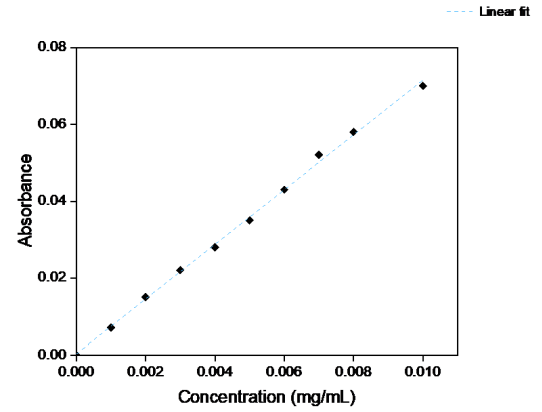
Samples were collected at 2, 4, 6, 24, and 48 hours of immersion. At each time point, the immersion solution was analyzed using UV-Vis spectrophotometry, and pH values at 37°C were recorded. All experimental data were processed using MatLab, and mean values with standard deviations were taken into consideration.

UV-Vis results and concentration of Riboflavin over time

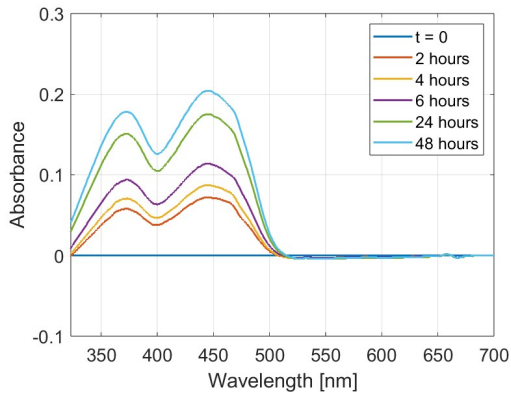
Figure 3.4 presents the UV-Vis spectra of the immersion solution after 2 to 48h of incubation.



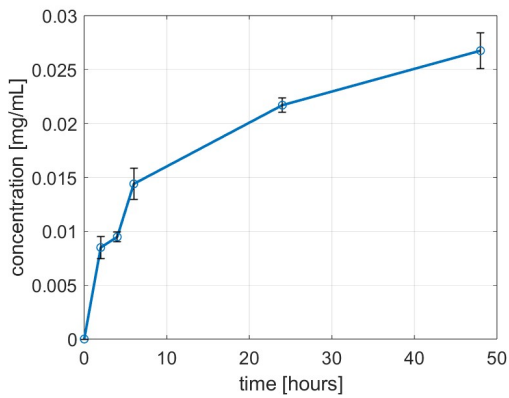
(a)



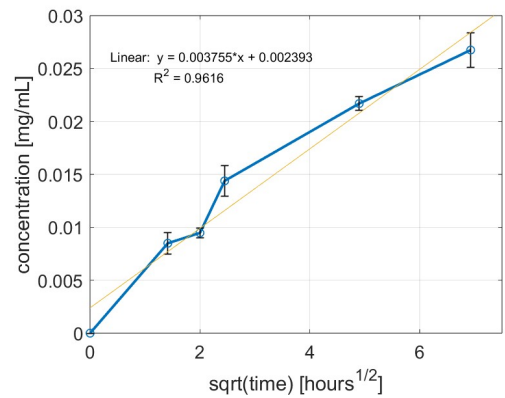
(b)



(c)



(d)



(e)

Figure 3.4: UV-Vis spectra of riboflavin in water at various concentration a) and intensity of the band at 372 nm, as a function of riboflavin concentration b). UV-Vis spectra of the TRIS solution after immersion of cements loaded with riboflavin for up to 48 hours of immersion c). Riboflavin concentration, in TRIS solution as a function of immersion time d) and square root of time e).

All absorption spectra exhibit 2 bands (at 372 nm and 444 nm) corresponding to the absorption band of the riboflavin as already reported in [121]. It is noticeable that the intensity of both bands progressively increases with increasing the degradation time. It is also important to note that the shape of the bands does not change over the testing period.

The riboflavin concentration increases rapidly for up to 6 hours and then the increase slows down. The increase in concentration appears, however, to be linear as a function of the square root of time ($R^2 = 0.9616$).

The standard curve for riboflavin in TRIS buffer solution is presented in Figure 3.4b. It was possible to plot the standard curve by taking into consideration the intensity of the absorbance at 372 nm by taking into account the absorbance peaks at different concentrations of riboflavin in aqueous solution:

$$y = 7.1212x + 0.0002 \quad (3.1)$$

$$R^2 = 0.9982 \quad (3.2)$$

As shown in Figure 3.4c, absorbance intensity increased consistently throughout the 48-hour period, reaching its maximum value at the final time point. This trend indicates a time-dependent release of Riboflavin into the surrounding medium.

