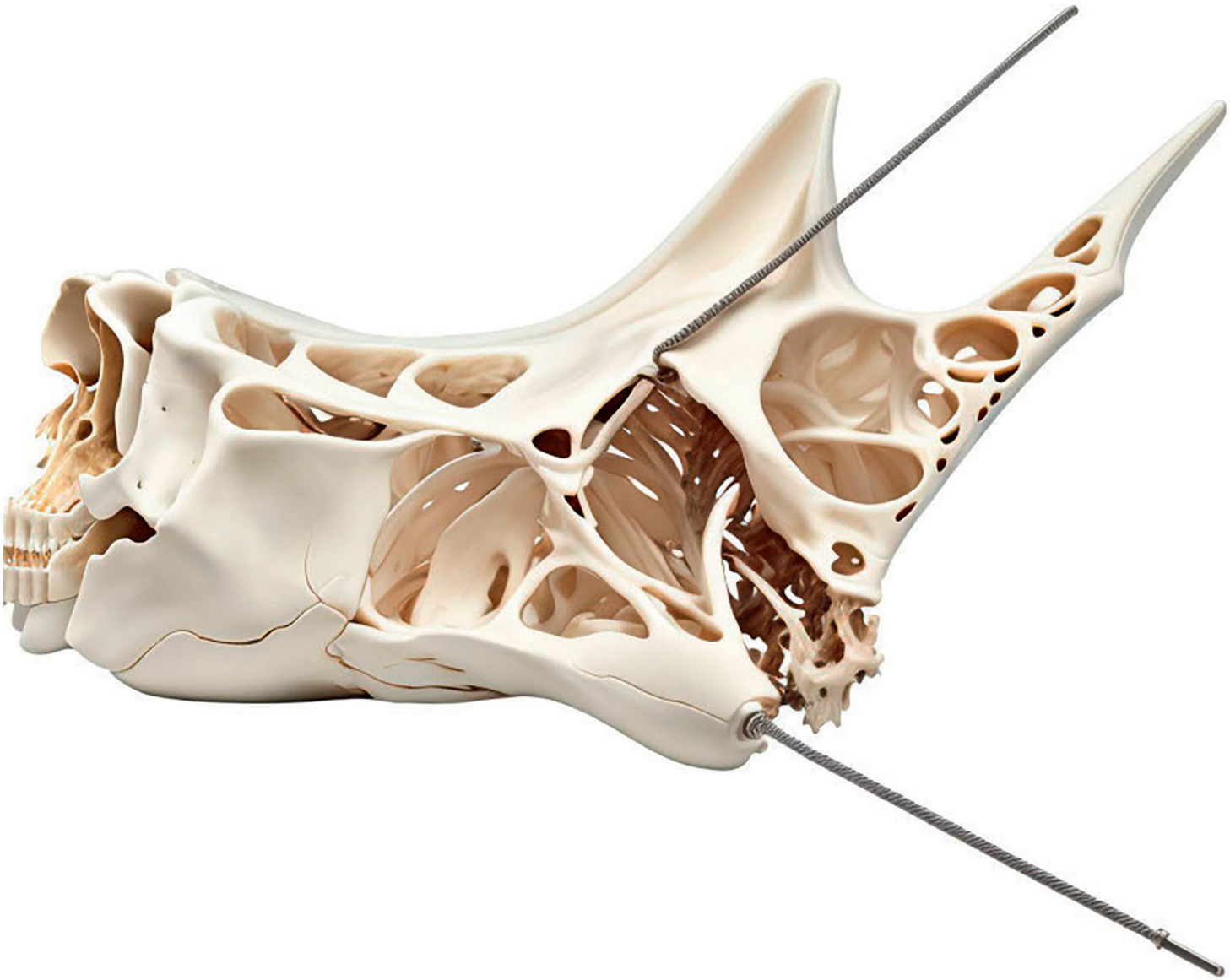
Advancements in Brushite cement formulations for bone repair

Morilla Espino, C.

Advancements in Brushite Cement Formulations for Bone Repair



CLAUDIA MORILLA ESPINO

Advancements in Brushite Cement Formulations for Bone Repair

CLAUDIA MORILLA ESPINO

The research described in this thesis was performed at the Department of Radiology, Leiden University Medical Center, the Netherlands.

The research described in this thesis was financially supported by Department of Radiology, Leiden University Medical Center, the Netherlands.

ISBN: 978-94-6522-634-7

Lay-out: Claudia Morilla Espino

Cover design: Claudia Morilla Espino

Thesis printing: Ridderprint

Advancements in Brushite cement formulations for bone repair

Proefschrift

ter verkrijging van

de graad van doctor aan de Universiteit Leiden,

op gezag van rector magnificus prof. dr. ir. H. Bijl,

volgens besluit van het college voor promoties

te verdedigen op dinsdag 11 november 2025

klokke 14:30 uur

door

Claudia Morilla Espino

geboren te Havana, Cuba

in 1994

Promotors:

prof. dr. L.F. de Geus-Oei

prof. dr. ir. L. van der Weerd

Co-Promotor:

dr. ir. JJP van den Beucken

Leden promotiecommissie

- prof. dr. M. van de Sande
- prof. dr. M.J. Goumans
- dr. D. Gawlitta (UMC Utrecht, Nederland)
- prof. dr. ir. A. Zadpoor (Delft University of Technology, Nederland)

To those who walked this path with me, near or far.

“La escritura es un acto de amor, como lo es también el estudio, la búsqueda del conocimiento.”
“Writing is an act of love, just like studying and the pursuit of knowledge.”
— Isabel Allende

Contents

Chapter 1: GENERAL INTRODUCTION

Introduction

Bone regeneration is a complex process involving well-orchestrated biological events, including bone induction and conduction, that mimic skeletal development and repair [1]. While bone has an intrinsic capacity for regeneration, large defects caused by trauma, infection, tumour resection, or skeletal abnormalities often exceed the body's self-healing potential and require external intervention [1]. Additionally, compromised conditions such as osteoporosis and avascular necrosis further hinder regeneration [1]. The reconstruction of bone defects, whether congenital or acquired, poses significant challenges due to the lengthy and unpredictable nature of the healing process [2]. Factors influencing the success of bone repair include the size and location of the defect, the soft tissue environment, and patient-specific circumstances such as age and chronic disease [3]. Despite advancements, there is no consensus on the diagnosis and treatment of "critical-size bone defects," (i.e. the smallest size intra-osseous wound in a particular bone and species that will not heal spontaneously during the lifetime of the organism [4]) reflecting the complexity and variability of these cases in clinical practice [3].

Composition of bone tissue

The bone extracellular matrix (ECM) and its associated cells play critical roles in bone formation and regeneration (Figure 1) [5]. During bone regeneration, mesenchymal stem cells (MSCs) home to the site of injury and differentiate into osteoblasts, which produce the ECM and facilitate osteoid mineralization, leading to the formation of terminally differentiated osteocytes [5]. The ECM is a non-cellular, three-dimensional structure secreted by cells into the extracellular space, composed of proteins and polysaccharides, with a unique composition and topology that supports tissue development, integrity, and homeostasis [5]. In bone tissue engineering, the ECM is considered a critical component for development [6].

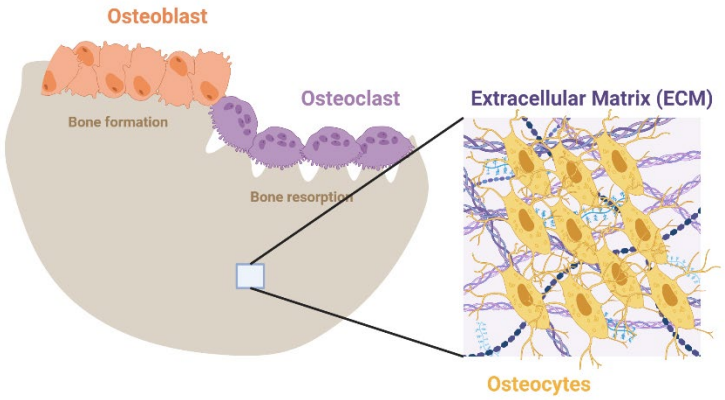


Figure 1. The bone extracellular matrix (ECM) and its associated cells osteoblasts, osteoclasts and osteocytes. (Created in https://BioRender.com)

The bone ECM consists of organic (40%) and inorganic (60%) components, with its exact composition varying by sex, age, ethnicity, and health conditions. The main inorganic elements include calcium-deficient apatite and trace minerals [5]. Osteoblasts, derived from MSCs, play a vital role in bone remodelling by synthesizing the ECM and ensuring its proper mineralization. These cells depend on collagen-based surfaces to produce mechanically stable and structured bone tissue [5]. Osteocytes, the terminally differentiated cells embedded within the bone matrix, communicate with each other and with bone lining cells to support bone growth and repair through processes like perilacunar/canalicular remodelling, which involves continuous resorption and deposition of the matrix [7]. Osteoclasts, which are multinucleated cells formed by the fusion of monocyte/macrophage precursors, are responsible for bone resorption. Their activity is regulated by macrophage colony-stimulating factor (M-CSF) and the receptor activator of nuclear factor κB ligand (RANKL), which are secreted by osteoblasts [5]. Together, the interactions between ECM components and cellular activities ensure bone regeneration, homeostasis, and repair.

Regeneration of bone tissue

Bone tissue regeneration relies on various grafting techniques, with autografts currently considered the gold standard due to their superior osteogenic potential [8]. Autografts involve harvesting bone from the patient, typically from sites like the iliac crest, and are highly effective in promoting bone regeneration. However, they present significant drawbacks, including donor-site morbidity, increased risk of wound infection, prolonged hospital stays, and higher costs, making them less favourable in some cases [9]. These challenges have driven the development and use of alternative grafting methods, including allogeneic, xenogeneic, and alloplastic scaffolds.

Allogeneic bone grafts, derived from human donors other than the patient, are the most commonly used alternative to autografts. While they eliminate the need for a second surgical site, allogeneic grafts pose risks of disease transmission, immune rejection, and resorption [9]. Alloplastic grafts, composed of synthetic materials, and xenogeneic scaffolds, derived from animal sources, offer additional alternatives in regenerative dentistry and bone reconstruction. These substitutes reduce the reliance on autografts, but ongoing research aims to address their limitations, such as varying osteogenic capabilities and integration within the host tissue [8]. Despite advancements, no single substitute has yet fully matched the effectiveness and reliability of autografts.

Calcium phosphate cements (CPCs)

Powder and liquid composition of CPCs

Calcium phosphate cements (CPCs) are biocompatible materials made from calcium phosphate powders that, when combined with a liquid phase, create a mouldable paste capable of hardening at room or body temperature. Once set, CPCs form a solid structure resembling the mineral makeup of natural bone, which makes them ideal for applications in bone repair and regeneration [10, 11]. The powders, often including alpha-tricalcium phosphate (α-TCP) or β-tricalcium phosphate (β-TCP), determine the CPC's reactivity and final composition based on their crystallinity and particle size [12-14]. Lower crystallinity and smaller particle sizes increase reactivity, enhancing the CPC setting properties [12, 14, 15]. The liquid phase typically contains water, but additives like organic acids (e.g., citric acid, sodium citrate) are frequently incorporated to regulate the setting reaction, handling properties, or injectability [16, 17]. These components enable the formulation of injectable CPC pastes that adapt perfectly to bone defect dimensions, facilitating minimally invasive surgical applications [18-20].

Unlike many other biomaterials, CPCs can support long-term bone defect repair by promoting new bone formation in harmony with their own gradual degradation [21-23]. CPCs are also biocompatible and osteoconductive [24-26]. The most common CPCs on the market include those based on apatite (aCPCs) (Norian SRS® (Synthes, USA) [27], Kyphos® (Kyphon, USA) [28], or Biopex-R® (Hoya Corp., Japan) [29]) [30, 31] and those based on brushite (bCPCs) [18] (ChronOs® Inject (Synthes, USA) [32], Eurobone (Groupe FH ORTHO, France) (Figure 2) [33]).

Figure 2. Commercial bCPC Eurobone (Groupe FH ORTHO, France). This is a 2-chamber device to separate powder and liquid; the plunger allows to break the separation and mix the components into a paste that then can be injected via the needle. (Reprinted from the "Eurobone 2 Injectable macroporous substitutes to fill bone defects. Documentation" provided by the company web page. [34])

Setting Reaction

The setting reaction of CPCs is a dissolution-precipitation process occurring in three stages: (1) dissolution of reactants to release calcium and phosphate ions; (2) nucleation of a new phase

(brushite or apatite crystals, depending on pH); and (3) growth of these crystals to form hardened cement [21, 35]. bCPCs form when the pH is below 4.2, while aCPCs (e.g., hydroxyapatite or calcium-deficient hydroxyapatite) form at pH levels above 4.2 [16, 21, 36]. For clinical use, the setting time is crucial: initial setting time (3–8 minutes) allows for manipulation, and final setting time (15–18 minutes) ensures proper hardening within a realistic timeframe for a clinical procedure [37-40].

Properties of set CPCs

Set CPCs exhibit biocompatibility, osteoconductivity, and the ability to degrade at rates conducive to new bone formation [23-25]. bCPCs are metastable under physiological conditions and resorb faster than aCPCs, which are more stable and degrade more slowly [35, 41]. aCPCs, characterized by low crystallinity, mimic the bone mineral phase and are preferred for long-term stability [35, 42]. bCPCs, while less mechanically robust, are advantageous for applications requiring rapid resorption [36, 41] (Table 1). Injectability and mechanical properties of CPCs are properties of variable magnitude depending on the clinical application, but it is evident that these are critical factors for clinical success.

Table 1. Comparison between bCPCs and Apatite Calcium Phosphate Cements (CPCs), highlighting their differences in chemical composition, formation conditions, solubility, resorption rates, mechanical properties, and clinical applications

Aspect	Brushite (bCPCs)	Apatite (e.g., HA, CDHA)
pH of Formation	Formed when pH < 4.2	Formed when pH > 4.2
Main Composition	Dicalcium phosphate dihydrate (DCPD)	Hydroxyapatite (HA), calcium-deficient hydroxyapatite (CDHA), or carbonated apatite
Solubility	Metastable under physiological conditions; resorbed faster than apatite	More stable and less soluble under physiological conditions
Setting Kinetics	Faster precipitation; brushite forms more rapidly than monetite	Slower precipitation compared to brushite
Resorption Rate	Faster resorption, which can promote faster remodelling	Slower resorption, ensuring longer stability in bone voids
Transformation	Can transform into apatite in vivo under physiological conditions	Does not transform; remains stable as apatite
Mechanical Properties	Generally weaker and more brittle	Stronger and more durable
Clinical Use	Preferred when faster resorption or remodelling is needed (e.g., temporary fillers)	Preferred for applications requiring long-term stability
Key Reference Studies	[43-45]	[43, 46, 47]

Brushite calcium phosphate cements (bCPCs)

bCPC is a type of calcium phosphate cement that forms dicalcium phosphate dihydrate (DCPD) (Table 1), commonly known as brushite, upon setting. It is created by mixing calcium phosphate

powders, such as β-tricalcium phosphate (β-TCP) and monocalcium phosphate monohydrate (MCPM), with water. [16, 21, 48].

Disadvantages of Brushite CPCs

bCPCs, while advantageous for fast bone resorption, have significant limitations. Their short setting time (30–60 seconds) is often impractical for surgical applications and requires the use of additives like citric acid to delay hardening [16, 17]. They exhibit lower mechanical strength (Figure 3) compared to cancellous bone, restricting their use in load-bearing applications [49]. Additionally, injectability issues, such as liquid phase separation during injection, result in uneven material composition and compromised in vivo behaviour [36, 50]. These challenges, combined with brittleness, limit their use despite their favourable resorption properties.

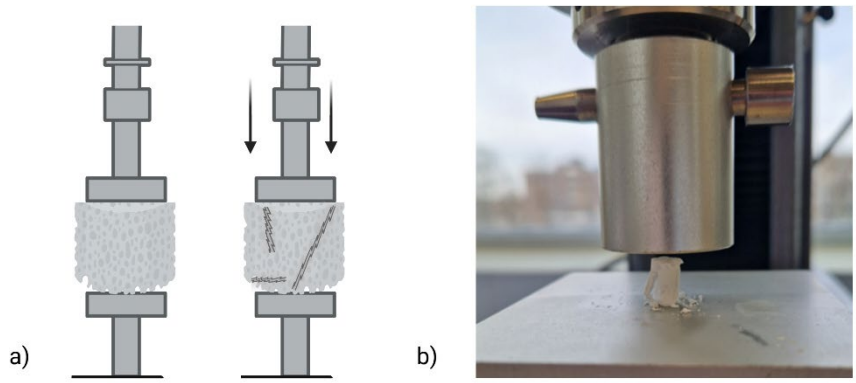


Figure 3. Mechanical test: a) Schematic representation of a compressive strength test, the cement is compressed until the point of fracture; b) Real picture of the compression strength set-up (Created in BioRender.com).

Brushite CPCs as drug-delivery systems

bCPCs have shown significant potential as drug-delivery systems, particularly for treating bone infections such as osteomyelitis. Osteomyelitis, caused by bacteria, mycobacteria, or fungi, is often challenging to treat due to the inability to maintain high antibiotic concentrations at the infection site. Incorporating antibiotics directly into CPCs for localized delivery has emerged as a promising approach to overcome this challenge and ensure effective treatment [51] (Figure 4). One key advantage of CPCs is their low setting temperature, which enables the incorporation of heat-sensitive drugs without denaturation. Furthermore, the intrinsic porosity of CPCs facilitates drug elution, enhancing the release profile to achieve the desired clinical outcomes [20]. This porosity, combined with isothermal setting, helps maintain the structural integrity of loaded drugs. Additionally, CPCs are evaluated for their ability to enhance bone regeneration and osseointegration, critical for restoring mechanical function, reducing failure risks, and promoting long-term stability of the material [20].

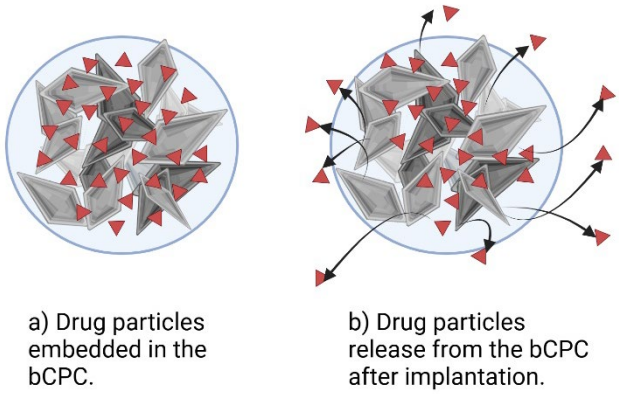


Figure 4. bCPCs as drug delivery systems: a) Drug molecules loaded in the bCPC are embedded in the matrix of the cement; b) After implantation, the drug starts to release from the bCPC mainly by diffusion [35] (Created in BioRender.com).

Objectives of the thesis

bCPCs are advantageous due to their rapid bone resorption; however, they are hindered by several significant drawbacks. Their short setting time (30–60 seconds) poses challenges during surgical procedures. Additionally, bCPCs exhibit low mechanical strength, limiting their suitability for load-bearing applications. Furthermore, issues with injectability, such as liquid phase separation, can lead to inconsistent material properties.

To address these limitations, this thesis aimed to enhance the mechanical properties of bCPCs and evaluate their effectiveness as a local antibiotic delivery system, focusing on their potential for improved clinical applications in bone regeneration therapies.

In more detail, this thesis addressed the following research questions:

1) How can the synergy between brushite calcium phosphate cements (bCPCs) and drug delivery mechanisms be optimized to advance bone regenerative therapies and improve clinical outcomes? *(Chapter 2)*
2) What is the effect of varying precursor proportions and the addition of collagen on the formulation, biocompatibility, and bioactivity of bCPCs? *(Chapter 3)*
3) How does the incorporation of sodium alginate in bCPC formulations influence tetracycline release and cell viability? *(Chapter 4)*
4) What strategies can improve the handling characteristics, mechanical strength, and antimicrobial efficacy of bCPCs, and how does the addition of α-tricalcium phosphate (α-TCP) and silk fibroin (SF) impact these properties along with tetracycline release dynamics? *(Chapter 5)*

References

[1] R. Dimitriou, E. Jones, D. McGonagle, and P. V. Giannoudis, "Bone regeneration: current concepts and future directions," *BMC medicine,* vol. 9, pp. 1-10, 2011.

[2] G. Arealis and V. S. Nikolaou, "Bone printing: new frontiers in the treatment of bone defects," *Injury,* vol. 46, pp. S20-S22, 2015.

[3] A. Nauth, E. Schemitsch, B. Norris, Z. Nollin, and J. T. Watson, "Critical-size bone defects: is there a consensus for diagnosis and treatment?," *Journal of orthopaedic trauma,* vol. 32, pp. S7-S11, 2018.

[4] A. Vajgel, N. Mardas, B. C. Farias, A. Petrie, R. Cimões, and N. Donos, "A systematic review on the critical size defect model," *Clinical oral implants research,* vol. 25, no. 8, pp. 879-893, 2014.

[5] X. Lin, S. Patil, Y.-G. Gao, and A. Qian, "The bone extracellular matrix in bone formation and regeneration," *Frontiers in pharmacology,* vol. 11, p. 757, 2020.

[6] S. Ravindran *et al.*, "Biomimetic extracellular matrix-incorporated scaffold induces osteogenic gene expression in human marrow stromal cells," *Tissue Engineering Part A,* vol. 18, no. 3-4, pp. 295-309, 2012.

[7] N. S. Dole *et al.*, "Osteocyte-intrinsic TGF-β signaling regulates bone quality through perilacunar/canalicular remodeling," *Cell reports,* vol. 21, no. 9, pp. 2585-2596, 2017.

[8] M. Heitzer *et al.*, "In vitro comparison of the osteogenic capability of human pulp stem cells on alloplastic, allogeneic, and xenogeneic bone scaffolds," *BMC Oral Health,* vol. 23, no. 1, p. 56, 2023.

[9] H. G. Moghadam, G. K. Sándor, H. H. Holmes, and C. M. Clokie, "Histomorphometric evaluation of bone regeneration using allogeneic and alloplastic bone substitutes," *Journal of oral and maxillofacial surgery,* vol. 62, no. 2, pp. 202-213, 2004.

[10] M. P. F. Graça and S. R. Gavinho, "Calcium phosphate cements in tissue engineering," *Contemporary Topics about Phosphorus in Biology and Materials,* vol. 1, 2020.

[11] F. Ozdemir, I. Evans, and O. Bretcanu, "Calcium phosphate cements for medical applications," *Clinical applications of biomaterials: state-of-the-art progress, trends, and novel approaches,* pp. 91-121, 2017.

[12] G. Lewis, "Injectable bone cements for use in vertebroplasty and kyphoplasty: State-of-the-art review," *Journal of Biomedical Materials Research Part B: Applied Biomaterials: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials and the Korean Society for Biomaterials,* vol. 76, no. 2, pp. 456-468, 2006.

[13] M. Bohner, U. Gbureck, and J. Barralet, "Technological issues for the development of more efficient calcium phosphate bone cements: a critical assessment," *Biomaterials,* vol. 26, no. 33, pp. 6423-6429, 2005.

[14] T. J. Brunner, R. N. Grass, M. Bohner, and W. J. Stark, "Effect of particle size, crystal phase and crystallinity on the reactivity of tricalcium phosphate cements for bone reconstruction," *Journal of Materials Chemistry,* vol. 17, no. 38, pp. 4072-4078, 2007.

[15] M. Bohner, T. J. Brunner, and W. J. Stark, "Controlling the reactivity of calcium phosphate cements," *Journal of Materials Chemistry,* vol. 18, no. 46, pp. 5669-5675, 2008.

[16] K. Hurle *et al.*, "Osteogenic lithium-doped brushite cements for bone regeneration," *Bioactive Materials,* vol. 16, pp. 403-417, 2022.

[17] F. Tamimi, Z. Sheikh, and J. Barralet, "Dicalcium phosphate cements: Brushite and monetite," *Acta Biomaterialia,* vol. 8, no. 2, pp. 474-487, 2012, doi: 10.1016/j.actbio.2011.08.005.

[18] L. Ding *et al.*, "Preparation and characterizations of an injectable and biodegradable high-strength iron-bearing brushite cement for bone repair and vertebral augmentation applications," *Biomaterials Science,* vol. 11, no. 1, pp. 96-107, 2023.

[19] M. Parent, H. Baradari, E. Champion, C. Damia, and M. Viana-Trecant, "Design of calcium phosphate ceramics for drug delivery applications in bone diseases: A review of the parameters affecting the loading and release of the therapeutic substance," *Journal of Controlled Release,* vol. 252, pp. 1-17, 2017.

[20] G. Mestres *et al.*, "Changes in the drug release pattern of fresh and set simvastatin-loaded brushite cement," *Materials Science and Engineering,* vol. 58, pp. 88-96, 2016.