Mass loss

Mass loss measurements were performed for all scaffold types at each time point to evaluate degradation behavior. Figure 3.5 presents the mass loss as a function of time for all samples of investigations (mean value $\pm$ SD; n=3).

$$MassLoss = \frac{W_o - W_t}{W_o} * 100 \quad (3.3)$$

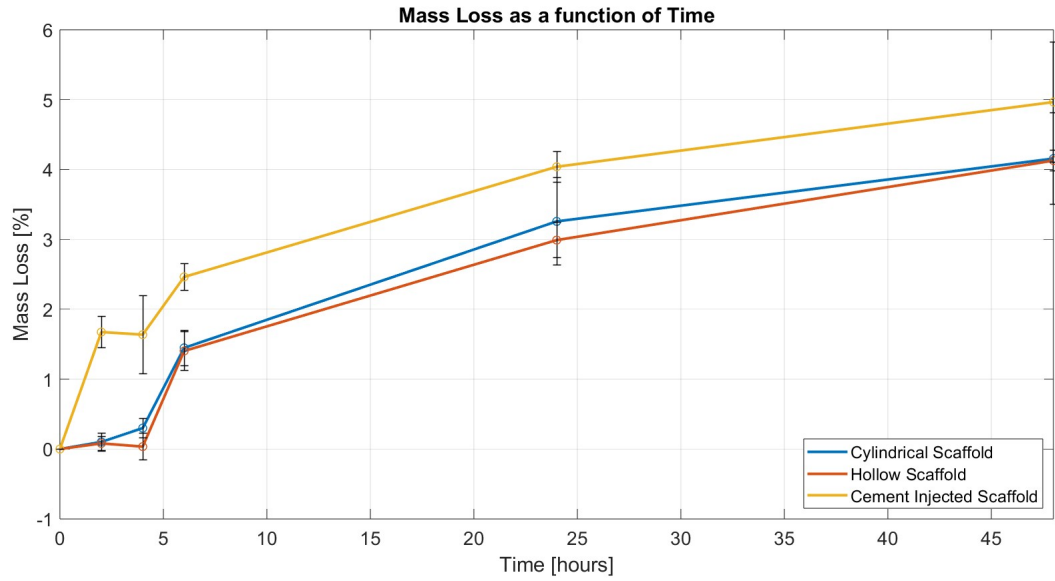


Figure 3.5: Samples mass loss as a function of immersion time.

As shown in Figure 3.5, all samples exhibit a similar mass loss profile. However, it should be noted that while the cylindrical and hollow scaffolds have identical trends, scaffolds containing the cements exhibit an earlier onset of mass loss and a slightly higher overall mass loss.

ICP-OES results

Inductively Coupled Plasma Optical Emission Spectroscopy (ICP–OES) analysis was performed to quantify the ionic release from the 1393B20 bioactive glass scaffolds during immersion in TRIS buffer. The release profiles of Silicon (Si), Boron (B), Calcium (Ca), Magnesium (Mg), Sodium (Na) Potassium (K), and Magnesium (Mg), Sodium (Na), Phosphorus (P), and Silicon (Si) were evaluated as a function of immersion time. TRIS was used as the control solution.