[21] A. A. Mirtchi, J. Lemaitre, and N. Terao, "Calcium phosphate cements: study of the b-tricalcium phosphate - monocalcium phosphate system," *Biomaterials,* vol. 10, pp. 475-480, 1989, doi: 10.1016/0142-9612(89)90089-6.
[22] L. Borkowski *et al.*, "Fluorapatite ceramics for bone tissue regeneration: Synthesis, characterization and assessment of biomedical potential," *Materials Science and Engineering: C,* vol. 116, p. 111211, 2020.
[23] L. Cui *et al.*, "A novel tissue-engineered bone graft composed of silicon-substituted calcium phosphate, autogenous fine particulate bone powder and BMSCs promotes posterolateral spinal fusion in rabbits," *Journal of Orthopaedic Translation,* vol. 26, pp. 151-161, 2021.
[24] C.-L. Ko, J.-C. Chen, C.-C. Hung, J.-C. Wang, Y.-C. Tien, and W.-C. Chen, "Biphasic products of dicalcium phosphate-rich cement with injectability and nondispersibility," *Materials Science and Engineering: C,* vol. 39, pp. 40-46, 2014, doi: 10.1016/j.msec.2014.02.033.
[25] D. L. Alge, W. S. Goebel, and T.-M. Gabriel Chu, "In vitro degradation and cytocompatibility of dicalcium phosphate dihydrate cements prepared using the monocalcium phosphate monohydrate/hydroxyapatite system reveals rapid conversion to HA as a key mechanism," *J Biomed Mater Res Part B: Appl Biomater,* vol. 100B, pp. 595-602, 2012, doi: 10.1002/jbm.b.31938/full.
[26] C.-H. D. Chen, C.-C. Chen, M.-Y. Shie, C.-H. Huang, and S.-J. Ding, "Controlled release of gentamicin from calcium phosphate/alginate bone cement," *Materials Science and Engineering: C,* vol. 31, no. 2, pp. 334-341, 2011.
[27] D. Apelt *et al.*, "In vivo behavior of three different injectable hydraulic calcium phosphate cements," *Biomaterials,* vol. 25, no. 7-8, pp. 1439-1451, 2004.
[28] R. Gumpert *et al.*, "Demineralization after balloon kyphoplasty with calcium phosphate cement: a histological evaluation in ten patients," *European Spine Journal,* vol. 23, pp. 1361-1368, 2014.
[29] M. Yomoda, S. Sobajima, A. Kasuya, and M. Neo, "Calcium phosphate cement–gelatin powder composite testing in canine models: Clinical implications for treatment of bone defects," *Journal of Biomaterials Applications,* vol. 29, no. 10, pp. 1385-1393, 2015.
[30] T. Kurien, R. G. Pearson, and B. E. Scammell, "Bone graft substitutes currently available in orthopaedic practice," *The Bone & Joint Journal,* vol. 95-B, pp. 583-597, 2013.
[31] L. Schröter, F. Kaiser, S. Stein, U. Gbureck, and A. Ignatius, "Biological and mechanical performance and degradation characteristics of calcium phosphate cements in large animals and humans," *Acta Biomaterialia,* vol. 117, pp. 1-20, 2020.
[32] F. Theiss *et al.*, "Biocompatibility and resorption of a brushite calcium phosphate cement," *Biomaterials,* vol. 26, no. 21, pp. 4383-4394, 2005.
[33] G. Cama, "Calcium phosphate cements for bone regeneration," in *Biomaterials for Bone Regeneration*, S. V. Vlierberghe Ed.: Woodhead Publishing, 2014, pp. 3-25.
[34] F. ORTHO. "Eurobone 2." https://www.fhortho.com/en/our-products/bone-substitutes/eurobone-2/ (accessed 05-11, 2024).
[35] M.-P. Ginebra, C. Canal, M. Espanol, D. Pastorino, and E. B. Montufar, "Calcium phosphate cements as drug delivery materials," *Advanced Drug Delivery Reviews,* vol. 64, pp. 1090-1110, 2012.
[36] S. V. Dorozhkin, "Self-Setting Calcium Orthophosphate Formulations," *J. Funct. Biomater,* vol. 4, no. 4, pp. 209-311, 2013, doi: https://doi.org/10.3390/jfb4040209.
[37] I. Khairoun, M. Boltong, F. Driessens, and J. Planell, "Limited compliance of some apatitic calcium phosphate bone cements with clinical requirements," *Journal of Materials Science: Materials in Medicine,* vol. 9, no. 11, pp. 667-671, 1998.
[38] A. Vezenkova and J. Locs, "Sudoku of porous, injectable calcium phosphate cements–Path to osteoinductivity," *Bioactive Materials,* vol. 17, pp. 109-124, 2022.
[39] B. van Oirschot, A. G. Mikos, Q. Liu, J. J. van den Beucken, and J. A. Jansen, "Fast Degradable Calcium Phosphate Cement for Maxillofacial Bone Regeneration," *Tissue Engineering Part A,* vol. 29, no. 5-6, pp. 161-171, 2023.
[40] Q. Liu *et al.*, "Tunable calcium phosphate cement formulations for predictable local release of doxycycline," *Materialia,* vol. 28, p. 101769, 2023.
[41] S. Vahabzadeh, M. Roy, and S. Bose, "Effects of silicon on osteoclast cell mediated degradation, in vivo osteogenesis and vasculogenesis of brushite cement," *Journal of Materials Chemistry B,* vol. 3, no. 46, pp. 8973-8982, 2015.

[42] B. R. Constantz *et al.*, "Skeletal repair by in situ formation of the mineral phase of bone," *Science,* vol. 267, no. 5205, pp. 1796-1799, 1995.
[43] M. Bohner, "Reactivity of calcium phosphate cements," *Journal of Materials Chemistry,* vol. 17, no. 38, pp. 3980-3986, 2007.
[44] G. Vereecke and J. Lemaître, "Calculation of the solubility diagrams in the system Ca (OH) 2-H3PO4-KOH-HNO3-CO2-H2O," *Journal of Crystal Growth,* vol. 104, no. 4, pp. 820-832, 1990.
[45] A. Gisep, R. Wieling, M. Bohner, S. Matter, E. Schneider, and B. Rahn, "Resorption patterns of calcium-phosphate cements in bone," *Journal of Biomedical Materials Research Part A: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials and the Korean Society for Biomaterials,* vol. 66, no. 3, pp. 532-540, 2003.
[46] S. Larsson and T. W. Bauer, "Use of injectable calcium phosphate cement for fracture fixation: a review," *Clinical Orthopaedics and Related Research®,* vol. 395, pp. 23-32, 2002.
[47] J. Luo, I. Ajaxon, M. P. Ginebra, H. Engqvist, and C. Persson, "Compressive, diametral tensile and biaxial flexural strength of cutting-edge calcium phosphate cements," *Journal of the mechanical behavior of biomedical materials,* vol. 60, pp. 617-627, 2016.
[48] S. H. Dabiri, A. Lagazzo, B. Aliakbarian, M. Mehrjoo, E. Finocchio, and L. Pastorino, "Fabrication of alginate modified brushite cement impregnated with antibiotic: Mechanical, thermal, and biological characterizations," *Journal of Biomedical Materials Research Part A,* vol. 107, no. 9, pp. 2063-2075, 2019.
[49] Q. Liu, W. F. Lu, and W. Zhai, "Toward stronger robocast calcium phosphate scaffolds for bone tissue engineering: A mini-review and meta-analysis," *Biomaterials Advances,* vol. 134, p. 112578, 2022.
[50] A. T. Saleh, L. S. Ling, and R. Hussain, "Injectable magnesium-doped brushite cement for controlled drug release application," *Journal of materials science,* vol. 51, no. 16, pp. 7427-7439, 2016.
[51] K. Hurle, J. Oliveira, R. Reis, S. Pina, and F. Goetz-Neunhoeffer, "Ion-doped brushite cements for bone regeneration," *Acta Biomaterialia,* vol. 123, pp. 51-71, 2021.

Chapter 2: Innovations for Brushite Cements toward applications in Bone Regeneration and Drug Delivery

This chapter is based on:

C. M. Espino, G. F. Estévez, L. van der Weerd, L.F. de Geus-Oei and J. J. van den Beucken, *Ceramics International* (2024).

Introduction

Due to the increase in human life expectancy, a growing number of people are facing various health challenges, particularly those associated with aging [52-54]. In the Netherlands alone, >13,000 people were diagnosed with a bone disease in 2021 [55], while larger populations, such as that of the United States, have reported 124.1 million adults to be affected by a musculoskeletal medical condition in 2015 [56]. These numbers of patients are projected to reach 15-24% of the population over 65 years old by 2060 [55, 56]. Among all diseases and disorders patients face, bone-related problems have become one of the most severe conditions: traumatic fractures, deteriorating joints, age-related osteoporosis, and bone defects resulting from different pathologies have significantly and increasingly impacted individuals' daily lives. The treatment of large bone defects, which cannot heal spontaneously and hence require the implantation of suitable bone grafts at the defect site, has become a challenge [52, 53]. Aside from obvious orthopaedic examples comprising spinal and extremity bone defects, also oral surgeons are confronted with multiple pathologies causing bone defects in the oral and maxillofacial region that need grafting for treatment: alveolar bone reconstruction [57], continuity defects [58], midface/orbital reconstruction [59], and congenital craniofacial deformities like Treacher-Collins syndrome. In addition to only causing bone defects, these pathologies also affect aesthetics and functionality [60].

In surgical procedures, a range of bone substitutes are available for use, including autografts, allografts, and synthetic materials. Over the last decades, there has been a substantial increase in the number of new bone substitutes available as alternatives for autografts in bone regenerative treatment. Despite this expanded range of bone substitutes, autologous bone grafting is still considered the gold standard, as it provides the essential elements for bone growth to achieve the biological performance reflected by osteoconduction, osteoinduction, and bioactivity (Table 1).

Table 2. Biological performance parameters for bone grafting materials.

Parameter	Definition	Reference
Bioactivity	Ability of a material to achieve direct bone apposition without an intervening layer of soft tissue	[61, 62]
Osteoconduction	Capacity of a material to allow bone growth along its surface, which in the biomaterial field involves providing a biocompatible surface structure that promotes the migration of cells on it (for example: mesenchymal cells)	[63] [64]
Osteoinduction	Capacity of a material to initiate *de novo* bone formation	[64]
Osteogenesis	Synthesis of new bone by cells obtained from the graft or the host	[65]

In more detail, bone tissue is the unique material to use for bone regenerative treatment, as the tissue contains the 'house' (extracellular matrix (ECM) with the proper composition), 'guests' (all relevant cell types normally present in bone tissue), and 'guidance' (growth factors and instructive elements in ECM components) [1, 66]. A major disadvantage of autografts is donor site morbidity [67]. While the harvest of donor bone, commonly at the iliac crest, is considered a safe procedure, it can lead to chronic pain, superficial infection, bruising, and lateral femoral cutaneous nerve injury, resulting in paraesthesia and gait disturbances [67].

The associated morbidity and the limited supply of autografts has led to the search for synthetic materials as an alternative [30]. Among these alternatives, calcium phosphates (CaPs) have gained prominence due to their biocompatibility and bioactive properties, making CaPs a viable option in the field of bone regeneration [68, 69]. CaPs can be used in various applications of orthopaedic and maxillofacial surgery [70, 71], whether for bone substitution in bone defects, augmentation of the alveolar ridge, middle ear implants, or fusion of spinal vertebrae [72]. CaPs can be applied in different forms, such as granules, variously shaped blocks, or as cements that set during their application [73, 74]. Notably, calcium phosphate cements (CPCs) offer a distinct advantage, as these can be prepared as a paste that can be injected and sets in a few minutes. This means that such injectable pastes can be applied via minimally invasive surgical techniques and moulded to easily adapt to the shape of the bone defect [75]. On top of that, CPCs have shown their potential as drug delivery systems for a variety of active compounds, including antibiotics that are critical after any surgical intervention. Such local application avoids the challenges of systemic antibiotic treatments, which are less effective at specifically targeting the infected surgical site. [76]. CPCs with brushite as the main component (bCPCs) are even more advantageous than apatitic CPCs, as brushite is faster resorbed, which is interesting from a regenerative medicine perspective to achieve complete replacement of the material by newly formed bone tissue [77].

Bone

Bone is composed of a unique extracellular matrix and several types of bone cells therein. Hierarchically, bone is present either as dense, cortical bone, or as spongious, cancellous bone. The characteristics of the bone are given by the composition of the bone matrix [78]. The bone matrix consists mainly of two components: organic material (approximately 35 wt% of the matrix) and inorganic material (constituting about 65 wt% of the matrix). The organic material includes predominantly collagen and proteoglycans, providing flexibility and strength to the bone matrix. On the other hand, the inorganic component comprises predominantly a non-stoichiometric

hydroxyapatite [79] (stoichiometric formula: $Ca_{10}(PO_4)_6(OH)_2$) which is calcium-deficient and contains carbonate. This combination of organic and inorganic constituents is responsible for the main characteristics of bone. [78, 80]. Additionally, bone marrow occupies the intramedullary space and the intertrabecular space in cancellous bone and regulates the origin and activity of the bone cells [81]. The bone marrow contains mesenchymal stem cells that differentiate into bone-regenerative osteoblastic cells under the influence of specific bone growth factors, cytokines, and hormones [81]. There are three main types of specialized cells in bone tissue: osteoblasts, osteocytes, and osteoclasts, each with distinct functions and origins.

Osteoblasts are of mesenchymal origin and characterized by the presence of an extensive endoplasmic reticulum and numerous ribosomes. Their primary role is producing collagen and proteoglycans, essential components of the bone ECM. Additionally, osteoblasts form vesicles that accumulate calcium ions (Ca^{2+}), phosphate ions (PO_4^{3-}), and various enzymes, which are used to start and propagate the formation of hydroxyapatite crystals. These processes result in the formation of a mineralized bone matrix, in which the mineral aligns with collagen fibrils [82]. Osteoblasts actively participate in bone formation, known as ossification or osteogenesis.
Osteocytes, derived from osteoblasts, represent the fully matured form of osteoblasts, but with the function of orchestrating bone homeostasis: osteocytes are mechanosensitive and capable of transducing mechanical signals into chemical signals through their lacuna-canalicular system within the bone extracellular matrix [83] [84]. This interconnected system of osteocytes regulates both bone formation and resorption during bone remodelling, meaning that osteocytes control the activity of both osteoblasts and osteoclasts. The osteocytes form a large network of cells that are connected through canaliculi [85]. They respond to mechanical stimulation to balance bone remodelling and formation in such a way that they make the bone stronger where necessary.
Osteoclasts are large, multi-nucleated cells responsible for bone resorption or bone breakdown. Originating from hematopoietic stem cells, osteoclasts form by the fusion of 2 or multiple macrophages. In the areas where the plasma membrane of osteoclasts comes into contact with the bone matrix, they form ruffled borders. These borders facilitate the pumping of hydrogen ions through this edge and cause a local decrease in pH of a secluded area that triggers the decalcification of the bone matrix. Osteoclasts are also involved in the resorption of bone by releasing enzymes that normally break down the thin layer of organic matrix covering the bone. Once this layer is removed, the osteoclasts come into contact with the mineralized bone, accelerating resorption [78]. Recently, osteoclasts have been implicated with a role in stimulating bone formation via secretion of extracellular vesicles that signal to bone forming osteoblasts via receptors on the surface [86] or their cargo [87].

Bone repair process

Following tissue damage resulting in a bone defect, a repair process is initiated. This process consists of four basic stages, in which multiple cell types are involved (see Figure 1 for a schematic representation of the bone repair process).

1. Following a bone defect, the damage of the blood vessels provokes the formation of a localized mass of blood confined within an organ or space, known as a hematoma. Normally this blood forms a clot, which consists of fibrous proteins that stop bleeding, however, the tissue adjacent to the defect site is further affected due to inadequate blood circulation.
2. At the bone defect site, a callus is formed, surrounding the defect. The formation of this callus is established via blood vessels growing into the blood clot, disintegration of the blood clot, and fibroblasts producing collagen and other extracellular molecules to form granulation tissue. The chondroblasts begin to produce cartilage in the fibrous network, while the osteoblasts produce new bone that contributes to the internal callus.
3. Callus ossification occurs when the cartilage in the external callus is replaced by cancellous bone tissue. Finally, this process occurs in the internal callus, which is replaced by cancellous bone tissue that further stabilizing the bone defect.
4. Bone remodelling is a crucial step in the bone repair process. Even after the internal callus or cancellous tissue is formed, the bone remains structurally unstable, so the final process of bone repair is only completed when the cancellous tissue is replaced by cortical bone [53, 78, 88]. This remodelling process takes time, and even after a year, it may not be complete.

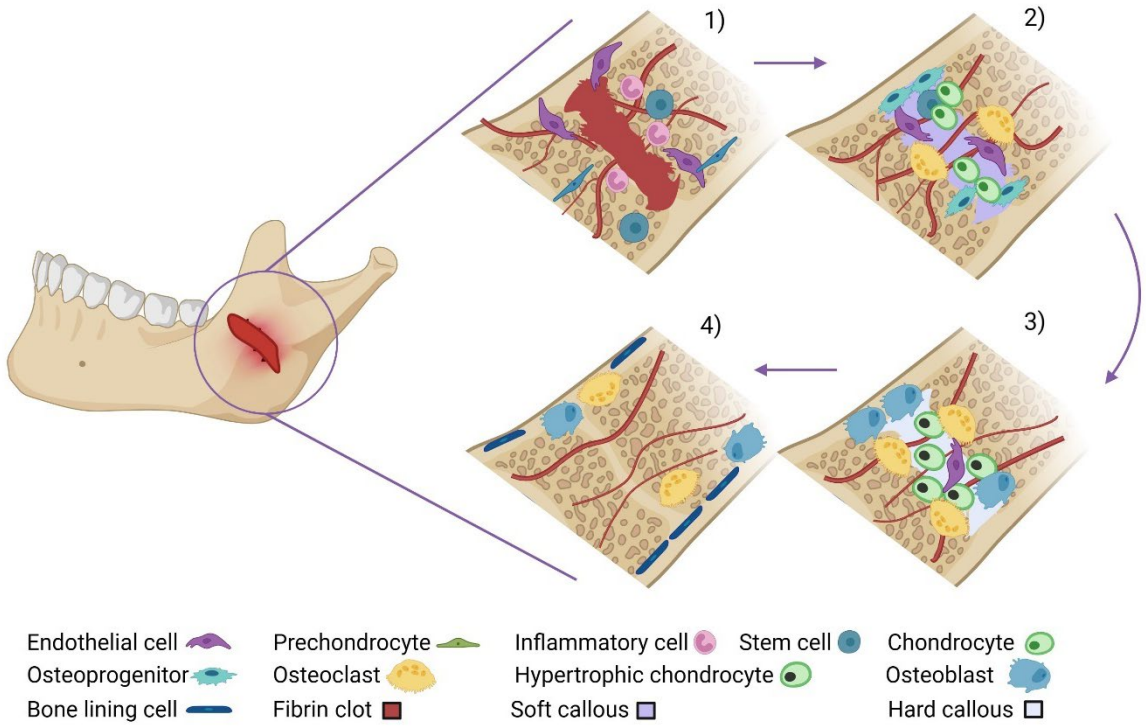


Figure 1. Schematic representation of the fundamental steps of bone healing and cellular activity therein: 1) Hematoma formation; 2) Soft callus formation; 3) Callus ossification; 4) Bone remodelling. (Created with BioRender.com)

Materials for bone regenerative applications

Regenerative materials for bone substitution are categorized as materials of natural origin and synthetic materials [89]. The natural materials, especially autografts, provide a conducive matrix for bone regeneration. One of the main natural origin materials used is demineralized bone matrix (DBM), which is an allograft [90] that consists of acid-treated bone that still contains the organic matrix and embedded growth factors. Due to the presence of the growth factors, DBM has shown the capacity to retain osteoinductive capacity [91]. However, the application of natural origin materials is limited by the relative scarcity of bone tissue, particularly for autografts [92]. On the other hand, in the case of allogeneic or xenogeneic bone tissue (i.e. human or animal, respectively), it also implies the possibility of immunological rejection, and can serve as a transmission route for diseases such as AIDS and hepatitis [93].

Significant research efforts have been dedicated to the development of synthetic materials that can effectively replace or repair bone defects by eliciting a favourable biological response and maintaining functionality. Among the multiple types of synthetic materials used in bone regeneration are calcium phosphate ceramics (CaPs) (Table 2), polymers, and CPCs. Synthetic materials used to regenerate bone defects must meet some essential minimal requirements to

obtain a favourable response from the body: biocompatibility and bioactivity. In addition, it can be required that the material possesses mechanical properties appropriate for the site where it will be implanted while maintaining the functionality of the skeletal system [94]. The material must be able to undergo osseointegration and complete resorption to facilitate full regeneration of the affected area in the most efficient way possible [95].

Table 3. Calcium phosphate compounds and their major properties (modified from [53])

Name and Abbreviation	Chemical Formula	Ca/P Ratio	Solubility at 25^{O}C (mg/L)
Amorphous calcium phosphate (ACP)	-	1.5	25.6-32.8
Hydroxyapatite (HA)	$Ca_{10}(PO_4)_6(OH)_2$	1.67	~0.3
Calcium-deficient hydroxyapatite (CDHA)	$Ca_{10-x}(HPO_4)_x(PO4)_{6-x}(OH)_{2-x}$	1.5-1.67	~9.4
Dicalcium phosphate anhydrous (DCPA)	$CaHPO_4$	1	~48
Dicalcium phosphate dihydrate (DCPD)	$CaHPO_4{\cdot}2H_2O$	1	~88
Monocalcium phosphate monohydrate (MCPM)	$Ca(H_2PO_4)_2{\cdot}H_2O$	0.5	~18,000
α-Tricalcium phosphate (α -TCP)	α -$Ca_3(PO_4)_2$	1.5	~2.5
β-Tricalcium phosphate (β -TCP)	β -$Ca_3(PO_4)_2$	1.5	~0.5
Fluorapatite (FAp)	$Ca_{10}(PO_4)_6F_2$.	5.5-6.0 [96]	~0.092 [96]

Calcium phosphate cements (CPCs)

CPCs have garnered significant interest for biomedical applications, particularly as bone regenerative materials. Unlike other biomaterials, CPCs can trigger the repair of bone defects in a more permanent way [21, 22] by promoting the formation of new bone tissue in tight balance with the CPC being degraded [23]. CPCs also present biocompatibility and osteoconductivity [24-26]. The most common CPCs on the market include HA-based (Norian SRS® (Synthes, USA) [27], Kyphos® (Kyphon, USA) [28], or Biopex-R® (Hoya Corp., Japan) [29]) [30, 31] and brushite-based CPCs [18] (ChronOs® Inject (Synthes, USA) [32], Eurobone (Group FH ORTHO, France) [33]).

Compared to other CaP ceramics, the use of CPCs offers several advantages. CPCs adapt perfectly to the bone defect dimensions regardless of their shape and can be injected into the body using minimally invasive techniques [16, 18, 19, 51]. However, the CPC formulation must be carefully chosen to ensure appropriate paste cohesion and proper injectability, which guarantees that the CPC moulds itself to the site where it is applied [20].

The final product of apatitic CPCs is hydroxyapatite (HA) or calcium-deficient hydroxyapatite (CDHA). This transformation occurs in an aqueous medium with a pH above 4.2. The resulting apatite, characterised by low crystallinity, closely mimics the mineral phase of bone [12, 35, 36]. Alpha-tricalcium phosphate (α-TCP) typically acts as the main constituent in these apatitic CPCs, where the particle size and crystallinity determine the degree of reactivity. Notably, lower crystallinity results in higher reactivity [13]. Reducing the particle size increases the contact surface, thereby enhancing the material's reactivity [12, 14, 15]. The solubility of the apatitic CPCs is relatively similar to the mineral component of bone. However, although they degrade faster than stoichiometric HA, the rate of degradation is slower than that exhibited by brushite CPCs [97, 98].

Another widely explored material in the field of dental treatment is fluorapatite (FAp) [99-101]. Fluoride ions are commonly included in toothpaste to enhance enamel strength and provide protection against cavities [99-101]. Several studies have shown evidence that the release of fluoride ions directly influences the differentiation, morphology and attachment of the osteoblast cells [102]. Furthermore, fluoride ions in the FAp crystal lattice structure have shown to prevent bacterial growth [102, 103].

Brushite CPCs

Contrary to apatite, brushite is characterised by being metastable in a physiological environment, which results in a much faster resorption [35, 41, 104]. CPCs are usually resorbed through two mechanisms: active or passive resorption. In the former, cells such as osteoclasts or macrophages intervene, while the latter is based on dissolution in body fluids [35].

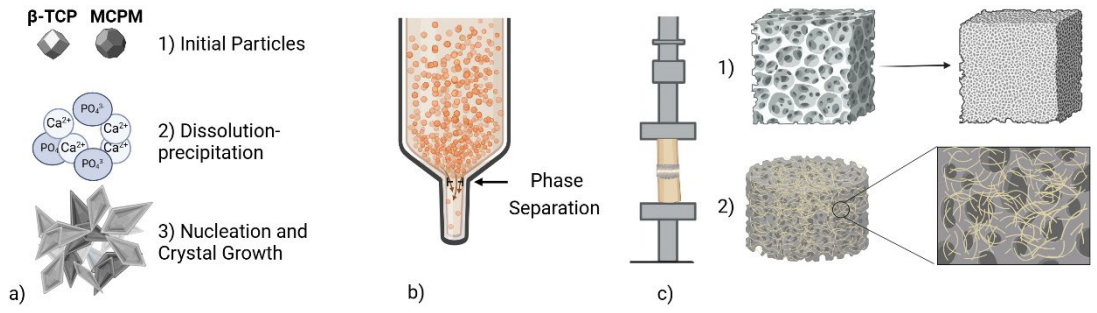


Figure 2. Handling properties of the bCPCs: a) Setting process from initial particles until the growth of the new phase crystals; b) Injectability (liquid separation during the extrusion process); c) Mechanical properties, methods to improve it, 1) Decreasing the CPC pores size; 2) Adding polymeric materials for reinforcement (Created with BioRender.com).