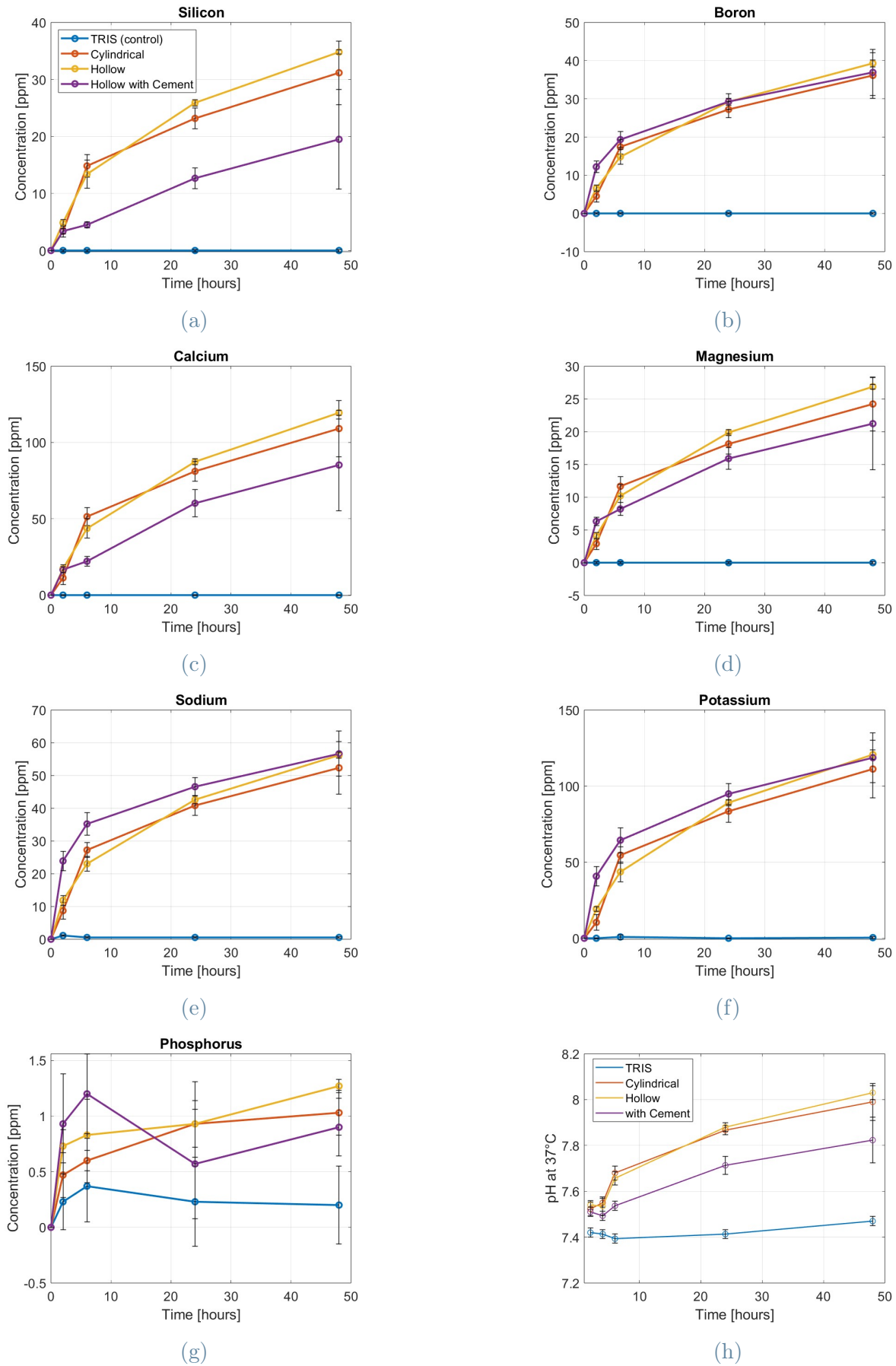


Figure 3.6: Si a), B b), Ca c), Mg d), Na e), K f) and P g) concentration in the TRIS solution over immersion time. pH of the TRIS solution as a function of time h).

Regardless of the samples, with increasing immersion time, an increase in all ions concentration is noticeable. Within the accuracy of the measurements one can also notice that the release of B, Mg, Na, K, and P are similar regardless of the shape of the scaffolds (cylindrical vs hollow) and regardless of the presence of the cement. However, while one would expect a faster degradation of the cement and, therefore a greater content of all ions, it is interesting to see that the Si and Ca concentration are lower in the case of samples loaded with cements. For all samples the pH of the solution increases with time. However, in agreement with the ion release the pH of the solutions containing the scaffolds loaded with the cements exhibit lower pH than the ones for the glass scaffolds alone.

3.1.3. Mechanical tests before and after immersion

Compression tests were performed on all scaffold types before immersion and after 48 hours of immersion in TRIS, followed by drying. This analysis aimed to evaluate the effect of immersion in TRIS on the mechanical performance of the scaffolds. Mean values and standard deviations of fracture strength and Young's modulus are reported.

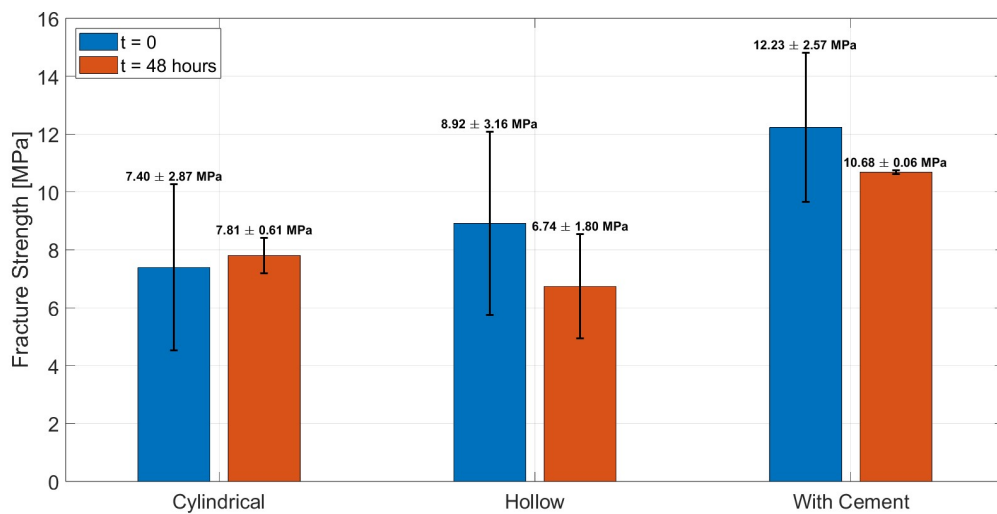


Figure 3.7: Fracture strength - before and after immersion in TRIS, for all three types of scaffolds.