Setting reaction of bCPCs

In 1989, Mirtchi *et al*. [21] mixed monocalcium phosphate monohydrate (MCPM) and β-tricalcium phosphate (β-TCP) with water, obtaining brushite, chemically known as dicalcium phosphate dihydrate (DCPD) (Equation 1) [16, 21, 48], with a tensile strength between 0.1 and 1.1 MPa.

$$\beta\text{-}Ca_3(PO_4)_{2\,(s)} + Ca(H_2PO_4)_2{\cdot}H_2O_{\,(s)} + 7H_2O = 4CaHPO_4{\cdot}2H_2O_{\,(s)} \quad (1)$$

The setting reaction that gives rise to the solid consistency of bCPC occurs in three stages: dissolution of the reagents, nucleation of a new phase (brushite) and the growth of crystals (Figure 2a). Consequently, setting of bCPC is a dissolution-precipitation reaction. During dissolution, calcium and phosphate ions are released which generates a supersaturation of the solution and when the critical concentration value is reached, nucleation occurs in a new phase, forming crystals, generally around the precursors particles [35, 36].

Setting time

In practical terms, the setting time is crucial as it reflects the amount of time a surgeon has to apply and manipulate a CPC prior to its setting. Therefore, for effective use of CPCs, the initial setting time (which indicates when the CPCs paste starts to lose its plasticity) should be between 3-8 minutes, while the final setting time (the complete hardening of the CPCs) should not exceed 15 to 18 minutes for proper clinical implementation [37-40].

In this regard, one of the main limitations of bCPCs is their short initial setting time, generally between 30 and 60 seconds. In order to increase this time for suitability in surgical procedures, several strategies have been adopted, usually in combination. The first approach involves increasing the liquid-to-powder ratio (L/P). This, however, can affect the mechanical properties of the material, as it is related to the porosity that the set bCPC will have (Figure 3). The second strategy engages the incorporation of a CPC setting retarder in the liquid phase, such as sodium pyrophosphate, sodium citrate, pyrophosphoric acid, tartaric acid, or citric acid [16, 105]. These additives modify the setting time by delaying the formation of brushite crystals, affecting the dissolution of the reagents, or both. Organic acids such as citric and tartaric acid, have the ability to bind to calcium ions and inhibit the formation of crystals, slowing down the setting speed. On the other hand, citrate ions can interact with the β-TCP particles, limiting their dissolution, and hence delaying CPC setting [17]. The optimal balance between the L/P ratio, the concentration of the retarder, and the mechanical properties of the obtained bCPC has been extensively investigated. The results of studies on this topic have led to the commercialization of bCPCs, such as ChronOS™ Inject and Eurobone [33].

Injectability

Injectability is a significant property of bCPCs, as it enables a surgical application via minimally invasive procedures and ensures full conformity to the bone defect dimensions [36, 106]. The standard for measuring the injectability of a CPC relies on testing a paste's ability to be extruded through a syringe in a testing machine with a force of 100 N. Injectability is quantified by calculating the percentage by weight of the extruded paste [107-110].

The injectability of CPCs is limited by a phenomenon identified as liquid phase separation (Figure 2b), which results in the separation of the liquid phase from the solid phase during extrusion. Consequently, only the liquid passes through, leaving the majority of the solid material behind in the syringe [50, 75]. For bCPCs to be optimally injectable, it is important that the L/P ratio stays consistent during injection. It has been reported that several bCPC formulations exhibit phase separation upon injection, resulting in limited injectability [54, 107, 109]. This phase separation alters the final composition of the CPCs by changing the L/P ratio, which in turn impacts other properties [50, 111].

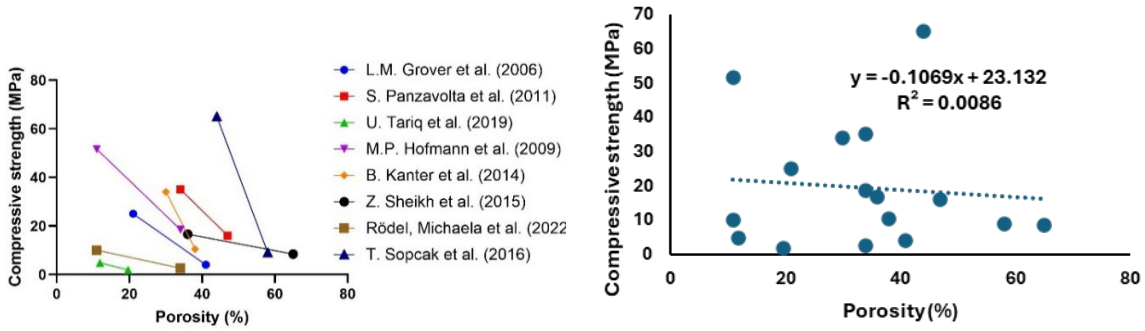
To enhance injectability, adjustments can be made both in the injection process and the bCPC composition. These include using different needle diameters or gauges [109], depending on the application. The factors associated with the composition of the bCPC encompass increasing the L/P ratio and the viscosity of the mixture, reducing particle-particle interactions or the particle size of the powder, and integrating sodium citrate or citric acid into the liquid phase [106]. Achieving a smaller particle size and a more spherical shape of the powder phase has proven to be effective [54]. However, these modifications may impact other important bCPCs properties. For example, an increased L/P ratio can significantly reduce the mechanical properties of the resulting bCPC after complete setting [112].

Mechanical properties and their optimization

Mechanical properties of bCPCs are known to be limited compared to cancellous bone [49]. Extensive research efforts have focused on improving mechanical properties. The use of several crystal growth retardants simultaneously causes higher compaction of the crystal, and hence an improvement in mechanical properties (Figure 2c), 1)) [113]. This densification comes with a reduction of porosity and several studies have probed an inverse relationship between porosity and mechanical properties (Figure 3). A search in PubMed[1] resulted in data used for the analyses on the relation between porosity and compressive strength are presented in Figure 3 and Figure

[1] PubMed search key words “brushite cements porosity” revealed a total of 49 results from which 40 articles were useful.

4 based on the criteria of showing numeric results of the porosity studies performed in each article.



A) B)

Figure 3. Correlation between porosity and mechanical properties of bCPCs within studies. A) Intra-study relation between porosity and compressive strength for bCPCs.)

Figure 3 shows the inverse correlation between porosity and mechanical properties for bCPCs across different studies. Each individual study shows that higher porosity leads to lower mechanical strength, which is a crucial consideration in the design and application of bCPCs [114-121]. In all retrieved data from the literature here, the main factor affecting the porosity and, ultimately the mechanical properties of the bCPCs, was the L/P ratio. However, this is not the only factor affecting porosity. After a wider search we found more elements to consider.

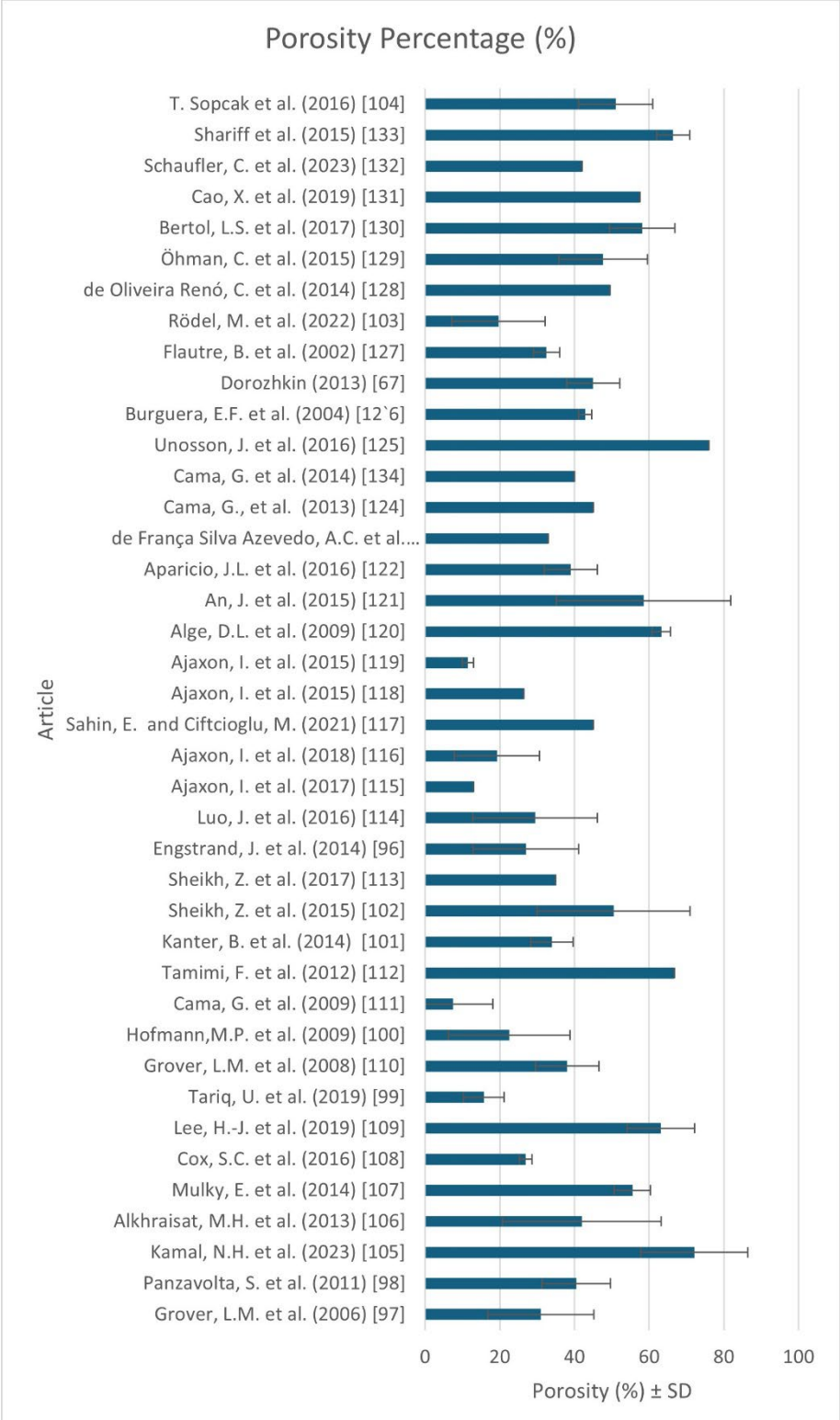


Figure 4. Porosity percentage in bCPCs analysed through a total of 40 articles. (Created consulting the references listed in the graph: [36, 47, 113-150], the percentages presented are the average of all porosity data for each publication, including standard deviation as error bar)

The average porosity across numerous studies ranges widely from 5% to 76% (Figure 4). This indicates significant variability in the porosity of bCPCs used in different contexts and

formulations. Many studies (e.g., Grover et al. [114], Panzavolta, et al. [115] , Tariq et al. [116]) consistently report that high porosity is related with low values of mechanical properties. This suggests a trade-off between porosity and mechanical strength that must be balanced depending on the application requirements. Additives such as chitosan-alginate, gentamicin, and adjustments in particle size significantly affect porosity. Lee et al. [126] found that addition of chitosan-alginate decreased porosity. Treatments like fiber reinforcement also impact porosity. For instance, Mulky et al. [124] observed a reduction in porosity after fiber reinforcement. Specific conditions like different degradation performance due to environmental exposure (e.g., medium been unstirred, stirred, or perfused) also influence porosity, as noted by An et al. [137].

Certain studies highlight ideal porosity levels for specific applications. For instance, de França Silva Azevedo et al. [139] found 33% porosity to be ideal for load-bearing applications in bone tissue injuries.

In general, we can infer that reducing the porosity of a bCPC improves the mechanical properties by increasing bCPC density. However, from a biological perspective, the reduction on the porosity could lead to a detrimental effect on cell migration into the inner parts of the bCPC.

Polymeric materials have been added to CPCs and specifically to bCPCs to improve some of their properties: injectability (as lubricants), cohesion, and mechanical properties (as reinforcement; Figure 2, c),2)) [18] [151]. Polymers, encompassing both natural and synthetic materials, are known for their versatile properties. Despite their structural complexity, polymers offer the advantage of being highly similar, and often nearly identical to the macromolecular substances found in the biological environment of the (bone) ECM [152]. They are or can be made degradable by the natural enzymes of living organisms, ensuring that permanent implants made from materials like cellulose, collagen, hyaluronic acid, and sodium alginate are metabolized through the body's physiological mechanisms [153]. Polymers can be integrated in bCPC in different states, as part of the liquid phase, or in the solid phase as powder, microparticles or fibers [154]. Several studies collectively demonstrate that the addition of specific polymers or polymeric fibers to CPCs/bCPCs significantly enhances their mechanical properties and suitability for medical applications such as vertebroplasty and kyphoplasty. Hyaluronic acid, especially of high molecular weight, improves the cohesion of bCPCs without negatively impacting their mechanical strength, particularly during the cement paste phase [155]. Other polymers, like xanthan and alginate, also contribute to better CPC cohesion by acting as flocculant agents, which influences properties like setting time and injectability, but increases the mechanical properties of CPCs [155, 156]. Composite bCPCs, particularly those reinforced with partially

acrylic matrices, provide a balance of mechanical strength and osteoconductivity, comparable to commercial bCPCs [157]. Additionally, the integration of poly(lactic-co-glycolic acid) (PLGA) fibers into bCPCs not only avoids adverse effects but also significantly boosts bone formation and mechanical properties, suggesting their potential for use in load-bearing bone defect treatments [158]. Poly(vinyl alcohol) (PVA) fiber reinforcement, particularly with decoated hydrophilic fibers, markedly improves the toughness and flexural strength of CPCs, introducing crack-arresting mechanisms that enhance their durability. However, the mechanical strength of these fiber-reinforced CPCs decreases over time due to biodegradation, although they continue to exhibit extensive plastic deformation [159, 160]. Finally, fiber-reinforced CPCs combined with carboxymethyl cellulose (CMC) demonstrate compressive strengths closer to natural vertebral bone, significantly outperforming traditional materials like PMMA, thereby making them more suitable for vertebral augmentation procedures [161]. Overall, these findings underscore the importance of selecting appropriate polymer or polymeric fibre additives to optimize the mechanical performance and clinical efficacy of CPCs in various orthopedic applications.

Collagen has been combined with bioceramics, hyaluronic acid and synthetic polymers on several occasions to improve mechanical properties and reduce susceptibility to degradation [162]. Collagen is the most abundant protein in mammalian tissues, making up a third of the entire protein mass in mammals. Its fibre forms the basis of the ECM in human bone, where minerals are incorporated in its structure. Additionally, collagen is a significant component of tendon and skin tissue, primarily providing mechanical strength to the tissue [162, 163]. This type of collagen is an organic component that makes up the ECM of bone and is widely accepted as a biocompatible substance that does not show any immunological effect [164-166]. For this reason, collagen has been widely used in support (biological scaffold) formulations including sponges, fibres, microspheres, and hydrogels. Its main applications are aimed at the regeneration of bone tissue, tendons, cartilage, and skin.

Sodium alginate has been utilized in CPCs due to its biocompatibility, biodegradability, and its ability to function as a reinforcement. The addition of sodium alginate improves both the cohesion and the injectability of a CPC paste, as well as its mechanical properties, raising the compressive strength to values of 20 MPa, compared to 14 MPa for the CPC without alginate [167]. Alginates are constituents of the cell wall of brown algae and are composed of linear block copolymers of β-D-mannuronic acid linked by 1-4 (M) and α-L-guluronic acid (G) [168]. Divalent ions form cross-links in the alginate joining the guluronic residues, which induces a sol-gel transition in the material [169]. Due to their abundance and affordability, alginates have been widely used in the food and pharmaceutical industries as thickeners, emulsifying agents,

binders, and disintegrating agents for tablet and capsule formulations. Due to their biocompatibility, alginates have been used in medical applications such as wound dressings, scaffolds for tissue engineering and hepatocyte cultures and surgical or dental impression materials, drug delivery, and cell transplantation, taking into account their ability to form a gel in the presence of divalent cations, for example, Ca^{2+} or Zn^{2+} [163, 169], forming what is called the egg-box model [170]. Alginate is commonly used as an encapsulation vehicle for cell xenotransplantation, particularly in therapies for conditions like diabetes, and as such serves as a drug delivery system. This acts as a semipermeable barrier that allows the diffusion of growth factors and other cellular signals to the host, although it protects the transplanted cells from immunological processes [171].

Degradation of bCPCs *in vivo*

Beside biocompatibility and adequate mechanical strength, CPCs should also possess the appropriate degradation rates for effective bone repair and regeneration. Numerous studies have shown that bCPCs can either remain stable or dissolve, releasing calcium and phosphate ions, depending on various conditions [172]. Table 3 provides characteristics of in vivo degradation studies of bCPCs resulting from a PubMed search[2].

Some bCPCs show rapid initial degradation within days to weeks, especially those modified with additives like PEG [173]. For instance, bCPCs implanted in vivo typically show substantial resorption within 8 weeks [105] (Table 3). However, complete resorption and replacement by newly-formed bone requires several months. Experimental work has shown nearly half resorption of the bCPC within 6 months [174].

bCPC degradation rates can vary depending on the implantation site and the specific conditions within the body, affecting the interaction with surrounding tissues and the rate of resorption [175]. bCPCs undergo bioresorption primarily through cell-mediated processes involving macrophages and osteoclasts [176]. This mechanism plays a crucial role in the gradual replacement of the bCPC with newly formed bone tissue. Understanding the degradation kinetics of bCPCs is essential for optimizing their clinical use. The ability to control degradation rates through formulation adjustments (e.g., additives) can impact the suitability of these bCPCs for specific applications, such as bone regeneration or drug delivery**.**

[2] A PubMed search with the key words “brushite cements degradation rate in vivo” retrieved a total of 10 results, from which 6 articles were selected. The selection process was based on presentation of quantitative results.

Table 4. In vivo degradation rate over time of bCPCs in different studies (for the calculations of the degradation rates per day, a constant degradation rate over time was assumed).

Study	Degradation rate (% per day)	Remaining amount of cement (% of total)	Time	Notes
Alkhraisat et al. (2010) [105]	1.5%	17% (bCPC with glycolic acid silica gel)	8 weeks	Slower resorption compared to bCPC with glycolic acid
	1.7%	3% (bCPC with glycolic acid)	8 weeks	Faster resorption
Rentsch et al. (2018) [174]	0.3%	53% (Cr 50)	6 months	Highest resorption among modified cements
	0.2%	72% (Unmodified cement)	6 months	Slower resorption
Kowalewicz et al. (2022) [177]	0.6%	4.63% (bCPC modified with Mg)	24 weeks	Significant material loss observed
	0.4%	35.14% (TCP)	24 weeks	Slower degradation
Gao, et al. (2024) [173]	7.4%	63% (bCPC@0.0% PEG)	5 days	Fastest degradation
	6.2%	69% (bCPC with 1.5% PEG)	5 days	Moderate degradation
	4.4%	78% (bCPC with PEG modified by carboxy acid)	5 days	Slowest degradation
Thao Le et al. (2022) [178]	0.5%	78% (CPC)	4 weeks	Lower degradation compared to CPC/CDHA20
	0.8%	85% (CPC/CDHA20)	4 weeks	Higher degradation
Jayasree et al. (2019) [176]	0.5%	86% (PB - normal bCPC)	28 days	Lower degradation compared to EB
	0.57%	84% (EB - egg shell bCPC)	28 days	Higher degradation

Drug delivery via bCPC

For several decades, the main forms of drug administration to the human body have included pills, capsules, potions, and injections [179]. Nonetheless, most of the treatments that aim to administer pharmaceutical compounds are based on the administration of these preparations systemically, resulting in abrupt fluctuations in drug levels in the bloodstream [180].

In recent years, considerable progress has been made in the development of new techniques for drug administration. These advancements not only control dosage and prolong therapeutic effects but also allow for precise targeting or "microlocation" of drug delivery to specific sites within the body [180]. This represents unquestionable advantages by drastically reducing the amount of drug delivered to the body and the repetition of unnecessary repetitive doses that could lead to an increase in adverse side effects in the patient as well as trigger the resistance of bacteria to antibiotics [76, 181].

CPCs are appealing as local delivery systems for drugs partially due to the fact that CPCs harden without requiring high temperatures, thereby preventing heat-induced drug denaturation [35, 38] [182]. Additionally, the porosity of CPCs helps to contain substances, including drugs, biologically active molecules and cells, and release them in the biological environment [20, 106] [49, 183]. For instance, CPCs can be used to locally deliver drugs that stimulate bone regeneration or to direct the action of the material to specific disorders or pathologies [35, 38]. To use CPCs as a drug delivery system, a wide range of factors must be considered, including the solubility of the drug and interactions with the CPC matrix, possible deterioration of this matrix, and the microstructure of the material, i.e. permeability, porosity and specific surface area [35].

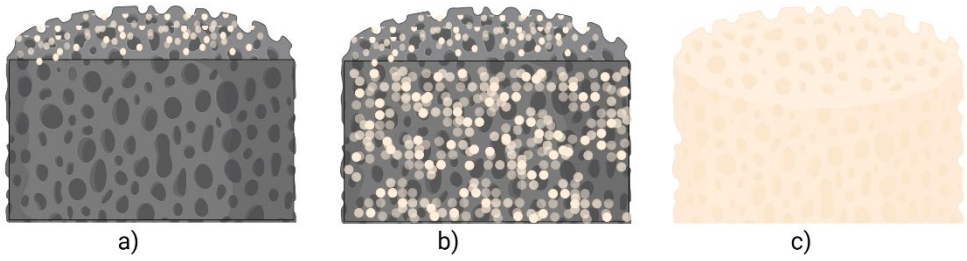


Figure 5. Distribution of drug within a CPC material. a) Only on the surface of the CPC, b) Distributed only in the inside of the CPC, generally while added in the solid phase, and c) Complete homogeneous distribution in all the CPC, generally while added in the liquid phase. (Created with BioRender.com)

The method of drug incorporation into the CPCs presents a challenge as it determines both the drug's distribution and its interaction with the matrix [184]. Typically, drugs are incorporated into CPCs either by mixing the drug powder with the solid phase or by dissolving it in the liquid phase. In both methods, the drug is distributed throughout the entire volume of the material, though incorporating it in the liquid phase tends to result in a more uniform distribution. [19, 35, 185] (Figure 5).

On the other hand, the addition of a drug can influence the setting reaction of the CPCs, whether in the liquid or solid phase, which affects the setting time and the development of the CPCs microstructure [35]. Obviously, the microstructure of the CPCs can also affect how the drug will be released after implantation [184].

Moreover, the mechanical properties of CPCs can be significantly affected by the introduction of a drug, potentially restricting their applications. However, this impact varies depending on the chemical nature of the drug molecule, making it difficult to predict the final effect on the material's mechanical properties [35]. Other properties can also be affected, such as release kinetics, as changes can occur in crystallization, crystal size and porosity [35].

Finally, the stability of the drug must be evaluated since the precipitation dissolution processes that occur during the CPCs setting process can affect the local pH and ionic concentrations of the solution. These changes may affect the drug's functionality and, consequently, influence its release process [35].

Another crucial aspect to consider is the drug release kinetics in CPCs, particularly because the release rate is often significantly lower than the rate of drug release in other systems. It is generally assumed that the release process occurs primarily through diffusion within the CPC matrix [35, 184]. A determining issue in the release kinetics is the distribution of the drug in the CPCs. It is not expected that the drug will be incorporated into the interior of the crystal lattice of the precipitated crystals. Only a minimal amount of drug would be incorporated in this manner, ensuring that the majority of the drug becomes entrapped within the entangled crystals [35, 184].

As any surgical intervention imposes the risk of bacterial infection, every intervention requires the use of antibiotics, generally in high doses and for prolonged times. Antibiotics are used prophylactically in the post-surgical period to combat potential infectious conditions, such as osteomyelitis, periodontal diseases or dental caries [186, 187]. Conventional approaches for antibiotic administration cause a range of side effects and an increase in bacterial resistance. These aspects have led to explorations on the use of CPCs as delivery systems of antibiotics in surgery for bone replacement and the repair of small bone defects in the field of traumatology or orthodontics [186]. Among the antibiotics most studied for these applications are the families of aminoglycosides, cephalosporins, glycopeptides, quinolones, and tetracyclines. For prophylactic treatment, it is important that the release of the antibiotic is fast enough so that it remains above the minimum inhibitory concentration (MIC), although the MIC is generally determined using *in vitro* experimental conditions that do not resemble clinical antibiotic treatment of infections. Concentrations below the MIC should be avoided for extended periods of time, as this can cause bacterial resistance. In contrast to acute release for prophylactic purposes, the treatment of infections such as osteomyelitis and periodontitis requires a long-lasting and sustained release period [35].

The incorporation of antibiotics into the CPC affects the physical, chemical, and mechanical properties. This causes prolonged setting times, and a decrease in mechanical properties in some cases. More specifically, tetracyclines can cause a decrease in the size of the crystals, affecting the microstructure of the CPCs [35]. Consequently, it is crucial to achieve a balance between the drug load and release rate to ensure effective bacterial eradication without compromising the overall performance of the CPCs.

For the use of bCPCs as local antibiotic drug delivery systems, Table 4 shows the results of a PubMed search[3]

Table 5. Antibiotics loaded in bCPCs and their release profiles.

Study	Antibiotic	Method of incorporation of the antibiotic in the bCPCs	Release Profile Summary
Taha et al. (2017) **[188]**	Gentamicin sulphate, Amoxicillin, Ampicillin trihydrate	Powder phase	Initial release: 47-65%. Strontium doping increased release to 73-96% within 72 hours. Bimodal release: burst followed by sustained release.
Cabrejos-Azama et al. (2016) **[189]**	Vancomycin	Method 1: Adsorption	Fast initial burst, then levels off over 6+ days. Cements with magnesium-modified TCP showed slightly faster release, with up to 81% release after 140 hours.
		Method 2: Powder phase	Two-stage release: high initial, then sustained. Magnesium modification resulted in a faster release, up to 98% within 72 hours.
Cox et al. (2016) **[125]**	Gentamicin sulphate	Liquid phase	~37% release from cement without implant. Lower burst release with antibiotic-loaded cement inserted in the implant.
Hofmann et al. (2009) **[117]**	Vancomycin, Ciprofloxacin	Powder phase	High porosity: 60-80% release within 24 hours. Lower porosity: slower, more sustained release.
Ren et al. (2021) **[190]**	Vancomycin, Tobramycin	Polymeric gel	Vancomycin: sustained release up to 28 days (~76%). Tobramycin: much lower release.
Jiang et al. (2010) **[191]**	Vancomycin Hydrochloride	Powder phase	~60% release in 120 hours.
Tamimi et al. (2008) **[192]**	Doxycycline	Liquid phase	80% release within 4 days, after an initial burst of 50% in 5 hours.
Dabiri et al. (2019) **[48]**	Gentamicin sulphate	Liquid and Powder phase	Three-phase release: initial burst (38-59%), secondary burst, plateau. ~91.5% release after 2 weeks.
Guardia et al. (2021) **[193]**	Erythromycin	Polymeric gel	Reduced burst release (32%), within the first 72 h. Sustained release up to 28 days (~90%).
Morilla et al. (2021) **[156]**	Tetracycline	Powder phase	The drug shows a burst release in the first 8 hours with the fastest release from the samples without alginate.

As shown in Table 4, different antibiotics have been incorporated into bCPCs, showing how different methods of incorporation and composition adjustments affect the drug release. For instance, Taha et al. [188] found that incorporating gentamicin sulphate, amoxicillin, and ampicillin trihydrate into the powder phase with strontium doping significantly increased the

[3] PubMed was searched with the key words “brushite cements antibiotic release;” this retrieved a total of 16 results from which 10 articles were included. The rest of the articles were discarded due to the use of other antibacterial agents, not traditional antibiotics.

release rates compare to the non-doped formulation, with gentamicin sulphate reaching 96% release within the first 72 hours. Cabrejos-Azama et al. [189] explored vancomycin release, noting that incorporation into the powder phase led to a two-stage release pattern with a rapid burst release followed by a sustained release, which varied depending on magnesium content. Similarly, Cox et al. [125] observed that gentamicin sulphate had a reduced initial burst release when incorporated via the liquid phase. Studies like those by Hofmann et al. [117] and Ren et al. [190] highlighted how porosity and the use of polymeric gels, respectively, influence release kinetics, with higher porosity leading to faster release and polymeric gels providing a more sustained release.

Adding tetracycline to bCPCs has several notable effects. Gbureck et al. (2007) [194] investigated the adsorption and desorption behaviour of various antibiotics, including tetracycline hydrochloride, with different CaP-based materials. They found that bCPC exhibited higher tetracycline loading (13 mg/sample), compared to monetite and HA. bCPC released about 25% of the drug over 5 days, following an initial burst release. The tetracycline forms stable chelates complexes with calcium ions, leading to a prolonged release compared to other antibiotics [156]. This release behaviour is influenced by physical properties like porosity and surface area, with brushite showing the highest release rate among the materials studied due to the fast degradation. Additionally, Tamimi et al. (2008) [192] reported that the addition of doxycycline to bCPC affects the final setting time by inhibiting crystallization, which can be beneficial for applications requiring extended working times. Doxycycline release patterns showed a substantial burst release (~50%), followed by a slower during 4 days. Still, 30% of the doxycycline demonstrated retention in the bCPC matrix due to chelate formation.

Overall, these studies demonstrate that antibiotics incorporated into bCPCs typically exhibit an initial burst release followed by a slower, sustained release, which can be tailored by altering the CPC's composition, porosity, or the incorporation method. This customization potential allows for bCPCs to be optimized for specific clinical needs, whether for rapid infection control or sustained antibiotic delivery.

Key Findings

Bone regeneration materials often encounter significant challenges in achieving optimal healing and complete defect regeneration. Among these materials, bCPCs stand out due to their notable biocompatibility and bioactivity. There has been growing interest in bCPCs, particularly because of their rapid resorption rates, which are considered to potentially enhance long-term clinical outcomes.

However, they also face significant challenges that have persisted over time, including rapid setting times that can compromise injectability, and suboptimal mechanical properties that may not meet the demands of bone regeneration. A range of strategies has been employed to improve these properties. These include the use of setting retarders to modulate the hardening process and the integration of polymers to enhance both mechanical integrity and handling characteristics. Moreover, the potential of bCPCs as drug delivery systems has been increasingly recognized. This feature is particularly advantageous for addressing complications related to surgical interventions, such as the risk of bacterial infections.

The ability of bCPCs to serve as localized drug delivery systems offers a strategic advantage, especially in the context of rising global concerns over antibiotic resistance. By enabling targeted antibiotic delivery locally at the surgical site, bCPCs reduce the need for systemic antibiotic therapy, thereby decreasing the potential for adverse side effects and mitigating the risk of developing antibiotic resistance.

In summary, the rapid resorption rates, and drug delivery capabilities of bCPCs significantly widen their importance in the field of bone regeneration. These properties not only support immediate post-surgical needs but also promise enhanced long-term outcomes through improved integration with host tissue and targeted therapeutic actions. As research progresses, optimizing the formulation and application of bCPCs remains pivotal to harnessing their full potential in clinical settings.

References

[1] H. H. Changsheng Liu, *Developments and Applications of Calcium Phosphate Bone Cements* (Springer Series in Biomaterials Science and Engineering). Springer 2018.

[2] A. Atala, R. Lanza, T. Mikos, and R. Nerem, *Principles of regenerative medicine*. Academic press, pp. 591-611, 2018.

[3] M. Bohner, "Design of ceramic-based cements and putties for bone graft substitution," *Eur Cell Mater,* vol. 20, no. 1, pp. 3-10, 2010.

[4] C. B. v. d. Statistiek. "Medisch Specialistische Zorg; DBC's naar diagnose (detail)." https://www.cbs.nl/nl-nl/cijfers/detail/82470NED?q=botziekte (accessed.

[5] T. B. o. M. D. i. t. U. States. United States Bone and Joint Initiative (USBJI). https://www.boneandjointburden.org/fourth-edition/i1/big-picture (accessed 22-11-2023, 2023).

[6] G. Şirin Sarıbal, N. Ersu, and E. M. Canger, "Effects of conventional complete dentures and implant-supported overdentures on alveolar ridge height and mandibular bone structure: 2-year and 6-year follow-up study," *Clinical Oral Investigations,* vol. 26, no. 9, pp. 5643-5652, 2022.

[7] P. Kämmerer, M. Klein, M. Moergel, M. Gemmel, and G. Draenert, "Local and systemic risk factors influencing the long-term success of angular stable alloplastic reconstruction plates of the mandible," *Journal of Cranio-Maxillofacial Surgery,* vol. 42, no. 5, pp. e271-e276, 2014.

[8] P. W. Kämmerer and B. Al-Nawas, "Bone reconstruction of extensive maxillomandibular defects in adults," *Periodontology 2000,* vol. 93, no. 1, pp. 340-357, 2023.

[9] H.-K. Lim, Y.-J. Choi, W.-C. Choi, I.-S. Song, and U.-L. Lee, "Reconstruction of maxillofacial bone defects using patient-specific long-lasting titanium implants," *Scientific Reports,* vol. 12, no. 1, p. 7538, 2022.

[10] A. J. Salinas and M. Vallet-Regí, "Bioactive ceramics: from bone grafts to tissue engineering," *RSC advances,* vol. 3, no. 28, pp. 11116-11131, 2013.

[11] J. Whitlow, A. Paul, and A. Polini, "Bioactive materials: definitions and application in tissue engineering and regeneration therapy," *Biocompatible Glasses: From Bone Regeneration to Cancer Treatment,* pp. 1-17, 2016.

[12] T. Albrektsson and C. Johansson, "Osteoinduction, osteoconduction and osseointegration," *European spine journal,* vol. 10, no. Suppl 2, pp. S96-S101, 2001.

[13] P. Kazimierczak and A. Przekora, "Osteoconductive and osteoinductive surface modifications of biomaterials for bone regeneration: A concise review," *Coatings,* vol. 10, no. 10, p. 971, 2020.

[14] S. N. Khan, F. P. Cammisa Jr, H. S. Sandhu, A. D. Diwan, F. P. Girardi, and J. M. Lane, "The biology of bone grafting," *JAAOS-Journal of the American Academy of Orthopaedic Surgeons,* vol. 13, no. 1, pp. 77-86, 2005.

[15] R. Dimitriou, E. Jones, D. McGonagle, and P. V. Giannoudis, "Bone regeneration: current concepts and future directions," *BMC medicine,* vol. 9, pp. 1-10, 2011.

[16] R. J. Klijn, G. J. Meijer, E. M. Bronkhorst, and J. A. Jansen, "A meta-analysis of histomorphometric results and graft healing time of various biomaterials compared to autologous bone used as sinus floor augmentation material in humans," *Tissue Engineering Part B: Reviews,* vol. 16, no. 5, pp. 493-507, 2010.

[17] P. D. Sawin, V. C. Traynelis, and A. H. Menezes, "A comparative analysis of fusion rates and donor-site morbidity for autogeneic rib and iliac crest bone grafts in posterior cervical fusions," *Journal of neurosurgery,* vol. 88, no. 2, pp. 255-265, 1998.

[18] T. Kurien, R. G. Pearson, and B. E. Scammell, "Bone graft substitutes currently available in orthopaedic practice," *The Bone & Joint Journal,* vol. 95-B, pp. 583-597, 2013.

[19] H. H. Xu *et al.*, "Calcium phosphate cements for bone engineering and their biological properties," *Bone research,* vol. 5, no. 1, pp. 1-19, 2017.

[20] I. Lodoso-Torrecilla, J. J. van den Beucken, and J. A. Jansen, "Calcium phosphate cements: Optimization toward biodegradability," *Acta biomaterialia,* vol. 119, pp. 1-12, 2021.

[21] V. Uskoković *et al.*, "Gold is for the mistress, silver for the maid: Enhanced mechanical properties, osteoinduction and antibacterial activity due to iron doping of tricalcium phosphate bone cements," *Materials Science and Engineering: C,* vol. 94, pp. 798-810, 2019.

[22] N. Nasrollahi, A. N. Dehkordi, A. Jamshidizad, and M. Chehelgerdi, "Preparation of brushite cements with improved properties by adding graphene oxide," *International Journal of Nanomedicine,* vol. 14, p. 3785, 2019.

[23] S. V. Dorozhkin, "Calcium Orthophosphate-Based Bioceramics," *Materials,* vol. 6, pp. 3840-3942, 2013, doi: https://doi.org/0.3390/ma6093840.
[24] H. Zhou and S. B. Bhaduri, "3.1 Brief introduction of calcium phosphates," *Biomaterials in Translational Medicine,* p. 37, 2018.
[25] Y. Lukina, T. Safronova, D. Smolentsev, and O. Toshev, "Calcium Phosphate Cements as Carriers of Functional Substances for the Treatment of Bone Tissue," *Materials,* vol. 16, no. 11, p. 4017, 2023.
[26] R. O'neill *et al.*, "Critical review: Injectability of calcium phosphate pastes and cements," *Acta biomaterialia,* vol. 50, pp. 1-19, 2017.
[27] N. Ribeiro *et al.*, "Improvement of a commercial calcium phosphate bone cement by means of drug delivery and increased injectability," *Ceramics International,* vol. 48, no. 22, pp. 33361-33372, 2022.
[28] M. Aqib *et al.*, "Metal-doped brushite cement for bone regeneration," *Journal of Bionic Engineering,* vol. 20, no. 6, pp. 2716-2731, 2023.
[29] R. R. Seeley, T. D. Stephens, and P. Tate, *Essentials of anatomy and physiology*. McGraw-Hill, 2002.
[30] B. Wang, Z. Zhang, and H. Pan, "Bone apatite nanocrystal: crystalline structure, chemical composition, and architecture," *Biomimetics,* vol. 8, no. 1, p. 90, 2023.
[31] K. Athanasiou, C.-F. Zhu, D. Lanctot, C. Agrawal, and X. Wang, "Fundamentals of biomechanics in tissue engineering of bone," *Tissue engineering,* vol. 6, no. 4, pp. 361-381, 2000.
[32] J. Compston, "Bone marrow and bone: a functional unit," *Journal of Endocrinology,* vol. 173, no. 3, pp. 387-394, 2002.
[33] A. K. Nair, A. Gautieri, S.-W. Chang, and M. J. Buehler, "Molecular mechanics of mineralized collagen fibrils in bone," *Nature communications,* vol. 4, no. 1, p. 1724, 2013.
[34] S. W. Shalaby and K. J. L. Burg, *Absorbable and Biodegradable Polymers* (Advances in Polymeric Biomaterials Series, no. III Series.). Taylor & Francis e-Library, 2005, p. 305.
[35] L. Qin, W. Liu, H. Cao, and G. Xiao, "Molecular mechanosensors in osteocytes," *Bone research,* vol. 8, no. 1, p. 23, 2020.
[36] K. Kusuzaki *et al.*, "Development of bone canaliculi during bone repair," *Bone,* vol. 27, no. 5, pp. 655-659, 2000.
[37] Y. Ikebuchi *et al.*, "Coupling of bone resorption and formation by RANKL reverse signalling," *Nature,* vol. 561, no. 7722, pp. 195-200, 2018.
[38] A. Faqeer *et al.*, "Cleaved SPP1-rich extracellular vesicles from osteoclasts promote bone regeneration via TGFβ1/SMAD3 signaling," *Biomaterials,* vol. 303, p. 122367, 2023.
[39] B. Safari, S. Davaran, and A. Aghanejad, "Osteogenic potential of the growth factors and bioactive molecules in bone regeneration," *International journal of biological macromolecules,* vol. 175, pp. 544-557, 2021.
[40] E. García-Gareta, M. J. Coathup, and G. W. Blunn, "Osteoinduction of bone grafting materials for bone repair and regeneration," *Bone,* vol. 81, pp. 112-121, 2015.
[41] G. I. Drosos, P. Touzopoulos, A. Ververidis, K. Tilkeridis, and K. Kazakos, "Use of demineralized bone matrix in the extremities," *World journal of orthopedics,* vol. 6, no. 2, p. 269, 2015.
[42] G. Fernandez de Grado *et al.*, "Bone substitutes: a review of their characteristics, clinical use, and perspectives for large bone defects management," *Journal of tissue engineering,* vol. 9, p. 2041731418776819, 2018.
[43] D. J. L. Paul Baldwin, Darryl A. Auston MD, Hassan S. Mir, Richard S. Yoon, Kenneth J. Koval, "Autograft, allograft, and bone graft substitutes: clinical evidence and indications for use in the setting of orthopaedic trauma surgery," *Journal of Orthopaedic Trauma,* 2019.
[44] J.-M. Bouler, P. Pilet, O. Gauthier, and E. Verron, "Biphasic calcium phosphate ceramics for bone reconstruction: A review of biological response," *Acta biomaterialia,* vol. 53, pp. 1-12, 2017.
[45] S. Tajvar, A. Hadjizadeh, and S. S. Samandari, "Scaffold degradation in bone tissue engineering: An overview," *International Biodeterioration & Biodegradation,* vol. 180, p. 105599, 2023.
[46] Z. Sheikh, "Mechanisms of in Vivo Degradation and Resorption of Calcium Phosphate Based Biomaterials," *Materials* vol. 8, pp. 7913-7925, 2015.
[47] Y. Zhu *et al.*, "A comparative study on the dissolution and solubility of hydroxylapatite and fluorapatite at 25 C and 45 C," *Chemical Geology,* vol. 268, no. 1-2, pp. 89-96, 2009.

[48] A. A. Mirtchi, J. Lemaitre, and N. Terao, "Calcium phosphate cements: study of the β-tricalcium phosphate - monocalcium phosphate system," *Biomaterials,* vol. 10, pp. 475-480, 1989, doi: 10.1016/0142-9612(89)90089-6.

[49] L. Borkowski *et al.*, "Fluorapatite ceramics for bone tissue regeneration: Synthesis, characterization and assessment of biomedical potential," *Materials Science and Engineering: C,* vol. 116, p. 111211, 2020.

[50] L. Cui *et al.*, "A novel tissue-engineered bone graft composed of silicon-substituted calcium phosphate, autogenous fine particulate bone powder and BMSCs promotes posterolateral spinal fusion in rabbits," *Journal of Orthopaedic Translation,* vol. 26, pp. 151-161, 2021.

[51] C.-L. Ko, J.-C. Chen, C.-C. Hung, J.-C. Wang, Y.-C. Tien, and W.-C. Chen, "Biphasic products of dicalcium phosphate-rich cement with injectability and nondispersibility," *Materials Science and Engineering: C,* vol. 39, pp. 40-46, 2014, doi: 10.1016/j.msec.2014.02.033.

[52] D. L. Alge, W. S. Goebel, and T.-M. Gabriel Chu, "In vitro degradation and cytocompatibility of dicalcium phosphate dihydrate cements prepared using the monocalcium phosphate monohydrate/hydroxyapatite system reveals rapid conversion to HA as a key mechanism," *J Biomed Mater Res Part B: Appl Biomater,* vol. 100B, pp. 595-602, 2012, doi: 10.1002/jbm.b.31938/full.

[53] C.-H. D. Chen, C.-C. Chen, M.-Y. Shie, C.-H. Huang, and S.-J. Ding, "Controlled release of gentamicin from calcium phosphate/alginate bone cement," *Materials Science and Engineering: C,* vol. 31, no. 2, pp. 334-341, 2011.

[54] D. Apelt *et al.*, "In vivo behavior of three different injectable hydraulic calcium phosphate cements," *Biomaterials,* vol. 25, no. 7-8, pp. 1439-1451, 2004.

[55] R. Gumpert *et al.*, "Demineralization after balloon kyphoplasty with calcium phosphate cement: a histological evaluation in ten patients," *European Spine Journal,* vol. 23, pp. 1361-1368, 2014.

[56] M. Yomoda, S. Sobajima, A. Kasuya, and M. Neo, "Calcium phosphate cement–gelatin powder composite testing in canine models: Clinical implications for treatment of bone defects," *Journal of Biomaterials Applications,* vol. 29, no. 10, pp. 1385-1393, 2015.

[57] L. Schröter, F. Kaiser, S. Stein, U. Gbureck, and A. Ignatius, "Biological and mechanical performance and degradation characteristics of calcium phosphate cements in large animals and humans," *Acta Biomaterialia,* vol. 117, pp. 1-20, 2020.

[58] L. Ding *et al.*, "Preparation and characterizations of an injectable and biodegradable high-strength iron-bearing brushite cement for bone repair and vertebral augmentation applications," *Biomaterials Science,* vol. 11, no. 1, pp. 96-107, 2023.

[59] F. Theiss *et al.*, "Biocompatibility and resorption of a brushite calcium phosphate cement," *Biomaterials,* vol. 26, no. 21, pp. 4383-4394, 2005.

[60] G. Cama, "Calcium phosphate cements for bone regeneration," in *Biomaterials for Bone Regeneration*, S. V. Vlierberghe Ed.: Woodhead Publishing, 2014, pp. 3-25.

[61] M. Parent, H. Baradari, E. Champion, C. Damia, and M. Viana-Trecant, "Design of calcium phosphate ceramics for drug delivery applications in bone diseases: A review of the parameters affecting the loading and release of the therapeutic substance," *Journal of Controlled Release,* vol. 252, pp. 1-17, 2017.

[62] K. Hurle, J. Oliveira, R. Reis, S. Pina, and F. Goetz-Neunhoeffer, "Ion-doped brushite cements for bone regeneration," *Acta Biomaterialia,* vol. 123, pp. 51-71, 2021.

[63] K. Hurle *et al.*, "Osteogenic lithium-doped brushite cements for bone regeneration," *Bioactive Materials,* vol. 16, pp. 403-417, 2022.

[64] G. Mestres *et al.*, "Changes in the drug release pattern of fresh and set simvastatin-loaded brushite cement," *Materials Science and Engineering,* vol. 58, pp. 88-96, 2016.

[65] M.-P. Ginebra, C. Canal, M. Espanol, D. Pastorino, and E. B. Montufar, "Calcium phosphate cements as drug delivery materials," *Advanced Drug Delivery Reviews,* vol. 64, pp. 1090-1110, 2012.

[66] G. Lewis, "Injectable bone cements for use in vertebroplasty and kyphoplasty: State-of-the-art review," *Journal of Biomedical Materials Research Part B: Applied Biomaterials: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials and the Korean Society for Biomaterials,* vol. 76, no. 2, pp. 456-468, 2006.

[67] S. V. Dorozhkin, "Self-Setting Calcium Orthophosphate Formulations," *J. Funct. Biomater,* vol. 4, no. 4, pp. 209-311, 2013, doi: https://doi.org/10.3390/jfb4040209.

[68] M. Bohner, U. Gbureck, and J. Barralet, "Technological issues for the development of more efficient calcium phosphate bone cements: a critical assessment," *Biomaterials,* vol. 26, no. 33, pp. 6423-6429, 2005.
[69] T. J. Brunner, R. N. Grass, M. Bohner, and W. J. Stark, "Effect of particle size, crystal phase and crystallinity on the reactivity of tricalcium phosphate cements for bone reconstruction," *Journal of Materials Chemistry,* vol. 17, no. 38, pp. 4072-4078, 2007.
[70] M. Bohner, T. J. Brunner, and W. J. Stark, "Controlling the reactivity of calcium phosphate cements," *Journal of Materials Chemistry,* vol. 18, no. 46, pp. 5669-5675, 2008.
[71] S. K. Sarkar *et al.*, "Brushite-based calcium phosphate cement with multichannel hydroxyapatite granule loading for improved bone regeneration," *Journal of Biomaterials Applications,* vol. 30, no. 6, pp. 823-837, 2016.
[72] M. Gallo, "In-vitro degradation of calcium phosphate bone substitutes: Coupled monitoring of the evolution of mechanical, microstructural and physico-chemical properties of DCPD and β-TCP samples," INSA de Lyon, 2015.
[73] A. Elghazel, R. Taktak, K. Elleuch, and J. Bouaziz, "Mechanical and tribological properties of tricalcium phosphate reinforced with fluorapatite as coating for orthopedic implant," *Materials Letters,* vol. 215, pp. 53-57, 2018.
[74] A. Kazuz *et al.*, "α-Tricalcium phosphate/fluorapatite based composite cements: Synthesis, mechanical properties, and biocompatibility," *Ceramics International,* vol. 46, no. 16, pp. 25149-25154, 2020.
[75] A. Kazuz *et al.*, "α-tricalcium phosphate/fluorapatite-based cement-promising dental root canal filling material," *Processing and Application of Ceramics,* vol. 16, no. 1, pp. 22-29, 2022.
[76] S. Seyedmajidi and M. Seyedmajidi, "Fluorapatite: A Review of Synthesis, Properties and Medical Applications vs. Hydroxyapatite," *Iran. J. Mater. Sci. Eng,* vol. 19, pp. 1-20, 2022.
[77] V. Stanić *et al.*, "Synthesis of fluorine substituted hydroxyapatite nanopowders and application of the central composite design for determination of its antimicrobial effects," *Applied Surface Science,* vol. 290, pp. 346-352, 2014.
[78] S. Vahabzadeh, M. Roy, and S. Bose, "Effects of silicon on osteoclast cell mediated degradation, in vivo osteogenesis and vasculogenesis of brushite cement," *Journal of Materials Chemistry B,* vol. 3, no. 46, pp. 8973-8982, 2015.
[79] J. C. Elliott, *Structure and chemistry of the apatites and other calcium orthophosphates*. Elsevier, 2013.
[80] S. H. Dabiri, A. Lagazzo, B. Aliakbarian, M. Mehrjoo, E. Finocchio, and L. Pastorino, "Fabrication of alginate modified brushite cement impregnated with antibiotic: Mechanical, thermal, and biological characterizations," *Journal of Biomedical Materials Research Part A,* vol. 107, no. 9, pp. 2063-2075, 2019.
[81] I. Khairoun, M. Boltong, F. Driessens, and J. Planell, "Limited compliance of some apatitic calcium phosphate bone cements with clinical requirements," *Journal of Materials Science: Materials in Medicine,* vol. 9, no. 11, pp. 667-671, 1998.
[82] A. Vezenkova and J. Locs, "Sudoku of porous, injectable calcium phosphate cements–Path to osteoinductivity," *Bioactive Materials,* vol. 17, pp. 109-124, 2022.
[83] B. van Oirschot, A. G. Mikos, Q. Liu, J. J. van den Beucken, and J. A. Jansen, "Fast Degradable Calcium Phosphate Cement for Maxillofacial Bone Regeneration," *Tissue Engineering Part A,* vol. 29, no. 5-6, pp. 161-171, 2023.
[84] Q. Liu *et al.*, "Tunable calcium phosphate cement formulations for predictable local release of doxycycline," *Materialia,* vol. 28, p. 101769, 2023.
[85] M. H. Alkhraisat *et al.*, "Effect of silica gel on the cohesion, properties and biological performance of brushite cement," *Acta Biomaterialia,* vol. 6, no. 1, pp. 257-265, 2010.
[86] F. Tamimi, Z. Sheikh, and J. Barralet, "Dicalcium phosphate cements: Brushite and monetite," *Acta Biomaterialia,* vol. 8, no. 2, pp. 474-487, 2012, doi: 10.1016/j.actbio.2011.08.005.
[87] Ö. Demir-Oğuz, A. R. Boccaccini, and D. Loca, "Injectable bone cements: What benefits the combination of calcium phosphates and bioactive glasses could bring?," *Bioactive Materials,* vol. 19, pp. 217-236, 2023.
[88] M. Bohner and G. Baroud, "Injectability of calcium phosphate pastes," *Biomaterials,* vol. 26, no. 13, pp. 1553-1563, 2005.