Immersion for 48h does not appear to significantly affect the maximum compressive strength of the scaffolds. As mentioned, earlier, the presence of the cements seemed to lead to a slight increase in the compressive strength. However, the change does not appear significant.

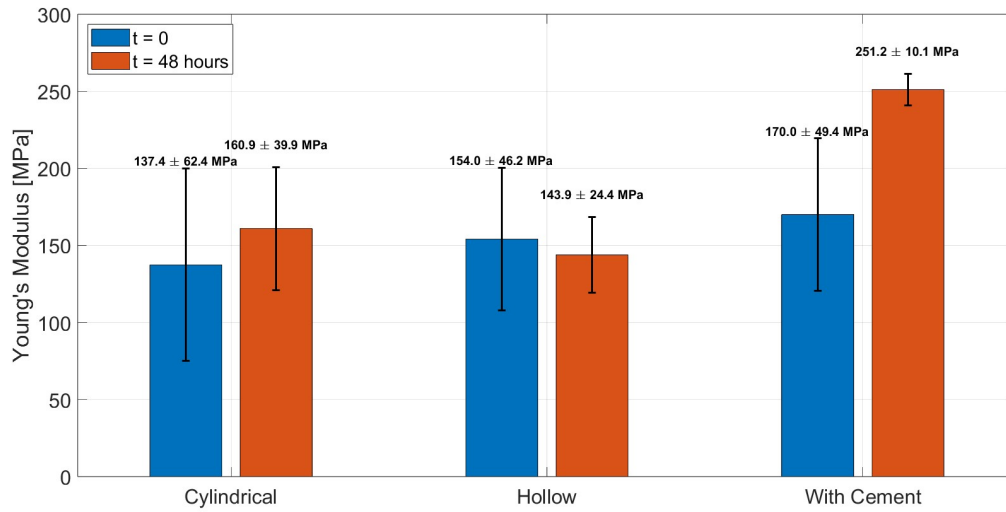


Figure 3.8: Young's modulus - Before and After immersion in TRIS, for all three types of scaffolds.

Conversely, while the young's modulus of the two glass scaffolds (without cements) do not change post immersion, samples loaded with the cement seem to show a slight increase, post immersion.

3.2. Cytocompatibility test: Live / Dead assay

Human adipose stem cells viability tests, using the Live/Dead assay, were performed to assess the cytotoxicity of the scaffolds, with particular focus on the hollow scaffolds containing cement with riboflavin. To evaluate cytotoxicity, the latter were compared to hollow scaffolds with cement without riboflavin. Human adipose stem cells, cultured in direct contact with the well plate, were used as the control. Two experimental conditions were considered: light and dark. Indeed, it is well known that riboflavin is light sensitive and, upon irradiation, photodegrade into lumichrome and lumiflavim. Lumiflavim is also known to be antimicrobial. Only the scaffolds with riboflavin-containing cement were evaluated under light irradiation. In the dark condition, both types of cement were studied with their respective controls.

For each condition triplicates were used.

Light exposure was applied every 2 hours for 15 minutes, with the well plates maintained inside the incubator at 37°C.

Initially, the tests were conducted in 24-well plates, with each well containing 2 mL of medium.

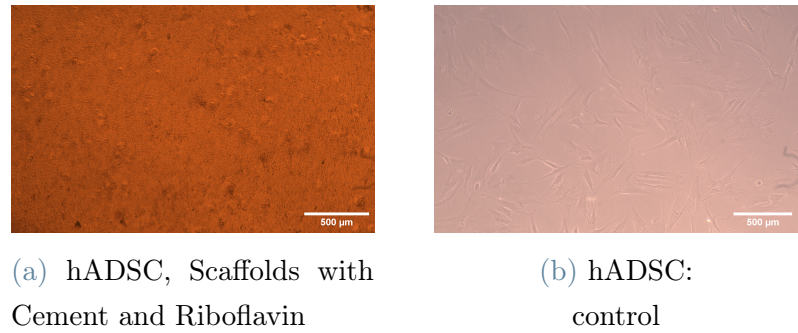


Figure 3.9: Optical microscope images of dead cells. Cells with: cement containing riboflavin scaffolds a), only hADSCs as control b), after incubation under light.