[89] I. Khairoun, M. Boltong, F. M. Driessens, and J. Planell, "Some factors controlling the injectability of calcium phosphate bone cements," *Journal of Materials Science: Materials in Medicine,* vol. 9, no. 8, pp. 425-428, 1998.
[90] E. F. Burguera, H. H. Xu, and L. Sun, "Injectable calcium phosphate cement: Effects of powder-to-liquid ratio and needle size," *Journal of Biomedical Materials Research Part B: Applied Biomaterials: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials and the Korean Society for Biomaterials,* vol. 84, no. 2, pp. 493-502, 2008.
[91] E. Montufar, Y. Maazouz, and M. Ginebra, "Relevance of the setting reaction to the injectability of tricalcium phosphate pastes," *Acta biomaterialia,* vol. 9, no. 4, pp. 6188-6198, 2013.
[92] A. T. Saleh, L. S. Ling, and R. Hussain, "Injectable magnesium-doped brushite cement for controlled drug release application," *Journal of materials science,* vol. 51, no. 16, pp. 7427-7439, 2016.
[93] M. Bohner, N. Doebelin, and G. Baroud, "Theoretical and experimental approach to test the cohesion of calcium phosphate pastes," *Eur Cell Mater,* vol. 12, no. 1473-2262, pp. 26-35, 2006.
[94] I. Ajaxon and C. Persson, "Mechanical properties of brushite calcium phosphate cements," *The World Scientific Encyclopedia of Nanomedicine and Bioengineering II: Bioimplants, Regenerative Medicine, and Nano-Cancer Diagnosis and Phototherapy,* vol. 3, pp. 285-300, 2017.
[95] Q. Liu, W. F. Lu, and W. Zhai, "Toward stronger robocast calcium phosphate scaffolds for bone tissue engineering: A mini-review and meta-analysis," *Biomaterials Advances,* vol. 134, p. 112578, 2022.
[96] J. Engstrand, C. Persson, and H. Engqvist, "The effect of composition on mechanical properties of brushite cements," *Journal of the Mechanical Behavior of Biomedical Materials,* vol. 29, pp. 81-90, 2014.
[97] L. M. Grover, U. Gbureck, A. J. Wright, M. Tremayne, and J. E. Barralet, "Biologically mediated resorption of brushite cement in vitro," *Biomaterials,* vol. 27, no. 10, pp. 2178-2185, 2006.
[98] S. Panzavolta, B. Bracci, K. Rubini, and A. Bigi, "Optimization of a biomimetic bone cement: role of DCPD," *Journal of Inorganic Biochemistry,* vol. 105, no. 8, pp. 1060-1065, 2011.
[99] U. Tariq, R. Hussain, K. Tufail, Z. Haider, R. Tariq, and J. Ali, "Injectable dicalcium phosphate bone cement prepared from biphasic calcium phosphate extracted from lamb bone," *Materials Science and Engineering: C,* vol. 103, p. 109863, 2019.
[100] M. Hofmann, A. Mohammed, Y. Perrie, U. Gbureck, and J. Barralet, "High-strength resorbable brushite bone cement with controlled drug-releasing capabilities," *Acta biomaterialia,* vol. 5, no. 1, pp. 43-49, 2009.
[101] B. Kanter, M. Geffers, A. Ignatius, and U. Gbureck, "Control of in vivo mineral bone cement degradation," *Acta biomaterialia,* vol. 10, no. 7, pp. 3279-3287, 2014.
[102] Z. Sheikh, Y. L. Zhang, L. Grover, G. E. Merle, F. Tamimi, and J. Barralet, "In vitro degradation and in vivo resorption of dicalcium phosphate cement based grafts," *Acta biomaterialia,* vol. 26, pp. 338-346, 2015.
[103] M. Rödel, J. Teßmar, J. Groll, and U. Gbureck, "Dual setting brushite—gelatin cement with increased ductility and sustained drug release," *Journal of Biomaterials Applications,* vol. 36, no. 10, pp. 1882-1898, 2022.
[104] T. Sopcak, L. Medvecky, M. Giretova, A. Kovalcikova, R. Stulajterova, and J. Durisin, "Phase transformations, microstructure formation and in vitro osteoblast response in calcium silicate/brushite cement composites," *Biomedical Materials,* vol. 11, no. 4, p. 045013, 2016.
[105] N. H. Kamal, L. A. Heikal, M. M. Ali, R. G. Aly, and O. Y. Abdallah, "Development and evaluation of local regenerative biomimetic bone-extracellular matrix scaffold loaded with nano-formulated quercetin for orthopedic fractures," *Biomaterials Advances,* vol. 145, p. 213249, 2023.
[106] M. H. Alkhraisat, J. Cabrejos-Azama, C. R. Rodríguez, L. B. Jerez, and E. L. Cabarcos, "Magnesium substitution in brushite cements," *Materials Science and Engineering: C,* vol. 33, no. 1, pp. 475-481, 2013.
[107] E. Mulky, G. Yazgan, K. Maniura-Weber, R. Luginbuehl, G. Fortunato, and A.-M. Bühlmann-Popa, "Fabrication of biopolymer-based staple electrospun fibres for nanocomposite applications by particle-assisted low temperature ultrasonication," *Materials Science and Engineering: C,* vol. 45, pp. 277-286, 2014.
[108] S. C. Cox *et al.*, "Adding functionality with additive manufacturing: Fabrication of titanium-based antibiotic eluting implants," *Materials Science and Engineering: C,* vol. 64, pp. 407-415, 2016.

[109] H.-J. Lee, B. Kim, A. R. Padalhin, and B.-T. Lee, "Incorporation of chitosan-alginate complex into injectable calcium phosphate cement system as a bone graft material," *Materials Science and Engineering: C,* vol. 94, pp. 385-392, 2019.
[110] L. M. Grover, M. P. Hofmann, U. Gbureck, B. Kumarasami, and J. E. Barralet, "Frozen delivery of brushite calcium phosphate cements," *Acta biomaterialia,* vol. 4, no. 6, pp. 1916-1923, 2008.
[111] G. Cama *et al.*, "Preparation and properties of macroporous brushite bone cements," *Acta biomaterialia,* vol. 5, no. 6, pp. 2161-2168, 2009.
[112] F. Tamimi, D. Le Nihouannen, H. Eimar, Z. Sheikh, S. Komarova, and J. Barralet, "The effect of autoclaving on the physical and biological properties of dicalcium phosphate dihydrate bioceramics: Brushite vs. monetite," *Acta biomaterialia,* vol. 8, no. 8, pp. 3161-3169, 2012.
[113] Z. Sheikh, Y. L. Zhang, F. Tamimi, and J. Barralet, "Effect of processing conditions of dicalcium phosphate cements on graft resorption and bone formation," *Acta biomaterialia,* vol. 53, pp. 526-535, 2017.
[114] J. Luo, I. Ajaxon, M. P. Ginebra, H. Engqvist, and C. Persson, "Compressive, diametral tensile and biaxial flexural strength of cutting-edge calcium phosphate cements," *Journal of the mechanical behavior of biomedical materials,* vol. 60, pp. 617-627, 2016.
[115] I. Ajaxon *et al.*, "Elastic properties and strain-to-crack-initiation of calcium phosphate bone cements: Revelations of a high-resolution measurement technique," *Journal of the mechanical behavior of biomedical materials,* vol. 74, pp. 428-437, 2017.
[116] I. Ajaxon, A. Holmberg, C. Öhman-Mägi, and C. Persson, "Fatigue performance of a high-strength, degradable calcium phosphate bone cement," *Journal of the mechanical behavior of biomedical materials,* vol. 79, pp. 46-52, 2018.
[117] E. Şahin and M. Çiftçioğlu, "Compositional, microstructural and mechanical effects of NaCl porogens in brushite cement scaffolds," *journal of the mechanical behavior of biomedical materials,* vol. 116, p. 104363, 2021.
[118] I. Ajaxon, Y. Maazouz, M.-P. Ginebra, C. Öhman, and C. Persson, "Evaluation of a porosity measurement method for wet calcium phosphate cements," *Journal of biomaterials applications,* vol. 30, no. 5, pp. 526-536, 2015.
[119] I. Ajaxon, C. Öhman, and C. Persson, "Long-term in vitro degradation of a high-strength brushite cement in water, PBS, and serum solution," *BioMed research international,* vol. 2015, no. 1, p. 575079, 2015.
[120] D. L. Alge, G. Santa Cruz, W. S. Goebel, and T.-M. G. Chu, "Characterization of dicalcium phosphate dihydrate cements prepared using a novel hydroxyapatite-based formulation," *Biomedical Materials,* vol. 4, no. 2, p. 025016, 2009.
[121] J. An, S. C. Leeuwenburgh, J. G. Wolke, and J. A. Jansen, "Effects of stirring and fluid perfusion on the in vitro degradation of calcium phosphate cement/PLGA composites," *Tissue Engineering Part C: Methods,* vol. 21, no. 11, pp. 1171-1177, 2015.
[122] J. L. Aparicio *et al.*, "Effect of physicochemical properties of a cement based on silicocarnotite/calcium silicate on in vitro cell adhesion and in vivo cement degradation," *Biomedical Materials,* vol. 11, no. 4, p. 045005, 2016.
[123] A. C. de Franca Silva Azevedo *et al.*, "Brushite bone cement containing polyethylene glycol for bone regeneration," *Bio-Medical Materials and Engineering,* vol. 33, no. 3, pp. 221-233, 2022.
[124] G. Cama *et al.*, "A novel method of forming micro-and macroporous monetite cements," *Journal of Materials Chemistry B,* vol. 1, no. 7, pp. 958-969, 2013.
[125] J. Unosson, E. B. Montufar, H. Engqvist, M. P. Ginebra, and C. Persson, "Brushite foams—the effect of T ween® 80 and P luronic® F-127 on foam porosity and mechanical properties," *Journal of Biomedical Materials Research Part B: Applied Biomaterials,* vol. 104, no. 1, pp. 67-77, 2016.
[126] E. F. Burguera, F. Guitian, and L. C. Chow, "A water setting tetracalcium phosphate–dicalcium phosphate dihydrate cement," *Journal of Biomedical Materials Research Part A: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials and the Korean Society for Biomaterials,* vol. 71, no. 2, pp. 275-282, 2004.
[127] B. Flautre, C. Maynou, J. Lemaitre, P. Van Landuyt, and P. Hardouin, "Bone colonization of β–TCP granules incorporated in brushite cements," *Journal of Biomedical Materials Research: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials and the Korean Society for Biomaterials,* vol. 63, no. 4, pp. 413-417, 2002.

[128] C. de Oliveira Renó, N. C. Pereta, C. A. Bertran, M. Motisuke, and E. de Sousa, "Study of in vitro degradation of brushite cements scaffolds," *Journal of Materials Science: Materials in Medicine,* vol. 25, pp. 2297-2303, 2014.
[129] C. Öhman, J. Unosson, E. Carlsson, M. P. Ginebra, C. Persson, and H. Engqvist, "Porosity prediction of calcium phosphate cements based on chemical composition," *Journal of Materials Science: Materials in Medicine,* vol. 26, pp. 1-8, 2015.
[130] L. S. Bertol, R. Schabbach, and L. A. Loureiro dos Santos, "Different post-processing conditions for 3D bioprinted α-tricalcium phosphate scaffolds," *Journal of Materials Science: Materials in Medicine,* vol. 28, pp. 1-12, 2017.
[131] X. Cao *et al.*, "3D plotting in the preparation of newberyite, struvite, and brushite porous scaffolds: using magnesium oxide as a starting material," *Journal of Materials Science: Materials in Medicine,* vol. 30, pp. 1-12, 2019.
[132] C. Schaufler *et al.*, "Physicochemical degradation of calcium magnesium phosphate (stanfieldite) based bone replacement materials and the effect on their cytocompatibility," *Biomedical Materials,* vol. 18, no. 1, p. 015022, 2022.
[133] K. A. Shariff, K. Tsuru, and K. Ishikawa, "Fabrication of interconnected pore forming α-tricalcium phosphate foam granules cement," *Journal of Biomaterials Applications,* vol. 30, no. 6, pp. 838-845, 2016.
[134] G. Cama, B. Gharibi, J. Knowles, S. Romeed, L. DiSilvio, and S. Deb, "Structural changes and biological responsiveness of an injectable and mouldable monetite bone graft generated by a facile synthetic method," *Journal of the royal society interface,* vol. 11, no. 101, p. 20140727, 2014.
[135] H. Mabroum, H. Noukrati, B. Lefeuvre, H. Oudadesse, and A. Barroug, "Physicochemical, setting, rheological, and mechanical properties of a novel bio-composite based on apatite cement, bioactive glass, and alginate hydrogel," *Ceramics International,* vol. 47, no. 17, pp. 23973-23983, 2021.
[136] S. Saurav *et al.*, "Harnessing Natural Polymers for Nano-Scaffolds in Bone Tissue Engineering: A Comprehensive Overview of Bone Disease Treatment," *Current Issues in Molecular Biology,* vol. 46, no. 1, pp. 585-611, 2024.
[137] N. Iqbal, A. S. Khan, A. Asif, M. Yar, J. W. Haycock, and I. U. Rehman, "Recent concepts in biodegradable polymers for tissue engineering paradigms: A critical review," *International Materials Reviews,* vol. 64, no. 2, pp. 91-126, 2019.
[138] R. A. Perez, H.-W. Kim, and M.-P. Ginebra, "Polymeric additives to enhance the functional properties of calcium phosphate cements," *Journal of tissue engineering,* vol. 3, no. 1, p. 2041731412439555, 2012.
[139] M. Alkhraisat *et al.*, "The effect of hyaluronic acid on brushite cement cohesion," *Acta biomaterialia,* vol. 5, no. 8, pp. 3150-3156, 2009.
[140] C. Morilla *et al.*, "Effect of the addition of alginate and/or tetracycline on brushite cement properties," *Molecules,* vol. 26, no. 11, p. 3272, 2021.
[141] S. Aghyarian *et al.*, "Characterization of a new composite PMMA-HA/Brushite bone cement for spinal augmentation," *Journal of biomaterials applications,* vol. 29, no. 5, pp. 688-698, 2014.
[142] S. Maenz *et al.*, "Enhanced bone formation in sheep vertebral bodies after minimally invasive treatment with a novel, PLGA fiber-reinforced brushite cement," *The spine journal,* vol. 17, no. 5, pp. 709-719, 2017.
[143] S. de Lacerda Schickert, J. A. Jansen, E. M. Bronkhorst, J. J. van den Beucken, and S. C. Leeuwenburgh, "Stabilizing dental implants with a fiber-reinforced calcium phosphate cement: an in vitro and in vivo study," *Acta Biomaterialia,* vol. 110, pp. 280-288, 2020.
[144] N. W. Kucko, D.-G. Petre, M. de Ruiter, R.-P. Herber, and S. C. Leeuwenburgh, "Micro-and macromechanical characterization of the influence of surface-modification of poly (vinyl alcohol) fibers on the reinforcement of calcium phosphate cements," *journal of the mechanical behavior of biomedical materials,* vol. 109, p. 103776, 2020.
[145] S. de Lacerda Schickert, J. C. Pinto, J. Jansen, S. C. Leeuwenburgh, and J. J. van den Beucken, "Tough and injectable fiber reinforced calcium phosphate cement as an alternative to polymethylmethacrylate cement for vertebral augmentation: a biomechanical study," *Biomaterials science,* vol. 8, no. 15, pp. 4239-4250, 2020.
[146] A. N. Renth and M. S. Detamore, "Leveraging ‘‘Raw Materials’’ as Building Blocks and Bioactive Signals in Regenerative Medicine," *Tissue Engineering: Part B,* vol. 18, no. 5, pp. 341-362, 2012.

[147] D. Shi, *Introduction to Biomaterials*. Beijing , P. R. China: World Scientific Publishing Co. Pte. Ltd., pp. 159-165, 2006.
[148] I. Palmer, J. Nelson, W. Schatton, N. J. Dunne, F. J. Buchanan, and S. A. Clarke, "Biocompatibility of calcium phosphate bone cement with optimized mechanical properties," *Journal of Biomedical Materials Research Part B: Applied Biomaterials,* vol. 104, no. 2, pp. 308-315, 2016.
[149] G. Tomoaia *et al.*, "Scaffolds made of nanostructured phosphates, collagen and chitosan for cell culture," *Powder Technology,* vol. 238, pp. 99-107, 2013, doi: http://dx.doi.org/10.1016/j.powtec.2012.05.023.
[150] Y. J. Hong, J.-S. Chun, and W.-K. Lee, "Association of collagen with calcium phosphate promoted osteogenic responses of osteoblast-like MG63 cells," *Colloids and Surfaces B: Biointerfaces,* vol. 83, no. 2, pp. 245-253, 2011, doi: http://dx.doi.org/10.1016/j.colsurfb.2010.11.028.
[151] J. Czechowska, A. Zima, D. Siek, and A. Ślósarczyk, "Influence of sodium alginate and methylcellulose on hydrolysis and physicochemical properties of α-TCP based materials," *Ceramics International,* 2018.
[152] L. Pereira and J. Cotas, "Introductory chapter: Alginates-A general overview," *Alginates-recent uses of this natural polymer,* 2020.
[153] C. Xu, X. Wang, J. Zhou, Z. Huan, and J. Chang, "Bioactive tricalcium silicate/alginate composite bone cements with enhanced physicochemical properties," *Journal of Biomedical Materials Research Part B: Applied Biomaterials,* vol. 106, no. 1, pp. 237-244, 2018.
[154] L. Agüero *et al.*, "Synthesis and characterization of polyelectrolyte complex microparticles for drug release," *Journal of Applied Polymer Science,* vol. 128, no. 6, pp. 3548-3554, 2013.
[155] J. Venkatesan, I. Bhatnagar, P. Manivasagan, K.-H. Kang, and S.-K. Kim, "Alginate composites for bone tissue engineering: A review," *International Journal of Biological Macromolecules,* vol. 72, pp. 269-281, 1// 2015, doi: http://dx.doi.org/10.1016/j.ijbiomac.2014.07.008.
[156] Y. Shu *et al.*, "Degradation in vitro and in vivo of β-TCP/MCPM-based premixed calcium phosphate cement," *Journal of the Mechanical Behavior of Biomedical Materials,* vol. 90, pp. 86-95, 2019.
[157] W. Gao, H. Wang, R. Liu, X. Ba, K. Deng, and F. Liu, "Simultaneous Regulation of the Mechanical/Osteogenic Capacity of Brushite Calcium Phosphate Cement by Incorporating with Poly (ethylene glycol) Dicarboxylic Acid," *ACS Biomaterials Science & Engineering,* vol. 10, no. 4, pp. 2062-2067, 2024.
[158] B. Rentsch *et al.*, "Trivalent chromium incorporated in a crystalline calcium phosphate matrix accelerates materials degradation and bone formation in vivo," *Acta biomaterialia,* vol. 69, pp. 332-341, 2018.
[159] G. Schneider, K. Blechschmidt, D. Linde, P. Litschko, T. Körbs, and E. Beleites, "Bone regeneration with glass ceramic implants and calcium phosphate cements in a rabbit cranial defect model," *Journal of Materials Science: Materials in Medicine,* vol. 21, pp. 2853-2859, 2010.
[160] R. Jayasree, T. S. Kumar, R. Venkateswari, R. P. Nankar, and M. Doble, "Eggshell derived brushite bone cement with minimal inflammatory response and higher osteoconductive potential," *Journal of Materials Science: Materials in Medicine,* vol. 30, pp. 1-14, 2019.
[161] K. Kowalewicz *et al.*, "Comparison of degradation behavior and osseointegration of 3D powder-printed calcium magnesium phosphate cement scaffolds with alkaline or acid post-treatment," *Frontiers in Bioengineering and Biotechnology,* vol. 10, p. 998254, 2022.
[162] T. T. Thao Le, P. Makkar, G. Tripathi, and B. T. Lee, "Porous CDHA microspheres laden brushite-based injectable bone substitutes for improved bone regeneration," *Journal of Biomedical Materials Research Part B: Applied Biomaterials,* vol. 110, no. 8, pp. 1771-1779, 2022.
[163] A. Alamgir and A. Alamgir, "Classification of drugs, nutraceuticals, functional food, and cosmeceuticals; proteins, peptides, and enzymes as drugs," *Therapeutic Use of Medicinal Plants and Their Extracts: Volume 1: Pharmacognosy,* pp. 125-175, 2017.
[164] J. Lucas-Aparicio *et al.*, "Silicon-calcium phosphate ceramics and silicon-calcium phosphate cements: Substrates to customize the release of antibiotics according to the idiosyncrasies of the patient," *Materials Science and Engineering: C,* vol. 106, p. 110173, 2020.
[165] N. Pajares-Chamorro, N. D. Hammer, and X. Chatzistavrou, "Materials for restoring lost Activity: Old drugs for new bugs," *Advanced Drug Delivery Reviews,* vol. 186, p. 114302, 2022.
[166] I. L. Balasooriya, J. Chen, S. M. Korale Gedara, Y. Han, and M. N. Wickramaratne, "Applications of nano hydroxyapatite as adsorbents: A review," *Nanomaterials,* vol. 12, no. 14, p. 2324, 2022.

[167] M. Fosca, J. V. Rau, and V. Uskoković, "Factors influencing the drug release from calcium phosphate cements," *Bioactive Materials,* vol. 7, pp. 341-363, 2022.
[168] A. Pylostomou, O. Demir-Oguz, and D. Loca, "Calcium phosphate bone cements as local drug delivery systems for bone cancer treatment," *Biomaterials Advances,* p. 213367, 2023.
[169] R. Belluomo, A. Khodaei, and S. A. Yavari, "Additively manufactured Bi-functionalized bioceramics for reconstruction of bone tumor defects," *Acta Biomaterialia,* vol. 156, pp. 234-249, 2023.
[170] C. Zhong, Y. Wu, H. Lin, and R. Liu, "Advances in the antimicrobial treatment of osteomyelitis," *Composites Part B: Engineering,* p. 110428, 2022.
[171] J. E. CILLO JR, "Dental implant infections," *Misch's Contemporary Implant Dentistry E-Book: Misch's Contemporary Implant Dentistry E-Book,* p. 341, 2020.
[172] A. Taha, M. Akram, Z. Jawad, A. Z. Alshemary, and R. Hussain, "Strontium doped injectable bone cement for potential drug delivery applications," *Materials Science and Engineering: C,* vol. 80, pp. 93-101, 2017.
[173] J. Cabrejos-Azama *et al.*, "Magnesium substitution in brushite cements: Efficacy of a new biomaterial loaded with vancomycin for the treatment of Staphylococcus aureus infections," *Materials Science and Engineering: C,* vol. 61, pp. 72-78, 2016.
[174] E. Ren, A. Guardia, T. Shi, P. Begeman, W. Ren, and R. Vaidya, "A distinctive release profile of vancomycin and tobramycin from a new and injectable polymeric dicalcium phosphate dehydrate cement (P-DCPD)," *Biomedical Materials,* vol. 16, no. 2, p. 025019, 2021.
[175] P. J. Jiang, S. Patel, U. Gbureck, R. Caley, and L. M. Grover, "Comparing the efficacy of three bioceramic matrices for the release of vancomycin hydrochloride," *Journal of Biomedical Materials Research Part B: Applied Biomaterials: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials and the Korean Society for Biomaterials,* vol. 93, no. 1, pp. 51-58, 2010.
[176] F. Tamimi *et al.*, "Doxycycline sustained release from brushite cements for the treatment of periodontal diseases," *Journal of Biomedical Materials Research Part A: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials and the Korean Society for Biomaterials,* vol. 85, no. 3, pp. 707-714, 2008.
[177] A. Guardia *et al.*, "Properties of erythromycin-loaded polymeric dicalcium phosphate dehydrate bone graft substitute," *Journal of Orthopaedic Research®,* vol. 39, no. 11, pp. 2446-2454, 2021.
[178] U. Gbureck, E. Vorndran, F. A. Müller, and J. E. Barralet, "Low temperature direct 3D printed bioceramics and biocomposites as drug release matrices," *Journal of Controlled Release,* vol. 122, no. 2, pp. 173-180, 2007.

Chapter 3: Synthesis and evaluation of a collagen–brushite cement as a drug delivery system.

This chapter is based on:

Morilla, C., Lima, Y. M., Fuentes, G., & Almirall, A. (2018). *International Journal of Materials Research*, *110*(4), 367-374.

Introduction

Calcium phosphates are well known biomaterials that promote bone regeneration and have excellent biocompatibility and bioactivity, which have caused a significant increase in their use in biomedical applications in recent years. These materials are used in various applications of orthopedic and maxillofacial surgery, either for filling bone defects, increase of the alveolar ridge, implants of the middle ear, fusion of spinal vertebrae or in the coating of metal prostheses. They are applied in different forms: as granulates, blocks of different shapes or as cements that solidify during their application [1]. The calcium phosphate cements (CPCs) have the advantage that they are prepared as a paste that sets in a few minutes and can easily adapt to the shape of the bone defect, which facilitates its application [2, 3]. These materials are used not only as dental cements, but also as bone reabsorbable implants. Particularly, and unlike other materials, these biomaterials can repair bone defects in a permanent way [4], promoting the formation of new bone tissue during the cement degradation [5] due to their osteoconductivity [6, 7]. In addition, the characteristics of calcium phosphate cements make them an excellent alternative for drugs release [8, 9].

Mirtchi and Lemaître described the brushite cements in 1989 [2, 10]. These materials are prepared by mixing water with a powder consisting of an acid calcium phosphate (monocalcium phosphate monohydrate, MCPM) and a basic calcium phosphate (β-tricalcium phosphate, β-TCP). The result of this mixture is a mouldable paste that solidifies by an exothermic reaction forming a hard material composed mainly of dicalcium phosphate dehydrate (DCPD), also known as brushite [4].

Subsequent studies showed that brushite cements are biocompatible; however, they are difficult to handle, harden too quickly (usually less than 30 s) and have poor mechanical properties. Several additives have been added to these cements to improve some of their properties: injectability [11], setting time [4], cohesion and mechanical properties [12]. The idea of using polymers to improve the mechanical properties of cements came from bone itself, which is a composite material made of an organic phase reinforced with hydroxyapatite crystals. Type I collagen is the main component in the organic phase of bone, and has a crucial role in the tensile strength of mineralized tissues [4], has been used as reinforcement for CPC [13] and promotes osteogenesis [14].

On the other hand, infection risk on surgical maxillofacial procedures requires the use of antibiotics, generally supplied in oral prescriptions in large quantities to ensure the appropriate therapeutic dosage on the treated site. A great number of bone implant infections are caused by

bacteria, which are very common in buccal cavity and are related to periodontal disease. Among other antibiotics, tetracycline is a well know an effective antibiotic with a broad- spectrum against bacterial infection, usually related to periodontal disease [15, 16].

In the present study, several brushite or DCPD bone cements for maxillofacial applications made from MCPM and *β*-TCP with or without collagen were prepared and evaluated as drug release systems for tetracycline. Although the use of CPCs as drug delivery system has been analysed, the study of more complex formulations that includes reinforcement materials, such as collagen, could have a significant impact on the development of more efficient bone regenerative biomaterials with the capability to be used as a drug delivery system.

Experimental procedure

Cements preparation

All chemicals employed were of analytical grade, used as received. *β*-TCP was synthetized by a wet neutralization reaction using CaO (Merck, Germany) and H_3PO_4 (Merck, Germany), following the method described by Carrodegüas et al. [17].

For the preparation of the CPCs, MCPM (Merck, Germany) and *β*-TCP were mixed in the solid phase and, depending on the experiment, 1 % collagen (type I collagen fibers from bovine Achilles tendon, commercial grade, Brazil), as reinforcement and 1 % tetracycline (Ningxia Qiyuan Pharmaceutical Co., China), as antibiotic, to determine their possible use as a drug release system, were added. A solution of 0.1 % citric acid (Merck, Germany) was used as a liquid phase of the cements and as a setting retarder. A comparative study to analyse the composition effect on compressive strength, drug release and antimicrobial activity was carried out. The effect of the independent variables, the quantity of *β*-TCP used and the addition or not of collagen and/or tetracycline, was studied through the experiments described in Table 1.

Table 1. Experimental design. Liquid phase = 0.35 ml g^{-1}.

Series	MCPM (%)	*β*-TCP (%)	Collagen (%)	Tetracycline (%)
M(1)	48	50	1	**1**
M(2)	49	50	1	**–**
M(3)	49	50	–	**1**
M(4)	43	55	1	**1**
M(5)	44	55	1	**–**
M(6)	**44**	**55**	**–**	**1**

X-ray diffraction (XRD)

Phase characterization was carried out by means of X-ray diffraction, in a Philips, PW 1710 diffractometer (UK) with Cu-Ka radiation (k = 1.54056 nm) and Ni filter. The scan were made in a 2h angular interval of 10 – 60° and scanning speed of 18 min^{-1}. The results were interpreted using the X'Pert HighScore PANalytical program database, version 2.0.

Fourier transform infrared spectroscopy (FTIR)

Infrared spectra were obtained in a Fourier transform infrared spectrometer Shimadzu IR Tracer 100 (Japan), with a resolution of 4 cm^{-1} and 21 scans per sample in a range of 400-4 000 cm^{-1}.

Scanning electronic microscopy (SEM)

The samples were coated with a 20 nm film of gold in a BAL-TEC MED 020 system and placed in a desiccator until analysis. A JEOL microscope, JSM-6360LV with Oxford EDX probe (USA), magnification of 5 – 300000, resolution of 3 nm and acceleration voltage of 20 kV, was used for the microstructural analysis.

Mechanical characterization

For the compressive strength of the material, 12 mm height and 6 mm diameter specimens were prepared. The samples were immersed in Ringer's solution at 37 °C and tested, after 24 h of the cement preparation, immediately after being extracted to maintain hydration. The study was carried out in a universal testing machine with load cell of 200 N and at 1 mm min^{-1} load application speed. The compressive strength (r_c) in MPa was determined by the following formula:

$$\sigma_c = \frac{F}{A_0} = \frac{4P}{\pi d^2} \cdot 10^{-6} \qquad (1)$$

where *P* is the maximum breaking load (N) and *d* the diameter of the specimen (m). Five specimens were tested for each formulation.

Drug release study

Test specimens of the cements of 6 mm in height and 12 mm in diameter loaded with tetracycline were used. The samples were immersed in 10 mL of Ringer's solution in glass bottles at (37.0 ± 0.5) °C throughout the study. The solution in contact with the specimens was completely extracted at the established times and replaced with 10 mL of fresh solution. The extractions were made every half hour until 5 h of the cement preparation and, after that, at 24 h up to seven days. Five specimen of each formulation were prepared and evaluated. The determination of the antibiotic released to the solution was carried out in a UV-Visible Spectrophotometer (Shimadzu,

Japan) at a wavelength of 276 nm; and the results were reported as a cumulative amount of the tetracycline released versus time [18].

Microbiological study

For the microbiological study, specimens of 6 mm in height and 12 mm in diameter were prepared. In order to evaluate the antimicrobial susceptibility of the composites, strains of *Escherichia coli* ATCC 10536 were used, at a strain concentration adjusted with a turbidimetric method employing as a reference a 0.5 MacFarland standard ($1 \cdot 10^8$ CFU mL^{-1}).

Subsequently, 500 μL of a previously prepared culture medium of Mueller-Hinton agar (Merck) was inoculated in Petri dishes. After a period of 20 min the test specimens were placed on top of the plates containing the culture medium and the bacterial suspension and were incubated at 37 ± 1 °C for a period of 72 h. Three specimens were tested for each formulation and the inhibition zone was measured with the software SCAN 500 Automatic Colony Counter Version 6.

Statistical calculations

Graphs, statistics and mathematical calculation of nonlinear models to evaluate release mechanisms were performed with OriginPro 2016.

Results and discussion

Once the cements were prepared, a malleable paste that set in about 2 to 3 min was obtained. The setting time delay regarding the values reported for brushite cements of less than 30 s [4, 19] was achieved with the addition of 0.1 % of citric acid to the liquid phase used as a reaction retarder [20]. The accepted mechanism for the setting reaction of the cement paste goes through the dissolution of the components, the formation of a gel and the nucleation and growth of brushite crystals, according to the reactions described in Eqs. (2 – 4) [4]. During the initial dissolution of MCPM a considerable decrease in pH occurred, followed by an increase during the dissolution of b-TCP due to the exposure to an acid environment. Finally, the precipitation and growth of interlocked brushite crystals resulted in a solid material. Equation (5) describes the complete setting reaction of the brushite cements.

$$Ca(H_2PO_4)_2 \bullet H_2O = Ca^{2+} + 2H_2PO_4^- + H_2O \quad (2)$$

$$\beta\text{-}Ca_3(PO_4)_2 + 4H^+ = 3Ca^{2+} + 2H_2PO_4^- \quad (3)$$

$$Ca^{2+} + H_2PO_4^- + 2H_2O = CaHPO_4 \bullet 2H_2O + H^+ \quad (4)$$

$$\beta\text{-}Ca_3(PO_4)_2 + Ca(H_2PO_4)_2 \bullet H_2O + 7H_2O = 4CaHPO_4 \bullet 2H_2O \quad (5)$$

Since, in aqueous solutions at physiological *pH*, DCPD is known as a precursor of hydroxyapatite that is more thermodynamically stable, the transformation of the cements once implanted should be expected. This transformation occurs through a dissolution–reprecipitation mechanism. Due to the relatively low solubility of brushite in water the presence of Ca^{2+} ions in the medium is needed to trigger the reprecipitation process [21], which occurs according to the following reaction (Eq. (6)) thanks to the Ca ions present in the body fluids.

$$CaHPO_4 \bullet 2H_2O + (3\text{-}x)Ca_3(PO_4)_2 = Ca_{10\text{-}x}(PO_4)_{6\text{-}x}(OH)_2 + H_3PO_4 \quad (6)$$

XRD and FTIR

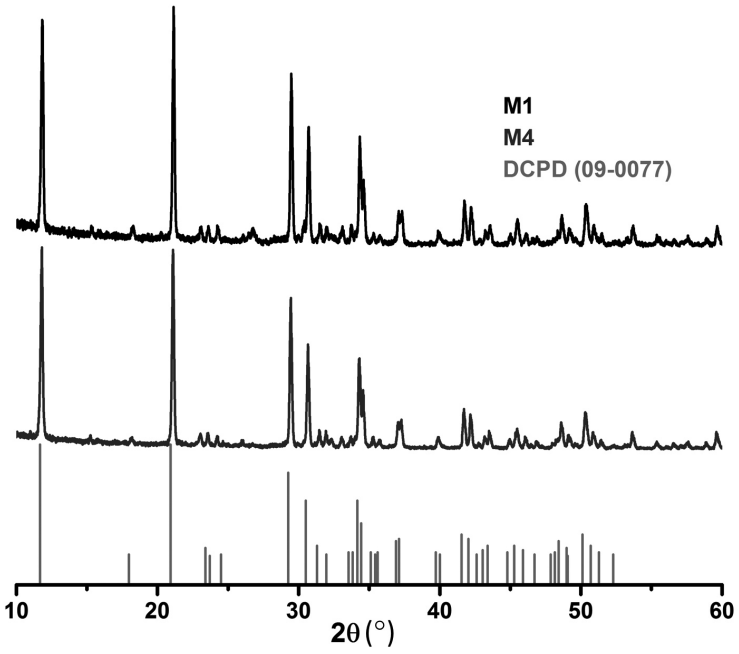


Figure 1. XRD pattern of samples M1 and M4, all the peaks corresponds to DCPD according to the ICDD PDF 9-0077 X-ray diffraction pattern. The maximum intensity DCPD peaks could be observed at 2h = 11.681° and 20.935°.

Figure 1 shows the DRX pattern of the cements samples M1 and M4 after 72 h of immersion in Ringer's solution. The most significant peaks of the pattern were compared with the ICDD PDF 9-0077 X-ray diffraction pattern of DCPD, which is the expected product of the setting reaction of the cement. A coincidence, in both, position and in intensity was observed, which confirms the occurrence of the setting reaction and the precipitation of brushite crystals. The results were very similar in all cements studied.

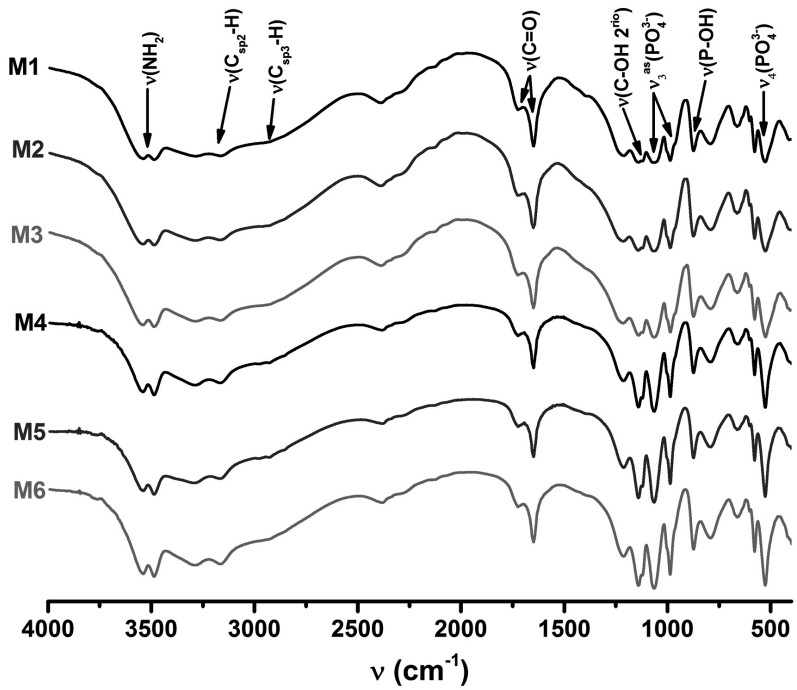


Figure 2. IR spectra of the samples

Figure 2 shows the FTIR spectra of the cements. The characteristic bands associated with the functional groups of calcium phosphates $\nu_3(PO_4^{3-})$ and the double signal of $\nu_4(PO_4^{3-})$ can be observed at 1119–1046, 605 and 570 cm^{-1} respectively In addition, some of the most relevant bands of the organic components, collagen and/or tetracycline, present in each formulation of the cements can be appreciated. Among them, the collagen bands corresponding to ν(C=O) and $\nu(Csp_3–H)$ at 1632 and 2 932 cm^{-1} can be identified. In the case of tetracycline, the ν(C=O), $\nu(Csp_2–H)$ and $\nu(NH_2)$ at 1 720, 3 148 and 3 280 cm^{-1}, respectively, can be observed. The spectra are very similar in all cases since the main component of the cement was the brushite obtained as a final setting reaction product, as discussed previously.

SEM

The morphology of the samples is shown in Figure 3. The presence of small pores of about 5 – 20 *μ*m can be seen; the samples with collagen (M1, M2, M4 and M5) showed the smallest size of the pores due to the more compact structure achieved with the presence of collagen and the swelling of its fibers by the absorption of water. In addition, in the samples with collagen the fibers can be observed and distinguished as flatter and more uniform areas than the rest of the crystalline framework of the cement, which is not observed in the samples without collagen (M3, M6). The decrease in the size of the pores in the samples with the presence of collagen and the integration of the fibers of this compound in the crystalline framework of the cement influence the mechanical properties.

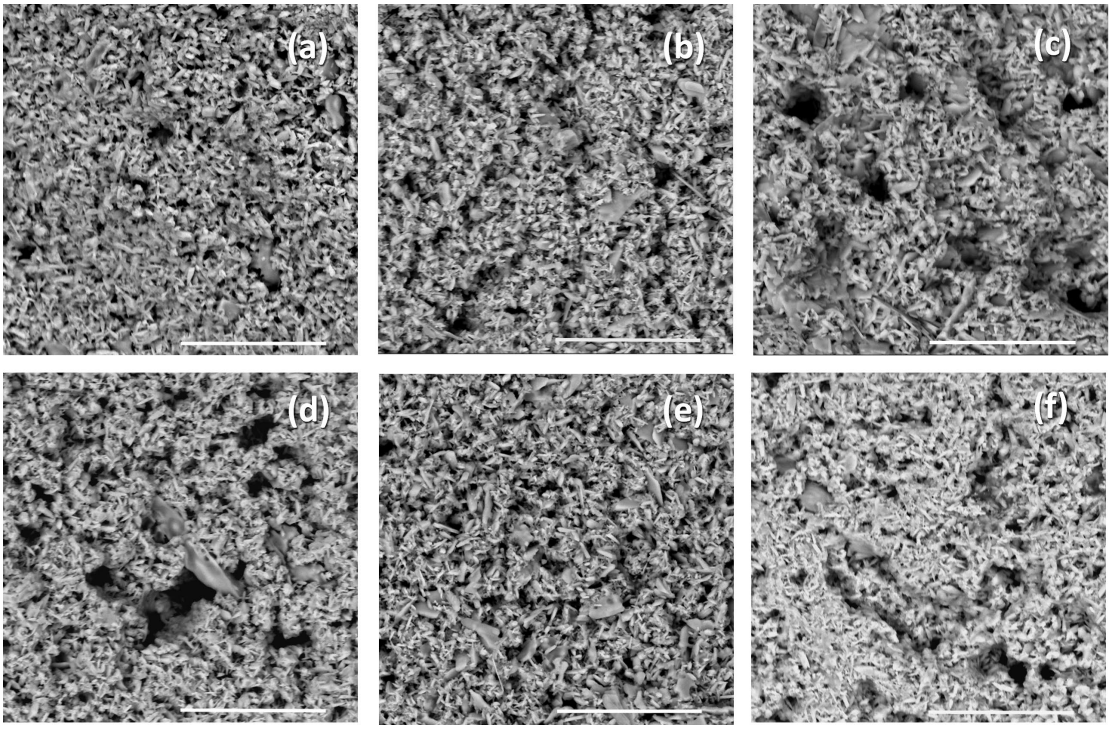


Figure 3. SEM micrographs of: (a) Cement M1, (b) Cement M2, (c) Cement M3, (d) Cement M4, (e) Cement M5, (f) Cement M6 (scale bar = 50 µm). Samples M1, M2 and M3 contain less β-TCP compared to M4, M5 and M6, respectively. M1, M2, M4 and M5 contain collagen.

Compressive strength

Table 2 shows the results of the evaluation of the mechanical properties. The values of the compressive strength of the samples vary between 0.8 and 1.7 MPa, similar values are reported in the literature for other brushite cements [10, 22]. It is also observed that the specimens that present tetracycline and collagen in their formulations, M1 and M4, reached the highest values, followed by those that contain collagen in the formulation (M2 and M5). As expected, the addition of collagen enhances the mechanical properties, this result corresponds to the effect reported in other investigations [4, 13].

Table 2. Compressive strength of the samples.

Samples	Compressive strength of the samples (MPa)
M1	1.7 ± 0.1
M2	1.3 ± 0.3
M3	1.0 ± 0.1
M4	1.4 ± 0.2
M5	0.9 ± 0.2
M6	0.8 ± 0.3

Drug release study

Normally when the release of drugs from biomaterials is studied, the interactions of the drug with the matrix must be taken into account, considering the molar mass of the active principle, its solubility, concentration and the porosity and mechanical properties of the support matrix.

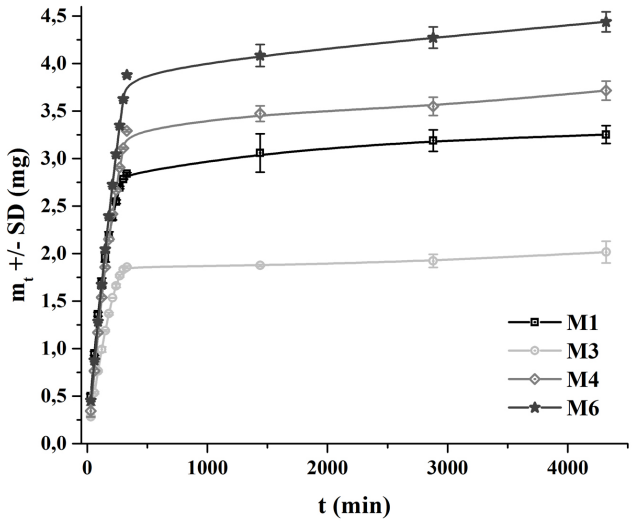


Figure 4. Release profiles of tetracycline. Samples M3 and M4 contain collagen. Samples M4 and M6 contain higher quantities of β-TCP compared to M1 and M3, respectively.

As it can be seen in Figure 4 the sample that released the most was M6, which did not contain collagen but had a higher proportion of *β*-TCP that did not react completely during the setting. When large quantities of *β*-TCP are used, part of it does not form a chemical bond with the crystalline framework and remains among the crystals; considering that this is one of the most soluble calcium phosphates, once in contact with an aqueous medium some gaps may be formed where *β* -TCP particles dissolve increasing the porosity of the cement. The liquid, Ringer's solution in this case, then penetrates easier into the material and extracts the drug. Therefore, when a higher proportion of this compound is introduced into the cement, more gaps are created, which enable the penetration of the liquid and therefore the release of the drug. In the case of samples containing natural polymer (M1 and M4), the material behaves more like a hydrogel that has the capability of retaining the liquid in its structure and regulates the release, therefore samples with collagen present a similar behaviour. However, once again the sample M4, which contains a greater quantity of *β*-TCP, is the one with the highest release because the increase in the porosity created by the *β*-TCP facilitates the drug release.

Calcium phosphate cement's capacity as controlled release systems has been reported to be dependent on diffusion during the first stages in accordance with the amount of drug on the surface layers of the matrices. However, as the study proceeds, the remaining tetracycline in the matrix is found in the most inaccessible areas of the fluid and the release mechanism must involve other processes besides the diffusion to allow the release of the drug.

The release mechanism of the drug of the formulations was studied through a series of mathematical models reported in the literature; the results obtained in the evaluation of the experimental data had a better fit to the logistic curve.

The generalized logistic function or curve, also known as the Richard curve, is a mathematical function that appears in various models of population growth, spread of epidemic diseases and dissemination in social networks. This function constitutes an extension of the sigmoid function for the growth of one magnitude [22], and is considered one of the best options to adjust dissolution curves [23].

The pharmacological variant, given by Eq. (8), was used:

$$Y = A2 + \frac{A1-A2}{1+\left(\frac{x}{x_0}\right)^p} \quad (8)$$

where *Y* is the amount of tetracycline released, *x* is time in minutes, *A*1 is the lower asymptote, *A*2 is the upper asymptote, x_0 is the value of the central node and *p* is the growth rate. Other interpretations, according to Adams et al. [23], lead to the Eq. (9):

$$X(t) = \frac{\alpha}{1+exp[(\beta-t)/\gamma]} \quad (9)$$

*A*1 – *A*2 is considered the *maximum* release percentage (α), β – *t* is *x* being β the time value for α/2, c is the time scale parameter which represents the distance between b and the point where the answer is $\alpha/(1 + e^{-1}) \approx 0.73\alpha$, equivalent to x_0. In this equation, Adams assumes the relationships of time and parameters as the argument of an exponential, while in the classical logistic equation used in our study the *p*-factor is the power, and the time relationships are then the basis of that power. The change in the magnitudes of the values from the mathematical point of view result in a change of the meaning of the parameters from the physical point of view in the pharmacokinetic phenomenon.

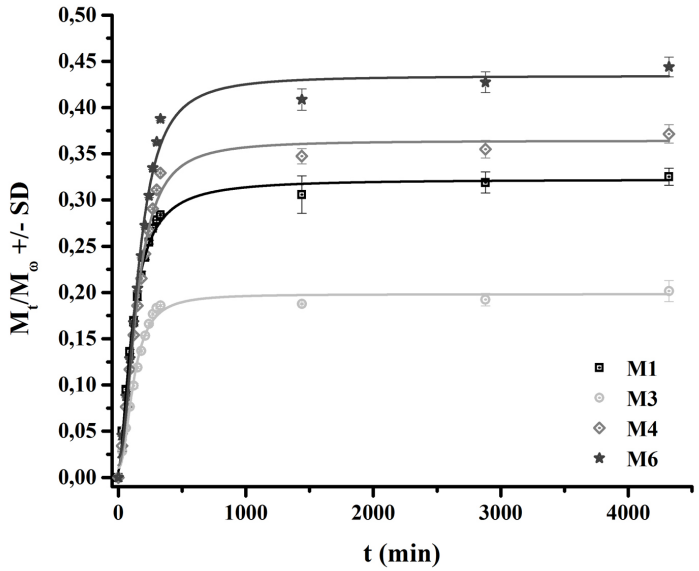


Figure 5. Adjustment to the logistic curve of the release profiles of the tetracycline.

Figure 5 shows the adjustment to the logistic curve of the release profiles. Coefficients of determination greater than 98 % were obtained, which confirms that this model represents the release process with accuracy. The values of the parameters for each sample are given in Table 3. The lower asymptote (*A*1) has values very close to zero, in the case of the superior asymptote (*A*2) the values obtained correspond to the maximum percentage of drug released by each sample. In addition, the values of *p* for each sample are approximately 2.

Table 3. Values of the parameters of the logistic curve for each sample.

Samples	*A*1	*A*2	x_0	*p*	χ^2_{red}	Adj. *R*-Square (%)
M1	0.007 ± 0.007	0.322 ± 0.004	111 ± 5	1.63 ± 0.09	5.310^{-5}	99.47
M3	0.011 ± 0.006	0.198 ± 0.004	120 ± 7	2.1 ± 0.2	6.210^{-5}	98.54
M4	0.013 ± 0.009	0.364 ± 0.007	145 ± 7	2.0 ± 0.2	1.510^{-4}	98.95
M6	0.02 ± 0.01	0.43 ± 0.01	159 ± 9	2.1 ± 0.2	2.910^{-4}	98.59

It can be concluded that the release of tetracycline from the matrices is governed by a mechanism of diffusion in the first hours, mainly due to the drug that is close to the edges of the matrix according to the geometric shape of matrices and the drug solubility. At the second stage, the release not only depends on the solubility of the drug, but also on other mechanisms such as the advance of the release front, the concentration of the drug and the diffusion medium. In this case, the logistic function that fits the release profiles manages to encompass not only the mechanisms of the second stage but also the diffusive mechanisms that govern the first stage, which is why it is able to describe the entire release process.