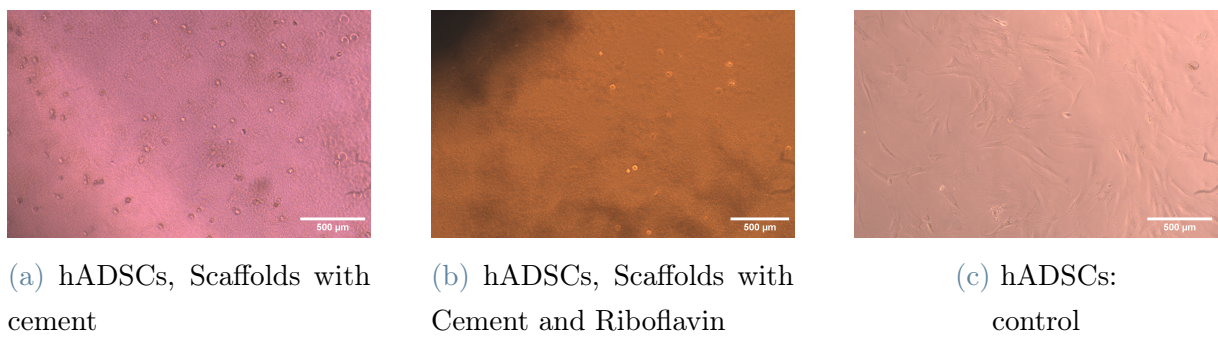


Figure 3.10: Optical microscope images of dead cells. Cells with: cement only scaffolds a), cement containing riboflavin scaffolds b), only hADSCs as control c), after incubation in dark condition.

As evidenced in Figures 3.9a, 3.10a, 3.10b, the samples exhibited cytotoxicity compared to their respective controls, which showed cell viability under both light and dark conditions. In order to understand whether this mortality was dependent on the high concentrations of ions released in the medium, more tests were performed using the 6-well plates. 4 mL of medium were used, instead of the initial 2 mL to have a more diluted environment for the cells.

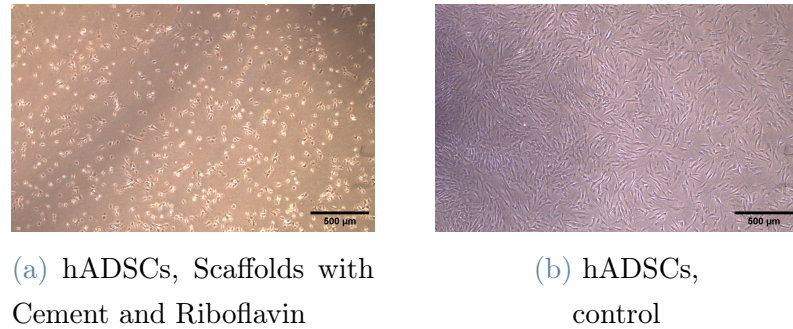


Figure 3.11: Optical microscope images of hADSCs. Cells with: cement containing riboflavin scaffolds a), only hADSCs as control b), after incubation under light.

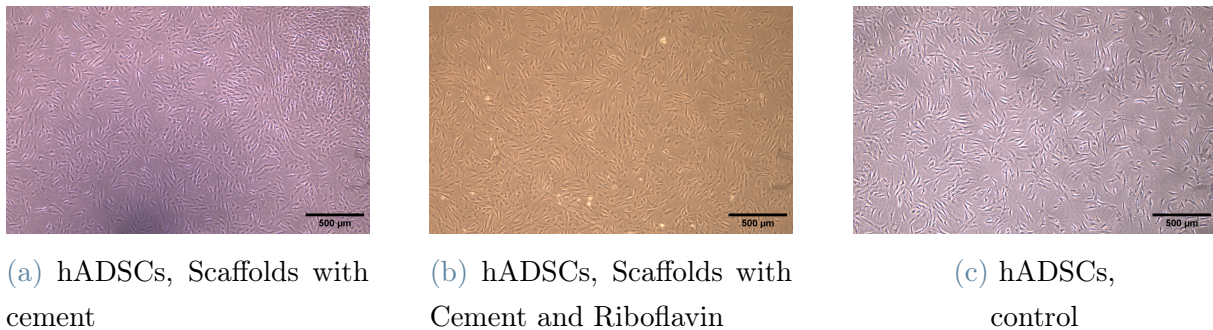


Figure 3.12: Optical microscope images of dead cells. Cells with: cement only scaffolds a), cement containing riboflavin scaffolds b), only hADSCs as control c), after incubation in dark condition.