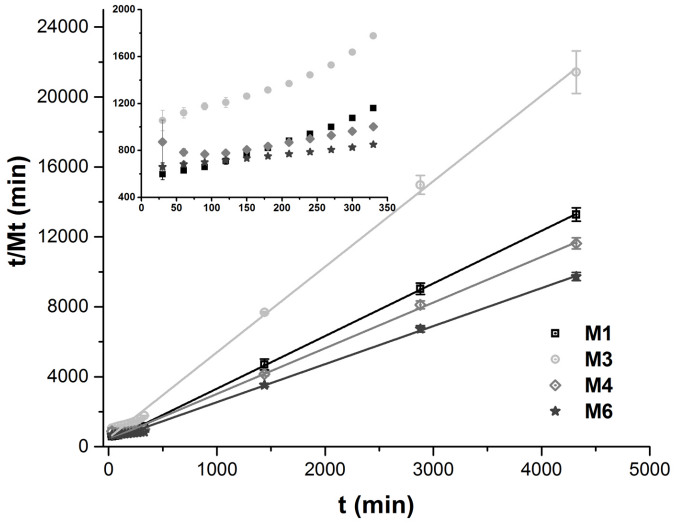


Figure 6. Prediction of the total drug release (M∞).

Another important analysis is the prediction of the total tetracycline released by the sample in an infinite time. For this purpose, the following equation was used:

$$\frac{t}{M_t} = \frac{1}{M_\infty} t + \frac{1}{k_{lib} M_\infty^2} \quad (10)$$

where t is time, M_t is the amount of drug released in time, M_∞ is the final amount of sample released for an infinite time and k_{lib} represents the kinetic constant of release [24]. The results of this analysis are shown in Figure 6 where the adjustment to the equation is observed, and the behaviour in early times (first day) can be seen in the inset. The differences in final tendencies of the sample release capacity are already appreciated after the first four hours; nonetheless, before that time, the samples present almost the same behaviour because the first stage is dependent solely on diffusion and the drug is equally homogeneously distributed on the surface of all samples due to the preparation method. When the release front advances, then it becomes more dependent on the actual composition of the cement and the characteristics arising from the release process such as increased porosity and pore size, the biodegradability of the material and presence of collagen.

Table 4 shows the comparison between the maximum values of release during the study ($A2$) and M_∞, which represents the prediction of the total drug released. The total release values indicate that the sample M6, which exhibited the best performance on release during the study, would be the one that releases the most, 46.0 %. It also, establishes that M3 with only 20.4 % would be the lowest release. In addition, it should be noted that this equation presents an adjustment of more than 99 % to the profiles obtained in the study.

Table 4. Prediction values of M_∞.

Samples	slope	*A2*	M_∞.	R^2 (%)
M1	3.01 ± 0.02	0.322 ± 0.004	0.332 ± 0.003	99.92
M3	4.89 ± 0.06	0.198 ± 0.004	0.204 ± 0.003	99.80
M4	2.61 ± 0.04	0.364 ± 0.007	0.383 ± 0.006	99.68
M6	2.18 ± 0.03	0.430 ± 0.01	0.460 ± 0.007	99.73

Microbiological study

Table 5 shows the final diameters of the inhibition growth halos within 72 h, where there was no bacterial growth due to the release of the drug to the culture medium. The behavior of inhibition on the *Escherichia coli* culture whit time is shown in Figure 7 by the measurements of the halos' diameters at 24, 48 and 72 h. In all cases, the tetracycline effect on the culture can be observed by the inhibition halo, ensuring that the drug preserved the biological activity after the setting of the different compositions cements. The sample with the largest inhibition halo (M6) correspond to the one with highest release of the drug, as expected.

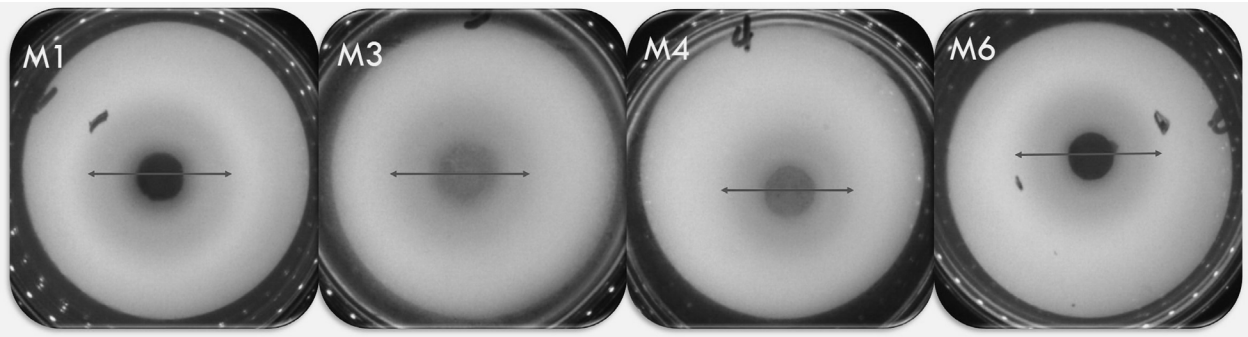


Figure 7. Final diameters of the inhibition halos at 72 h. From left to right, samples M1, M3, M4 and M6 (mm).

Table 5. Diameter of the inhibition growth halos on time.

Time (h)	inhibition halo diameter (mm)			
	M1	M3	M4	**M6**
24	16.3 ± 0.2	13.4 ± 0.2	13.9 ± 0.2	14.2 ± 0.2
48	29.9 ± 0.3	28.9 ± 0.1	27.7 ± 0.2	32.2 ± 0.3
72	43.3 ± 0.2	41.5 ± 0.3	40.1 ± 0.3	47.7± 0.3

Conclusions

Six formulations of a DCPD were prepared, in which a malleable paste that set after about 2 or 3 min was obtained. The addition of collagen was the most relevant variable on the increase of the compressive strength of the samples, which varies between 0.8 and 1.7 MPa, values that allow their application in small maxillofacial defects.

The drug release profiles of tetracycline from the different formulations can be described by the logistic curve and is governed by a mechanism of diffusion in the first hours, while the advance of the release front, which depends on the composition, is more relevant in the second stage. The incorporation of a greater proportion of *β*-TCP increases the release of the tetracycline. The microbiological evaluation of the cements showed their effective antimicrobial activity against strains of *Escherichia coli* in a period of 24 to 72 h.

The proposed CPC is suitable for use as a drug delivery system with bone regenerative capabilities. In addition, the study of the drug release allowed the understanding of the mechanisms of the release phenomenon and could be used to predict the overall release behaviour in a complex CPC matrix with polymeric reinforcement.

References

[1] Dorozhkin, S. V. (2013). Calcium orthophosphate-based bioceramics. Materials, 6(9), 3840-3942.

[2] Dorozhkin, S. V. (2013). Self-setting calcium orthophosphate formulations. Journal of functional biomaterials, 4(4), 209-311.

[3] JZhang, J., Liu, W., Schnitzler, V., Tancret, F., & Bouler, J. M. (2014). Calcium phosphate cements for bone substitution: chemistry, handling and mechanical properties. Acta biomaterialia, 10(3), 1035-1049.

[4] Tamimi, F., Sheikh, Z., & Barralet, J. (2012). Dicalcium phosphate cements: Brushite and monetite. Acta biomaterialia, 8(2), 474-487.

[5] Sheikh, Z., Abdallah, M. N., Hanafi, A. A., Misbahuddin, S., Rashid, H., & Glogauer, M. (2015). Mechanisms of in vivo degradation and resorption of calcium phosphate based biomaterials. Materials, 8(11), 7913-7925.

[6] Ko, C. L., Chen, J. C., Hung, C. C., Wang, J. C., Tien, Y. C., & Chen, W. C. (2014). Biphasic products of dicalcium phosphate-rich cement with injectability and nondispersibility. Materials Science and Engineering: C, 39, 40-46.

[7] Alge, D. L., Goebel, W. S., & Chu, T. M. G. (2012). In vitro degradation and cytocompatibility of dicalcium phosphate dihydrate cements prepared using the monocalcium phosphate monohydrate/hydroxyapatite system reveals rapid conversion to HA as a key mechanism. Journal of Biomedical Materials Research Part B: Applied Biomaterials, 100(3), 595-602.

[8] Ginebra, M. P., Canal, C., Espanol, M., Pastorino, D., & Montufar, E. B. (2012). Calcium phosphate cements as drug delivery materials. Advanced drug delivery reviews, 64(12), 1090-1110.

[9] Vorndran, E., Geffers, M., Ewald, A., Lemm, M., Nies, B., & Gbureck, U. (2013). Ready-to-use injectable calcium phosphate bone cement paste as drug carrier. Acta biomaterialia, 9(12), 9558-9567.

[10] Mirtchi, A. A., Lemaitre, J., & Terao, N. (1989). Calcium phosphate cements: study of the β-tricalcium phosphate—monocalcium phosphate system. Biomaterials, 10(7), 475-480.

[11] O'neill, R., McCarthy, H. O., Montufar, E. B., Ginebra, M. P., Wilson, D. I., Lennon, A., & Dunne, N. (2017). Critical review: Injectability of calcium phosphate pastes and cements. Acta biomaterialia, 50, 1-19.

[12] Engstrand, J., Persson, C., & Engqvist, H. (2014). The effect of composition on mechanical properties of brushite cements. Journal of the mechanical behavior of biomedical materials, 29, 81-90.

[13] O'Hara, R. M., Orr, J. F., Buchanan, F. J., Wilcox, R. K., Barton, D. C., & Dunne, N. J. (2012). Development of a bovine collagen–apatitic calcium phosphate cement for potential fracture treatment through vertebroplasty. Acta biomaterialia, 8(11), 4043-4052.

[14] Hong, Y. J., Chun, J. S., & Lee, W. K. (2011). Association of collagen with calcium phosphate promoted osteogenic responses of osteoblast-like MG63 cells. Colloids and Surfaces B: Biointerfaces, 83(2), 245-253.

[15] Bottino, M. C., Münchow, E. A., Albuquerque, M. T., Kamocki, K., Shahi, R., Gregory, R. L., ... & Pankajakshan, D. (2017). Tetracycline-incorporated polymer nanofibers as a potential dental implant surface modifier. Journal of Biomedical Materials Research Part B: Applied Biomaterials, 105(7), 2085-2092.

[16] Shahi, R. G., Albuquerque, M. T. P., Münchow, E. A., Blanchard, S. B., Gregory, R. L., & Bottino, M. C. (2017). Novel bioactive tetracycline-containing electrospun polymer fibers as a potential antibacterial dental implant coating. Odontology, 105, 354-363.

[17] García Carrodeguas, R., Morejón Alonso, L., García-Menocal, J. A. D., Morejón Alonso, L., Ginebra Molins, M. P., Martínez Manent, S., ... & Planell Estany, J. A. (2003). Hydrothermal method for preparing calcium phosphate monoliths. Materials Research, 6, 395-401.

[18] Peppas, N. A., & Narasimhan, B. (2014). Mathematical models in drug delivery: How modeling has shaped the way we design new drug delivery systems. Journal of Controlled Release, 190, 75-81.

[19] Mirtchi, A. A., Lemaître, J., & Hunting, E. (1989). Calcium phosphate cements: action of setting regulators on the properties of the β-tricalcium phosphate-monocalcium phosphate cements. Biomaterials, 10(9), 634-638.
[20] Barralet, J. E., Grover, L. M., & Gbureck, U. (2004). Ionic modification of calcium phosphate cement viscosity. Part II: hypodermic injection and strength improvement of brushite cement. Biomaterials, 25(11), 2197-2203.
[21] Cuneyt Tas, S.B. Bhaduri. Preparation of Brushite Powders and their in Vitro Conversion to Nanoapatites. In Bioceramics: Materials and Applications V. John Wiley & Sons (2012).
[22] Richards, F. J. (1959). A flexible growth function for empirical use. Journal of experimental Botany, 10(2), 290-301.
[23] Adams, E., Coomans, D., Smeyers-Verbeke, J., & Massart, D. L. (2002). Non-linear mixed effects models for the evaluation of dissolution profiles. International journal of pharmaceutics, 240(1-2), 37-53.
[24] Valles, E., Durando, D., Katime, I., Mendizábal, E., & Puig, J. E. (2000). Equilibrium swelling and mechanical properties of hydrogels of acrylamide and itaconic acid or its esters. Polymer bulletin, 44, 109-114.

Chapter 4: Effect of the addition of alginate and/or tetracycline on brushite cement properties.

This chapter is based on:

Morilla, C.; Perdomo, E.; Hernández, A.K.; Regalado, R.; Almirall, A.; Fuentes, G.; Campos Mora, Y.; Schomann, T.; Chan, A.; Cruz, L.J. *Molecules*, *26*(11), 3272. 2021.

Introduction

Calcium phosphates are biomaterials well known to stimulate bone regeneration and have excellent biocompatibility and bioactivity, which has led to a substantial increase in their use in biomedical applications in the last three decades.

These materials are widely used in various applications of orthopedic and maxillofacial surgery, whether for alveolar ridge augmentation, filling of bone defects, middle ear implants, fusion of spinal vertebrae or in the coating of metal prostheses. They are applied in different ways: as granules, blocks or as cements [1]. Due to their excellent biocompatibility, bioactivity and osteoconductivity, they can be reabsorbed by new bone, by the action of bone cells (osteoclasts and osteoblasts) responsible for bone remodelling [2, 3]. Since the first research conducted in the 1980s [4], calcium phosphate cements (CPC) have attracted significant interest as a bone substitute. Additionally, due to the malleability of CPC, they have the ability to adapt to bone defects and implant sites, and then harden in situ to provide stability and support [5]. Unlike other materials, these biomaterials can repair bone defects permanently [6], promoting the formation of new bone tissue during cement degradation [7] due to their osteoconductivity [8, 9]. In addition, the characteristics of calcium phosphate cements make them an excellent alternative for the release of drugs and other active ingredients, including growth factors and cells [10, 11].

Inorganic CPCs often have critical drawbacks that limit their possible clinical application, including a lack of injectability [12, 13] that is generally characterized by phase separation during injection, low mechanical properties for the loading requirements of the implantation site [14, 15] as well as a weak cohesion that results in the disintegration of the cement paste when in contact with physiological fluids [2, 13].

Brushite cements (dicalcium phosphate dehydrated, DCPD, $CaHPO_4 \cdot 2H_2O$) are prepared by mixing water with a powder consisting of an acid calcium phosphate (monocalcium phosphate monohydrated, MCPM, $CaHPO_4 \cdot H_2O$) and a basic calcium phosphate (β-tricalcium phosphate, β-TCP, β-$Ca_3(PO_4)_2$). The result of this mixture is a mouldable paste that solidifies by an exothermic reaction, forming a hard material. These materials were first described in 1989 by Mirtchi and Lemaître [6, 16, 17].

The first studies performed showed that brushite cements, despite their biocompatibility, are difficult to handle, their setting time is too short (usually less than 30 s) and they have poor mechanical properties [2, 12, 13, 18].

Different additives have been added to these cements to improve some of their properties like injectability [5], cohesion and mechanical properties [19], and the setting time [6]. Polymers have been proven to enhance the mechanical properties of cements due to the role they fulfil in the bone itself, which is a composite material made of an organic phase reinforced with hydroxyapatite crystals. Between the natural polymers, sodium alginate has been studied for many biomedical applications because it is biocompatible, biodegradable and able to form hydrogels. Sodium alginate hydrogels can be prepared under mild conditions by ionic crosslinking and shows a structural similarity to the extracellular matrices of living tissues, which leads to use in applications such as the administration of bioactive agents, the healing of wounds and in tissue engineering [13, 20-26].

Other concerns in maxillofacial surgical operations include the risk of infections that demand the use of antibiotics. A large number of infections in bone implants are caused by bacteria, which are very common in the oral cavity and are related to periodontal diseases. However, antibiotics generally have a negative effect on the mechanical properties of cements due to two effects: the increase in the porosity and the inhibitory effect on the setting reaction [10]. One of the most widely used antibiotics in stomatology is tetracycline, which is known as a very effective antibiotic, with a broad spectrum against bacterial infections, generally related to periodontal diseases [27, 28].

In our present study, several brushite or DCPD bone cements for maxillofacial applications made from MCPM and β-TCP with or without sodium alginate were prepared and evaluated as drug release systems for tetracycline. Although the use of CPC as a drug delivery system has been analysed, the study of more complex formulations that include reinforcement materials, such as sodium alginate, could have a significant impact on the development of more efficient bone regenerative biomaterials with the capability to be used as a drug delivery system and an injectable restoration biomaterial.

Materials and Methods

Cement Preparation

All chemicals employed were of analytical grade, used as received. β-TCP was synthetized by a wet neutralization reaction using CaO and H_3PO_4 (both: Merck KGaA, Darmstadt, Germany), following the method described by Carrodeguas and de Aza [29].

For the preparation of the CPC, MCPM (Merck KGaA, Darmstadt, Germany) and β-TCP were mixed in the solid phase and, depending on the experiment, 2 or 5% of sodium alginate was added to

the liquid phase as a reinforcement. In order to determine their possible uses as a drug release system, 1% tetracycline (Ningxia Qiyuan Pharmaceutical Co., Yinchuan, China) was added as an antibiotic. A solution of sodium citrate was used as a liquid phase of the cements and as a setting retarder. A comparative study to analyse the composition effect on compressive strength, injectability, drug release and antimicrobial activity was carried out. The effect of the independent variables, the quantity of β-TCP used and the addition or not of sodium alginate and/or tetracycline, was studied through the experiments described in Table 1.

Table 1. Experimental design, MCPM/β-TCP = 45/55%. Liquid phase = 0.5 mL/g.

Series	Sodium Alginate (% *w*/*w*)	Tetracycline (% *w*/*w*)
A0T0	0	0
A0T1	0	1
A2T0	2	0
A2T1	2	1
A5T0	5	0
A5T1	5	1

X-ray Diffraction (XRD)

Phase characterization was carried out by means of X-ray diffraction, in a Rigaku Rotaflex, RU200B diffractometer with Cu-K_α radiation (1.54056 nm). The scans were made in a 2 θ angular interval of 10–60° and a scanning speed of 0.02°/min. The results were interpreted using the X'Pert HighScore PANalytical program database, version 3.0 (PANalytical B. V. Almelo, The Netherlands). Crystal size was calculated using the Debye Scherrer tools of the software.

Electronic Microscopy

Scanning Electronic Microscopy (SEM)

The samples were coated with a 20 nm film of gold in a BAL-TEC MED 020 system and placed in a desiccator until analysis. A JEOL microscope, JSM-6360LV (Jeol Ltd., Tokyo, Japan) with Oxford EDX probe (Oxford Instruments, High Wycombe, UK), magnification of 5–300,000, resolution of 3 nm and acceleration voltage of 30 kV, was used for the microstructural analysis.

Transmission Electron Microscopy (TEM)

Samples were diluted in Milli-Q water. Subsequently, carbon-coated grids (Formvar/Carbon on 200 Mesh Copper; AGS162; Van Loenen Instruments; Zaandam, the Netherlands) were glow-discharged using the Emitech K950X Turbo Evaporator (Quorum Technologies; Ashford, UK) at 2 × 20^{-1} mbar and 20 mA for 1 min. Next, 3 µL of sample solution were applied on the freshly glow-discharged grid and allowed to adhere for 1 min. Afterwards, excess liquid was discarded by

blotting onto a filter paper and the sample was air-dried for 10 min. Grids were mounted on a room temperature holder and examined using a FEI T12 Spirit BioTwin (FEI Company; Hillsboro, OR, USA) equipped with an OneView Camera Model 1095 (Gatan; Pleasanton, CA, USA) at a voltage of 120 kV. Digital images were acquired and stored using DigitalMicrograph 3.4 (Gatan, Pleasanton, CA, USA).

Mechanical Characterization

For the compressive strength of the material, 12 mm height and 6 mm diameter specimens were prepared. The samples were immersed in Ringer's solution at 37 °C and tested after 24 h of the cement preparation, immediately after being extracted in order to maintain hydration. The study was carried out in a universal testing machine (TestCom-5, IBERTEST, Madrid, Spain) with a load cell of 200 N and at 1 mm min^{-1} load application speed. The compressive strength (σ_c) in MPa was determined by the following formula:

$$\sigma_c = \frac{F}{A_0} = \frac{4P}{\pi d^2} \cdot 10^{-6} \quad (1)$$

where P is the maximum breaking load (N) and d is the diameter of the specimen (m). Five specimens were tested for each formulation.

Injectability Study

The injectability of the samples was determined by extruding a certain quantity of the paste placed in a commercial plastic syringe of 5 mL capacity and with an exit diameter in the nozzle of 2 mm [30]. The extrusion was performed by placing the syringe in a universal testing machine (TestCom-5, IBERTEST, Madrid, Spain) using a compression speed of 15 mm/min until reaching a maximum load of 100 N [31]:

$$\%Injectability = \frac{\text{mass of injected material}}{\text{total mass of material}} \cdot 100\% \quad (2)$$

Drug Release Study

Test specimens of the cements of 6 mm in height and 12 mm in diameter loaded with tetracycline were used. The samples were immersed in 10 mL of Ringer's solution in glass bottles at (37.0 ± 0.5) °C throughout the study. The solution in contact with the specimens was completely extracted at the established times and replaced with 10 mL of fresh solution. The extractions were made every half hour until 5 h of the cement preparation and, after that, at 24 h up to seven days. Five specimens of each formulation were prepared and evaluated. The determination of the antibiotic released to the solution was carried out in a UV-Visible Spectrophotometer (Shimadzu,

Kyoto, Japan) at a wavelength of 276 nm and the results were reported as a cumulative amount of the tetracycline released versus time [32].

Microbiological Study

For the microbiological study, specimens of 6 mm in height and 12 mm in diameter were prepared. In order to evaluate the antimicrobial susceptibility of the composites, strains of Staphylococcus aureus Agar Tripton were used, at a strain concentration adjusted with a turbidimetric method employing as a reference a 0.5 MacFarland standard (1×10^8 CFU mL^{-1}). Subsequently, 500 µL of a previously prepared culture medium of Mueller-Hinton agar (Merck KGaA, Darmstadt, Germany) was inoculated in Petri dishes. After a period of 20 min the test specimens were placed on top of the plates containing the culture medium and the bacterial suspension and were incubated at 37 ± 1 °C for a period of 72 h. Three specimens were tested for each formulation and the inhibition zone was measured with the software SCAN 500 Automatic Colony Counter Version 6.

pH Study

For the pH study, the samples of 300 mg approximately, were immersed in 10 mL of PBS at 37 °C of temperature and the pH was measure (HI-83300 pH-meter, Hanna Instruments, Woonsocket, RI, USA) over 7 h of the first day and then at the 24 and 96 h.

Cell Viability

MTS assay. To further corroborate the cell viability results, an MTS assay was performed. This is a colorimetric technique in which (3-(4,5-dimethylthiazol-2-yl)-5-(3- arboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), in the presence of phenazine methosulfate (PMS), produces a formazan product that has an absorbance maximum at 490 nm in PBS. Scaffold samples with dimensions like a viability assay were loaded with osteoblastic MC3T3-E1 cells (density: 10^4 per well; 500 µL of cell suspension), and then incubated for 24, 48 and 72 h; 100 µL of the supernatant solution was extracted to a 96-well plate for reading into a tunable, spectrophotometric microplate reader (VersaMax, Molecular Devices, San José, CA, USA with Program Softmax Pro) and the absorbance (λ = 490 nm) was measured.

Statistical Calculations

Graphs and statistics were performed with OriginPro 2021 (OriginLab Corp., Northampton, MA, USA). Data are reported as mean ± standard deviation (SD), unless stated otherwise. Error bars represent the SD calculated from tests of triplicate measurements for each scaffold.

Results

All the cements prepared for our study formed a malleable paste that set in about 2 to 3 min. According to thermochemical studies of calcium phosphate minerals, the dissolution of MCPM in calcium and phosphate ions must be exothermic. In the presence of water, the MCPM tends to hydrolyse in diphosphate and calcium ions following the exothermic reaction of Equation (3). Simultaneously, the exothermic dissolution of β-TCP occurs as a result of its exposure to the acidic medium (Equation (4)). Following the initial dissolution of the reagents, the cement undergoes an increase in pH as a result of the exothermic precipitation of brushite crystals (Equation (5)). Finally, the overall reaction of the cement (Equation (6) = Equation (3) + Equation (4) + 4 × Equation (5)) is exothermic and the brushite cements are usually slightly heated in the final set reaction [6].

$$Ca(H_2PO_4)_2{\cdot}H_2O = Ca^{2+} + 2H_2PO_4^- + H_2O \quad (3)$$

$$\beta\text{-}Ca_3(PO_4)_2 + 4H^+ = 3Ca^{2+} + 2H_2PO_4^- \quad (4)$$

$$Ca^{2+} + H_2PO_4^- + 2H_2O = CaHPO_4{\cdot}2H_2O + H^+ \quad (5)$$

$$\beta\text{-}Ca_3(PO_4)_2 + Ca(H_2PO_4)_2{\cdot}H_2O + 7H_2O = 4CaHPO_4{\cdot}2H_2O \quad (6)$$

It is known that DCPD is a precursor of hydroxyapatite in aqueous solutions, which is thermodynamically more stable, by a dissolution–reprecipitation mechanism. This compound has a relatively low solubility. Therefore, just the presence of water is not enough to trigger the reprecipitation mechanism. However, the aqueous medium in which the DCPD is immersed contains Ca^{2+} ions. In the presence of Ca^{2+} ions, the process occurs according to Equation (7) [33].

$$6\ CaHPO_4{\cdot}2H_2O + (4 - x)\ Ca_3^{2+} = Ca_{10-X}(HPO_4) \times (PO_4)_{6-X}(OH)_{2-X} + (10 + x)\ H_2O + (8 - 2x)\ H^+ \quad (7)$$

X-ray Diffraction (XRD)

Figure 1 shows the XRD pattern of the cement samples A0T0, A0T1, A2T0, A2T1, A5T0 and A5T1 after 72 h of immersion in Ringer's solution. The most significant peaks of the pattern were compared with the XRD pattern of DCPD, which is the expected product of the setting reaction of the cement, using the ICDD PDF 9-0077. A congruence in both position and intensity of the peaks for all samples were observed, which confirms the occurrence of the setting reaction and the precipitation of brushite crystals. It can be observed that the addition of sodium alginate and/or tetracycline do not affect the setting reaction of the cements.