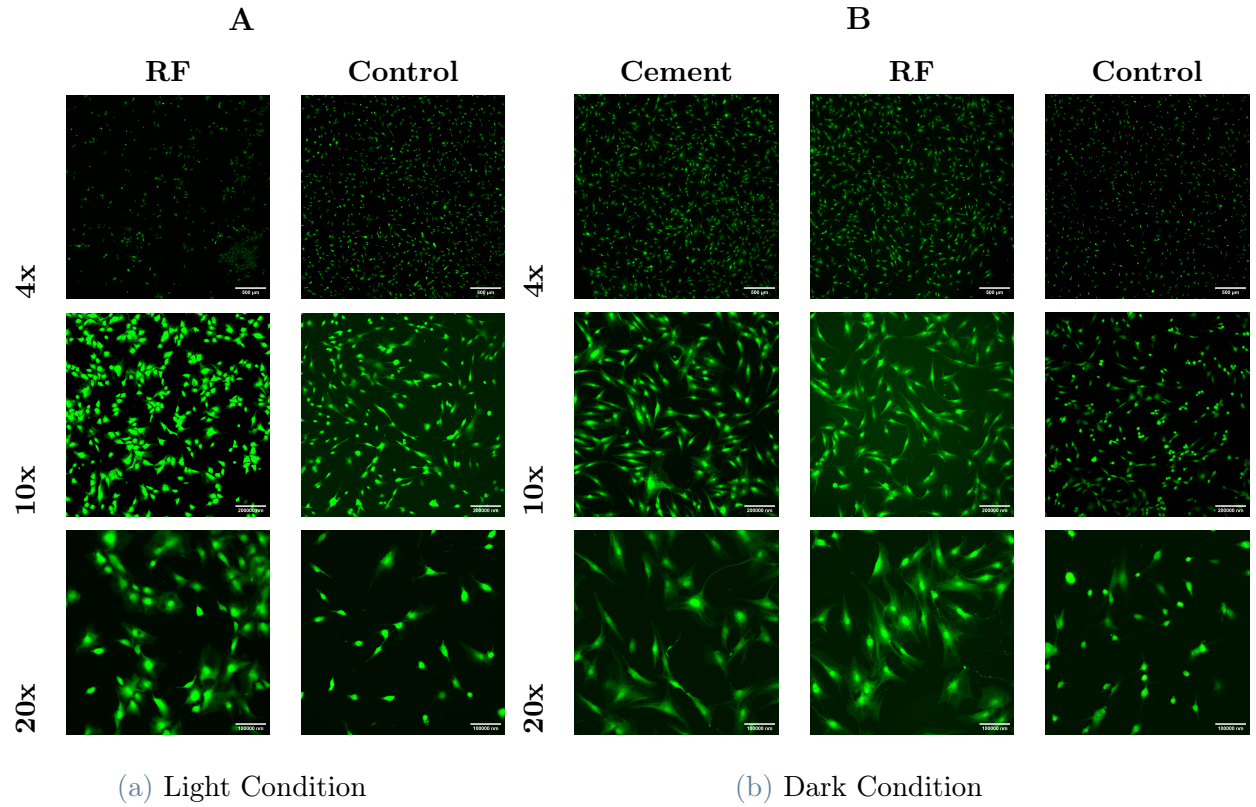


Figure 3.13: Confocal microscope images of cells after 24 hours in contact with the materials, exposed to light A) and not exposed to light B).

As expected, increasing the medium volume contributed to improve cell viability when cells are in direct contact with the scaffolds. In particular, the control group under light exposure showed high viability, ensuring that light exposure was non-toxic to the cells. Furthermore, cells in both light and dark conditions exhibited an elongated shape, characteristic behavior of this cell line.

4 | Discussion

This chapter presents a discussion of the experimental results obtained for the developed scaffolds for bone regeneration applications. The primary objective is to interpret how the addition of a drug-containing cement can influence the mechanical properties of the scaffolds, the dissolution rates, and the ion release behaviors.

Compression tests performed using the Instron Electropuls®E1000 and analyzed with Bluehill Universal®software and MatLab allowed the evaluation of the mechanical properties, such as the Young's modulus and fracture strength, of cylindrical scaffolds, hollow scaffolds, and hollow scaffolds injected with cement.

As reported in the Results chapter, the measured Young's modulus values are relatively low, in the range of MPa and while these values are relatively low compared to the gigapascal (GPa) range typically expected for cortical bone-replacement materials, they remain relevant when considering the porous nature of scaffolds intended to mimic cancellous bone [18]. Similarly, the fracture strength of all materials of investigation are similar, ranging from 7.40 ± 2.87 MPa to 12.23 ± 2.57 MPa, with no significant difference between material type. While the value of the fracture strength is much lower than value reported for cortical bone [17], values fall in the range reported for cancellous bone 0.1 - 100 MPa [18]. Vena et al. reported that in such type of scaffolds the mechanical properties, especially fracture strength, are dictated by defects within the struts rather than the overall geometry and structure of the scaffolds [122]. With regard to the Young's modulus, the strain is measured by the equipment. The strain appears high for a ceramic and may be related to the calibration of the equipment. However, it should be noted that the goal is to compare materials rather than provide a strict elastic modulus value. One hypothesis was that the cement would slightly increase the mechanical properties of the scaffold. This was not seen in this study and might be attributed to the visco-elastic nature of cements post processing [123]. Upon immersion in TRIS, the mechanical properties do not change drastically 3.7, 3.8, except for the Young's modulus of the samples loaded with the cement, that demonstrate a slight increase 3.8. The increase in Young's modulus, post immersion is most likely related to the hydration of the cements, known

to change the mechanical behavior of the cements from visco-elastic, before immersion, to brittle and stiff, once hydrolyzed [124].

Upon immersion in TRIS buffer solution, the bioactive glass scaffold, as well as the cement, were expected to degrade. The degradation of the materials was confirmed by mass loss measurements. As expected, all scaffold of investigation exhibited a progressive reduction in weight. The initial phase of immersion is characterized by a relatively rapid mass loss, which can be attributed to the dissolution of the glass particles located on the outer surfaces of the scaffold. Since these particles are in direct contact with the buffer, they undergo immediate hydration. A significant difference was observed between the cylindrical and hollow and those injected with cement. In the case of the injected scaffolds, thereby the overall mass loss is higher. The total mass loss is influenced by the higher overall bioactive glass content (scaffolds and particles present in the cements). Furthermore, the scaffold architecture plays a crucial role in this process.

The greater mass loss recorded for the scaffolds with cements was not solely attributable to the higher bioactive glass content. Indeed, ICP analysis tends to indicate that the ion release from all materials is fairly identical 3.6. This holds true for all elements, except for the silicon and calcium. It is therefore conceivable that the silica and the calcium remain trapped, during degradation, inside the chitosan matrix. This is supported by the literature where extended work has discussed the silica/chitosan hybrid formation, via electrostatic interaction between negatively charged silica and positively charged amino groups of the chitosan [125]. Calcium is also known to be used for the cross linking of chitosan chain via binding with amine and/or hydroxyl group [126]. In conclusion, while the mass loss is greater for the materials with cements, while the ion release remains fairly similar, the excess mass loss is mainly due to the chitosan degradation. The co-degradation of the glass and chitosan is also confirmed by pH change. Regardless of the material studied, the pH increased with increasing immersion time. However, while the pH was similar at each time point, for the scaffolds without cements, the increase in pH was lower for the scaffolds with cements. The increase in pH, is associated with the first stage of bioactive glass dissolution that involves the ion exchange between Na^+/K^+ and H^+ ions. The second stage of the glass dissolution is the silica layer formation, followed, in appropriate condition, to the precipitation of hydroxyapatite [59], [60]. Chitosan, however, is known to decrease the pH of the solution during degradation [127]. The combination of ions being trapped within the chitosan matrix and the impact of pH of the co-degradation of the chitosan and glass can explain the differences in pH change. Another point to keep into consideration is the presence of riboflavin within the cement. It is, first, important to note that the presence of the riboflavin does not impact significantly the degradation of

the bioactive glass, nor the chitosan, based on previous results obtained within the group (unpublished). Upon immersion, the riboflavin is released in solution. Initially the release is rapid and then it slows down, with full release obtained at 48 hours of immersion 3.6d. While the release is not linear as a function of time, it is linear as a function of the square root of time 3.6e, indicating that the riboflavin release is diffusion controlled [128]. From the UV-Vis spectra one can see that the absorption bands attributed to the riboflavin remain unchanged irrespective of the dissolution time, indicating that the riboflavin is not denaturated by the dissolution process nor by the release of ions from the bioactive glass.

To evaluate the biocompatibility and cytotoxicity of the scaffolds containing the cement with riboflavin, live/dead assays were performed using human Adipose-Derived Stem Cells (hADSCs). The Live/Dead assay at 1 day of culture provides a critical initial assessment of the material's cytotoxicity and the cells' ability to adhere to and survive on the scaffold-cement system. This stage of the study is essential to verify that neither the chemical composition of the 1393B20 glass nor the products of the cement's dissolution interfere with cellular viability. hADSCs were used for the cell tests for their advantageous characteristics such as the fact that they are easily harvested with non-invasive procedures and that they exhibit high proliferative capacities [129]. Furthermore, it is well known that riboflavin is light sensitive and upon photodegradation lumichrome and lumiflavin are formed. Lumiflavin is known to be antimicrobial. The impact of light excitation on the riboflavin degradation was studied in the group and can also be found in literature [130], [117].

As shown in Figure 3.9 and Figure 3.10, the preliminary cell tests resulted in significant cell mortality across the samples. This outcome was attributed to the experimental setup. The restricted volume (2 mL in 24-well plates) led to a too-high local concentration of ions, which surpassed the toxicity threshold for the hADSCs and compromised their viability.

To overcome this limitation and better understand the material's bio-compatibility, more tests were performed using 6-well plates. By increasing the medium volume to 4 mL per well, it was possible to achieve a higher dilution of the released ions, mitigating the toxic effects observed in the first trial. This transition confirmed that the scaffolds themselves were not inherently toxic; rather, the high ionic concentration in the initial 24-well plate setup was the primary cause of cell death.

In fact, as illustrated in the fluorescence microscopy images in Figures 3.11, 3.12 the qualitative analysis reveals a high density of viable cells (stained green) across all samples, with a notable absence of dead cells (stained red). Despite the lack of the morphology

assays, the images clearly show that the cells adopted an elongated shape, which is an indicator of successful cell adhesion and suggests that the cells found the scaffold environment favorable for proliferation. The transition from a rounded to an elongated phenotype is often associated with interaction between the stem cells and the bioactive substrate. This confirms that the material, including the incorporated Riboflavin, is non-toxic and supports the basic biological requirements for bone tissue engineering. Furthermore, not only the dissolution by-products are beneficial for cell behavior, but the released riboflavin, photodegraded or not, does not lead to significant changes in cells behavior.

It should be noted that this study focused on an initial 1-day assessment of cell viability. While these results are promising, a more comprehensive evaluation-including time points at 3 and 7 days would be necessary to fully assess the long-term proliferation, differentiation potential, and overall stability of the hADSCs on these scaffolds.

5 | Conclusions and future developments

The main objective of this study was to develop and characterize 3D-printed scaffolds made of 1393B20 bioactive glass incorporating a riboflavin-loaded cement to study mechanical stability and promote bone regeneration. The scaffolds were successfully fabricated via robocasting and sintering, preserving their designed porous architecture and central longitudinal channel for cement infiltration. The cement formulation, based on 1393B20 glass powder, riboflavin, and chitosan as a biocompatible binder, was successfully integrated into the scaffolds.

Mechanical testing indicated that the addition of cement did not significantly alter the compressive strength of the scaffolds, which remained within the range of trabecular bone. In vitro dissolution tests demonstrated not only structural stability of the composite system over 48 hours, but also successful release of riboflavin, confirming the potential of the scaffolds as a drug-delivery platform. Live/Dead assays further confirmed cytocompatibility, with control experiments showing that neither light exposure nor the cement alone induced cell death under the tested conditions.

Despite these promising results, some limitations were identified. The mechanical performance could be further optimized and biological evaluations were restricted to short-term viability (24 hours). Additionally, statistical analysis was not conducted due to the limited number of samples tested.

Overall, this study provides a promising platform for bioactive, drug-releasing scaffolds in bone tissue engineering and lays the groundwork for further evaluations. Future work could focus on enhancing the mechanical properties to approach those of cortical bone, optimizing the cement formulation to reduce potential cytotoxicity at lower volumes, and systematically investigating the release kinetics of drugs of different molecular sizes.

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List of Figures

1.1	Hierarchical system of the bone [13].	3
1.2	Organization in osteons of the cortical bone [16].	4
1.3	Cortical and Trabecular Bone [22].	5
1.4	Bone Cells: Osteogenic cell, Osteoblast, Osteocyte, Osteoclast [24].	6
1.5	Bone Repair Process [36].	10
2.1	1393B20 Glass melting protocol.	26
2.2	Annealing protocol.	27
2.3	Different size 1393B20 glass powder before sieving.	27
2.4	1393B20 Glass powder with size $<38\text{ }\mu\text{m}$	28
2.5	Cylindrical and hollow scaffolds before sintering.	29
2.6	1393B20 Glass scaffold sintering protocol.	30
2.7	Cement injected scaffolds.	31
2.8	Compression test.	32
3.1	Stress-strain curve obtained from mechanical testing of a cylindrical scaffolds, taken as an example, in compression	38
3.2	Fracture Strength of the scaffolds of investigation (means $\pm$ SD)	38
3.3	Young's modulus of the scaffolds of investigation (means $\pm$ SD)	39
3.4	UV-Vis spectra of riboflavin in water at various concentration a) and intensity of the band at 372 nm, as a function of riboflavin concentration b). UV-Vis spectra of the TRIS solution after immersion of cements loaded with riboflavin for up to 48 hours of immersion c). Riboflavin concentration, in TRIS solution as a function of immersion time d) and square root of time e).	41
3.5	Samples mass loss as a function of immersion time.	43
3.6	Si a), B b), Ca c), Mg d), Na e), K f) and P g) concentration in the TRIS solution over immersion time. pH of the TRIS solution as a function of time h).	44

3.7	Fracture strength - before and after immersion in TRIS, for all three types of scaffolds.	45
3.8	Young's modulus - Before and After immersion in TRIS, for all three types of scaffolds.	46
3.9	Optical microscope images of dead cells. Cells with: cement containing riboflavin scaffolds a), only hADSCs as control b), after incubation under light.	47
3.10	Optical microscope images of dead cells. Cells with: cement only scaffolds a), cement containing riboflavin scaffolds b), only hADSCs as control c), after incubation in dark condition.	47
3.11	Optical microscope images of hADSCs. Cells with: cement containing riboflavin scaffolds a), only hADSCs as control b), after incubation under light.	48
3.12	Optical microscope images of dead cells. Cells with: cement only scaffolds a), cement containing riboflavin scaffolds b), only hADSCs as control c), after incubation in dark condition.	48
3.13	Confocal microscope images of cells after 24 hours in contact with the materials, exposed to light A) and not exposed to light B).	49

List of Tables

2.1	Reagents and raw material weights.	25
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