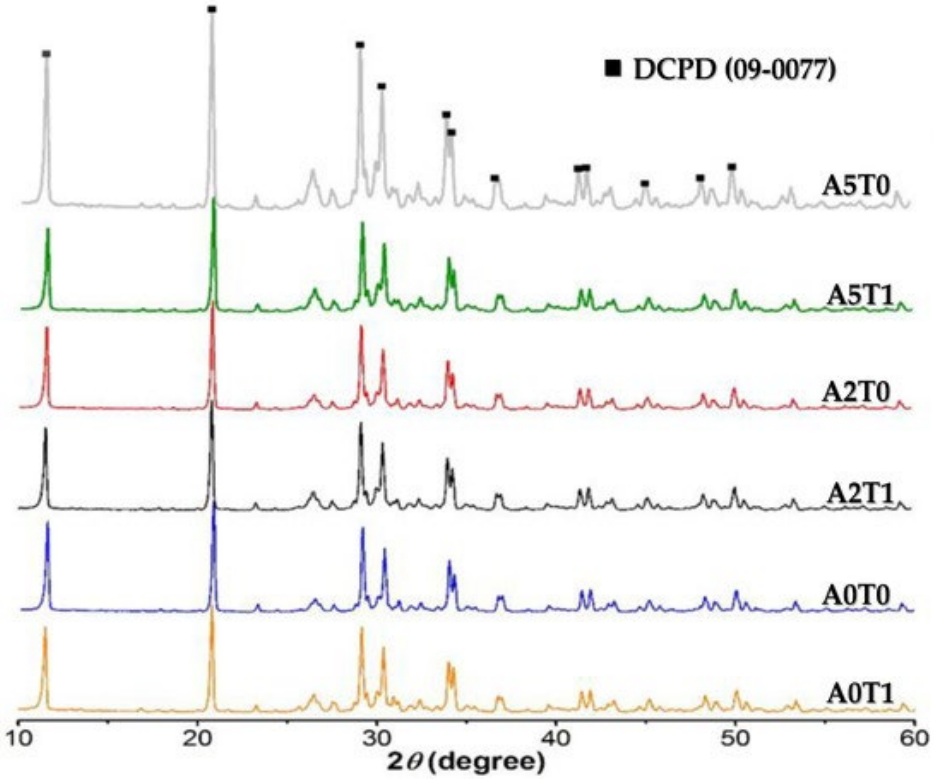


Figure 1. XRD pattern of samples. All peaks correspond to DCPD according to the ICDD PDF 9-0077 X-ray diffraction pattern. The six-maximum intensity DCPD peaks could be observed between 12°and 35° at 2 θ.

Table 2 shows the crystallite size of the cements. The addition of alginate causes a decrease in the size of the crystals while the incorporation of the drug shows the opposite behaviour, but not enough to compensate the effect of the polymer. In none of the cases was there a significant difference between any of the values, whether analysing the variation of sodium alginate or tetracycline.

Table 2. Crystallite size of the cements.

Samples	**A0T0**	**A0T1**	**A2T0**	**A2T1**	**A5T0**	**A5T1**
Crystallite Size (nm)	58 ± 7	57 ± 7	54 ± 4	56 ± 7	50 ± 4	58 ± 7

Morphology by Electronic Microscopy

Scanning Electronic Microscopy (SEM)

In Figure 2, the micrographs of the samples showed mostly the presence of small size pores, although the presence of macropores can also be observed. In the sample A2T0, we can detect that the presence of alginate causes a low porosity due to its uniform distribution among the calcium phosphate crystals. In A5T0, with the increase of the alginate concentration, the number of pores decreased, although there is a presence of a non-uniform and rough surface that was not observed in the A2T0.

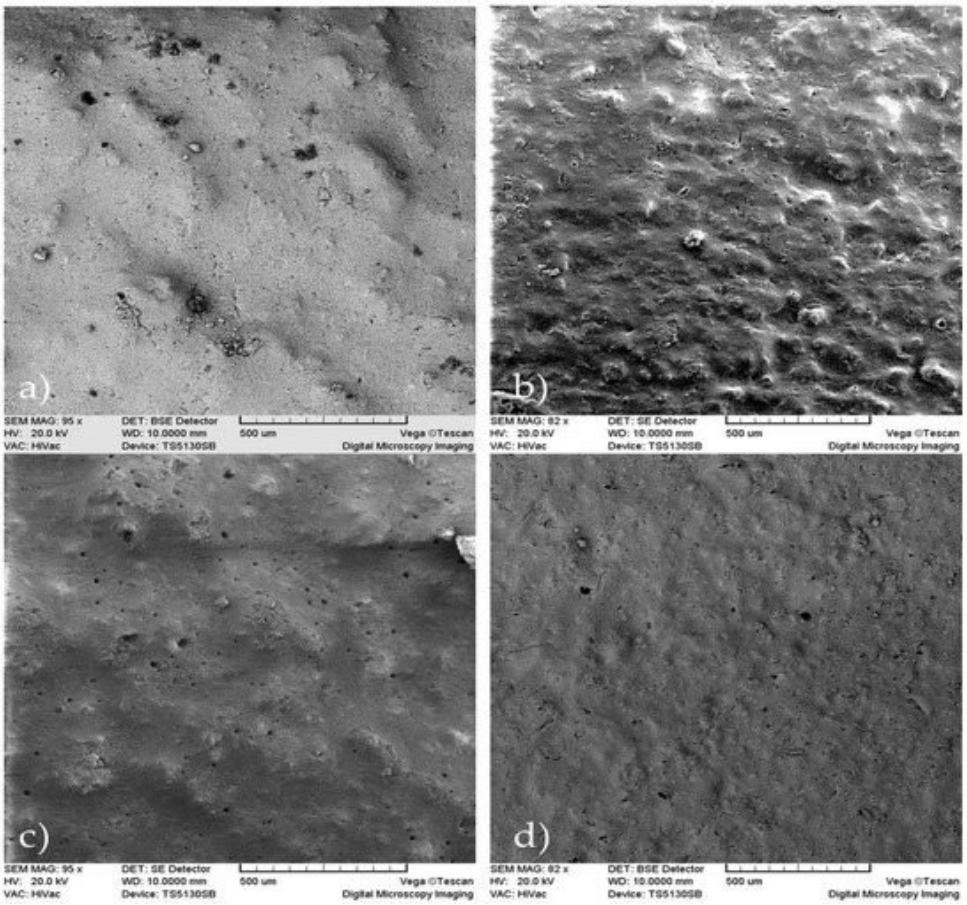


Figure 2. SEM micrographs of the samples: (a) A2T0, (b) A2T1, (c) A5T0 and (d) A5T1, (scale bar = 500 µm).

Comparing samples A2T0 and A2T1, it was determined that the presence of tetracycline causes a superficial uniformity, but at the same time there is an increased presence of macropores and deformities that are not observed in the sample lacking tetracycline. When comparing A5T0 with A5T1, a larger roughness of the surface with the presence of macropores is observed in A5T0 that was not formed in A5T1, which is characterized by a more uniform surface and is devoid of macropores.

In A2T1 and A5T1, both samples with tetracycline and different concentrations of alginate, a superficial roughness can be observed as well as a greater number of macropores in A2T1.

Transmission Electronic Microscopy (TEM)

In Figure 3, the transmission micrographs of the cements show agglomerates of spherical particles of around 50 nm in size. This particle size corresponds with the crystallite size calculated previously (Table 2), and is evidence that increases in the amount of alginate induces a decrease in the size of the crystals. The addition of tetracycline affects the particles size in the opposite way, but the influence is less marked, and cannot counteract the impact of the alginate.

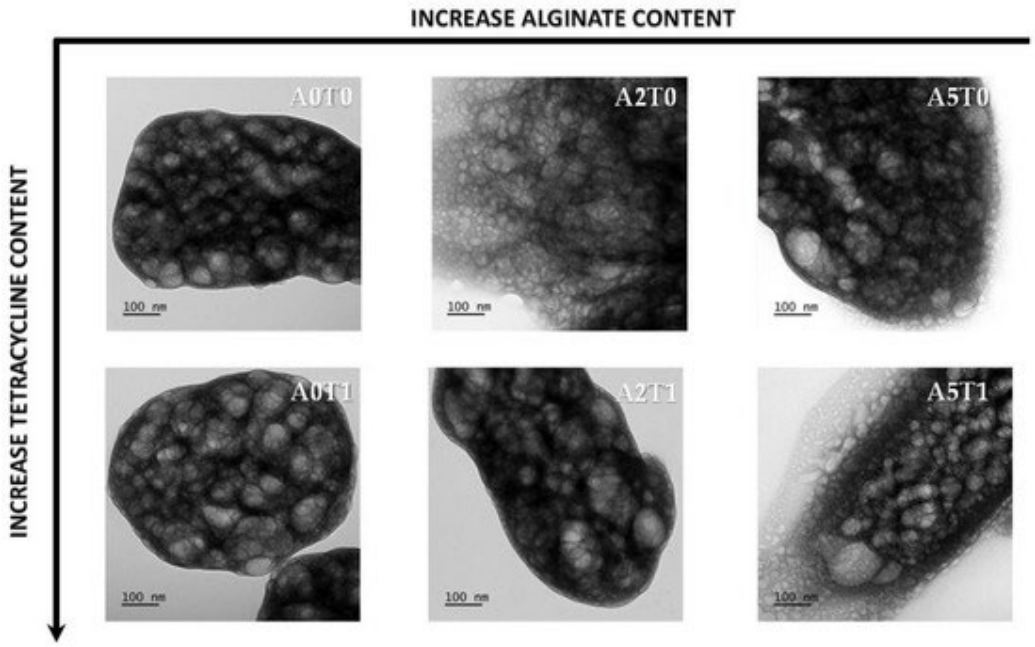


Figure 3. TEM micrographs of all samples at the same magnification.

Mechanical Properties

Table 3 shows the results of the mechanical property (compression strength) evaluation. Values between 1.6 and 2.6 MPa for the compressive strength of the samples were obtained, which corresponds to what is reported in the literature for this type of cement [13, 16, 21, 34].

Table 3. Compression strength (after 72 h of incubation) and injectability of the samples.

Samples	Compressive Strength (MPa)	Injectability (%)
A0T0	1.6 ± 0.5	72.56
A0T1	1.9 ± 0.3	87.07
A2T0	2.6 ± 0.2	41.71
A2T1	1.9 ± 0.5	93.04
A5T0	2.3 ± 0.7	4.78
A5T1	1.9 ± 0.4	93.43

It is also observed that the samples with sodium alginate and without the presence of tetracycline in their formulations (A2T0 and A5T0) were the ones that reached the highest values. As expected, the addition of sodium alginate increases the mechanical properties of the material; this result corresponds with the effect reported in other studies [34].

Injectability

The results of the injectability process are shown in Table 3, where it can be seen that the percentage of injectability ranges between 4.78% and 93.43%. When samples do not contain tetracycline, the addition of sodium alginate does not improve the injectability but makes the cement paste more difficult to handle in the first few minutes and therefore decreases the

injectability. In the case of sample A5T0, this effect is more marked by the high viscosity of the liquid phase that does not allow the preparation of the paste in the time required for the test. Thus, the measurement that is reported was made approximately 40 s later than the rest of the samples. In samples with tetracycline, the addition of sodium alginate improved the injectability.

Drug Release Study

In order to study the drug release mechanisms, the Peppas and Sahlin model [35] was used (Equation (8)). In this equation, the first term shows the fraction of drug release. K_1 is the kinetic constant related to the diffusion process and K_2 to the polymer chain relaxation process, and t is the time of release. The diffusional coefficient n is a measure of the diffusion type for a device of any geometric shape that exhibits a controlled release.

$$\frac{M_t}{M_\infty} = K_1 t^n + K_2 t^{2n} \quad (8)$$

The release profile for the first eight hours (around 75% of the release), as well as the calculated parameters for the Peppas and Sahlin Equation, are shown in Figure 3 and Table 4, respectively. In Figure 4, the inset shows the first eight hours of the release study that is governed by a diffusion mechanism.

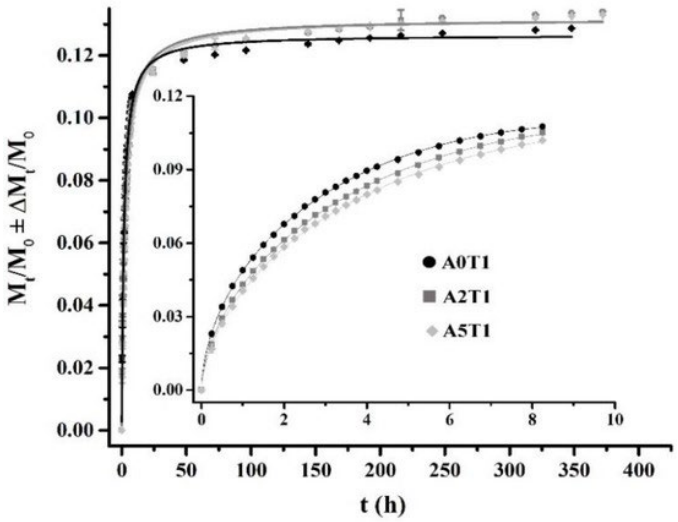


Figure 4. Tetracycline release profiles fitting to Equation (6) (the inset, first 8 h) and Equation (7), from the beginning to the end of the process.

Table 4. Values of the parameters of the Peppas and Sahlin Equation for each sample (inset Figure 3, first eight hours).

Plot	A0T1	A2T1	A5T1
K1	0.0559 ± 0.0001	0.0483 ± 0.0002	0.0452 ± 0.0002
K2	−0.00719 ± 0.00004	−0.00537 ± 0.00005	−0.00482 ± 0.00006
n	0.589 ± 0.003	0.610 ± 0.004	0.620 ± 0.006
R2	99.99%	99.98%	99.97%

It can be observed that as the amount of alginate increases, the system moves away from diffusion (n = 0.5) to enter a process where the diffusion governs in conjunction with the relaxation of the polymer chains (n > 0.5). The increase in the chain relaxation effect is also described by the increment of K_2, which is the part of the equation associated with the relaxation phenomenon, and the decrease of K_1 that represents the diffusion process (Table 4).

In the inset of Figure 4, it can be seen that the formulation A0T1 showed the highest release in that period of time due to the absence of sodium alginate in the sample, which causes a higher surface porosity and therefore facilitates the release of the drug. However, in the remaining days (full graphic, Figure 3) the other two formulations were the ones that were most released, since A0T1 had already expelled the highest amount of tetracycline in the first stages.

The generalized logistic function or curve, also known as the Richard's curve, is a mathematical function that appears in various models of population growth, and the spread of epidemic diseases and dissemination in social networks. This function constitutes an extension of the sigmoid function for the growth of one magnitude [36], and is considered one of the best options to adjust dissolution curves [37]:

$$y = A2 + \frac{A1-A2}{1+(\frac{x}{x_0})^p} \quad (9)$$

where y is the amount of tetracycline released, x is time in minutes, $A1$ is the lower asymptote, $A2$ is the upper asymptote, x_0 is the value of the central node and p is the growth rate.

The values of the parameters for each sample, based on the model of Equation (9), can be seen in Table 5. The asymptote $A1$ shows values very close to zero, while the upper asymptote $A2$ shows values that correspond to the maximum percentage of drug released by each sample. In addition, the p values for each sample are approximately 0.9. Coefficients of determination with a value greater than 99% were obtained for the fitting of all the release curves, which confirms that this model explains more than 99% of the variability in the amount of drug release.

Table 5. Values of the parameters of the logistic curve for each sample (full graphic, Figure 4, 15 days).

Plot	A0T1	A2T1	A5T1
A1	0.003 ± 0.002	0.00162 ± 0.00189	0.00142 ± 0.00144
A2	0.1266 ± 0.0008	0.1319 ± 0.0008	0.1319 ± 0.0006
x0	1.70 ± 0.07	2.27 ± 0.09	2.54 ± 0.08
p	0.98 ± 0.03	0.93 ± 0.03	0.92 ± 0.02
R2	99.58%	99.65%	99.80%

Microbiological Study

Figure 5 shows the inhibition zones where no bacterial growth occurred due to the release of the drug to the culture medium.

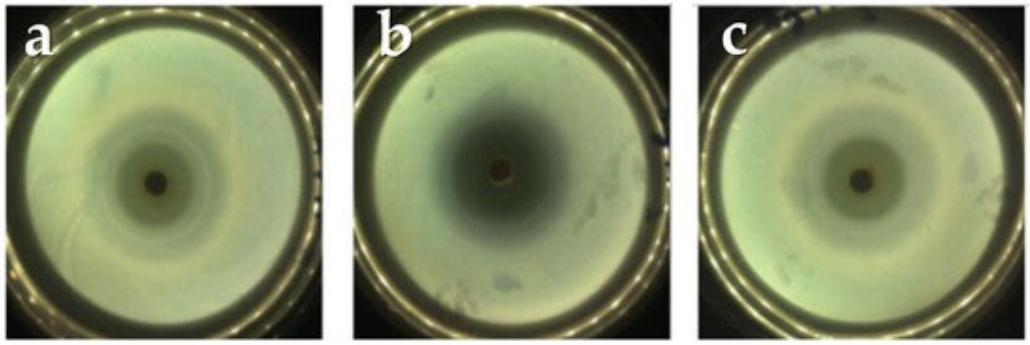


Figure 5. Diameters of the inhibition halos from the A2T1 sample at: (a) 24 h, (b) 48 h and (c) 72 h. The other sample (A5T1) shows a similar behavior.

The measurements of the halos at 24, 48 and 72 h respectively can be observed in Table 6. Here, it is important to notice that the sample A2T1 showed the greatest inhibition halo, which corresponds with the results obtained in the release study.

Table 6. Diameter of the growth inhibition halos (mm) on time.

Samples	24 h	48 h	72 h
A0T1	37 ± 2	37 ± 2	37 ± 2
A2T1	37.0 ± 0.6	38 ± 1	40.1 ± 0.5
A5T1	37.5 ± 0.5	37.6 ± 0.4	37.7 ± 0.3

pH Study

The pH values of the samples over time are shown in Table 7, where it can be observed that in the first minutes of the setting reaction, the pH remains basic but as the reaction progresses the medium acidifies in accordance with Equations (3)–(6). This effect is more significant with the increase in the sodium alginate contents of the formulations.

Table 7. Investigation of pH values of the samples.

t (min)	t (h)	A0T0	A0T1	A2T0	A2T1	A5T0	A5T1
5	0.08	7.24	7.24	7.26	7.22	7.19	7.23
60	1	7.06	6.87	7.13	7.16	7.11	7.13
120	2	6.92	6.37	7.01	6.69	6.84	6.54
185	3	6.84	6.28	6.79	6.63	6.35	6.42
240	4	6.78	5.93	6.74	6.44	5.98	6.33
300	5	6.74	5.81	6.50	6.22	5.70	6.17
360	6	6.72	5.71	6.41	6.18	5.58	6.12
420	7	6.67	5.68	6.36	6.10	5.49	6.08
1440	24	6.60	5.41	6.29	5.89	4.91	5.87
2304	96	6.47	5.01	6.05	5.60	4.74	5.45

Cell Viability Test

Figure 6 displays the cell viability percentages of the samples in the first 72 h of the initial setting. All samples show a percentage above 70, which means that the materials are not cytotoxic according to the standard ISO 10993-5. Although a trend toward improved cell viability was observed upon addition of sodium alginate and tetracycline, statistical significance was confirmed only for the A0T0 vs A0T1 group at 48 h ($p < 0.05$).

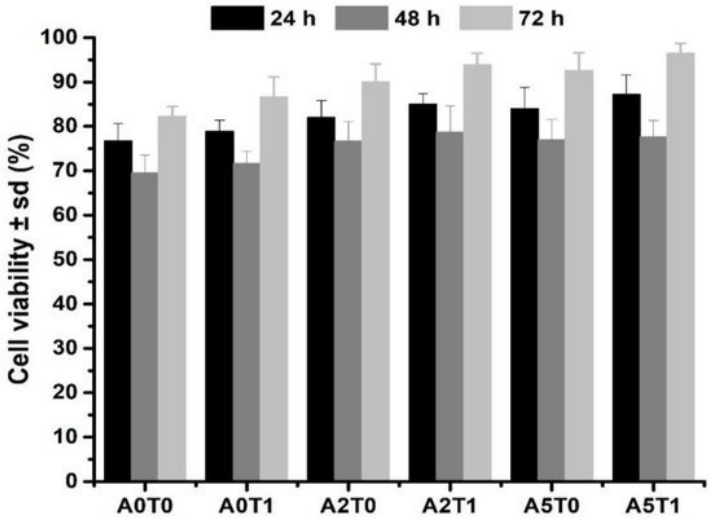


Figure 6. Cell viability assay of the samples at the 24, 48 and 72 h. Statistical analysis (unpaired t-test, $p < 0.05$) was performed between T0 and T1 groups for each alginate level at each time point. A significant difference was observed at 48 h for the A0T0 vs. A0T1 comparison ($p < 0.05$); no other comparisons reached statistical significance.

Discussion

The addition of sodium alginate interferes in the setting reaction, delaying the nucleation of the brushite crystals by making a substitution with the Ca^{2+} ions in the solution. This delay in the initial growth of the crystals causes a marked decrease in the final size of crystallites, as the amount of alginate increases. On the other hand, the presence of the drug also affects the initial growth of the crystals, as explained elsewhere. However, in the presence of alginate, the effect is opposite, but not enough to completely counteract the influence of the drug. The change to a higher value of the cation valence causes an increase in the stability of the alginate complexes that go from being inter- or intramolecular to only being intermolecular at higher valences. Compared with sodium (monovalent cation), the stability of the calcium chelate is remarkably higher than that of sodium, not only for the solubility or viscosity, but several thermodynamic evidences [38, 39].

In this study, we showed that the addition of tetracycline in combination with the alginate causes a decrease in the compressive strength of CPC. Tetracycline tends to form chelates with

Ca^{2+} ions, which causes a delay in the primary nucleation of the crystals [38, 39]. This produces a greater porosity and an increase in setting time, which affects the mechanical properties of cement [10, 16, 21, 23].

The decrease in compressive strength values is expected after 72 h of incubation. The solubility and high hydration capacity of MCPM and the biodegradability of β-TCP are conditioning factors for the low values of mechanical properties, even with the addition of alginate, widely reported and discussed, which should increase them, but competes with the drug's solubility that decreases it [10, 13, 21].

In the samples with tetracycline, the presence of alginate improved the injectability. Samples with a drug as a salt are more injectable, which is also evidence that the presence of antibiotics affects the setting reaction of the cement, causing it to be retarded. This allows the material to be more fluid for a longer period of time, promoting injectability, as has been reported in previous investigations [10, 12, 13].

The release of tetracycline in the matrices is guided by a diffusion mechanism in the first few hours, mainly due to the drug that is close to the edges of the matrix and according to the geometric shapes and the drug solubility. In the case of the samples that contain sodium alginate, the release is controlled by a diffusion mechanism in conjunction with the relaxation of the polymer chains. For a second stage, the release no longer depends only on the solubility of the drug, but also on other factors, such as the advance of the release front, the concentration of the drug and the diffusion medium added. In this case, the logistic function that fits the release profile manages to encompass not only the mechanisms of the second stage but also the diffusion mechanisms that govern the first stage, which is why it is able to describe the entire release process [10, 34, 36, 37, 40].

The effectivity of the cements as drug release systems was proven by the inhibition zones, within which there was no bacterial growth in the culture medium. The results of the microbiological study demonstrated that the antibiotic not only is released, but also maintains its pharmacological activity [34, 41, 42].

The pH study reveals that the increment of hydroxyl groups causes a drop in the pH around the cement due to the presence of alginate. The crosslinking among Ca^{2+} and alginate increases the viscosity of the medium in the first stages. In addition, the Ca^{2+} incorporated in the crosslinking process with alginate releases PO_4^{3-} into the medium, contributing to the possibility of a decrease

of pH values [24, 26, 43]. This is an important factor to take into account when viability tests of cells are made, since it is a determinant for the survival of the cells in the in vitro tests.

The addition of sodium alginate, a biocompatible natural polymer, provides a substrate that promotes cell growth, resulting in an increase in the cell viability [12, 25, 26, 44]. Moreover, tetracycline creates a safe nest, free from bacteria, which can positively affect the cell development in the implant.

Conclusions

Six DCPD formulations were obtained in which the influence of the addition of sodium alginate and/or tetracycline on magnitudes such as mechanical properties, release capacity, injectability, microbiological response and cell proliferation was studied. The addition of sodium alginate caused an increase in mechanical properties and cell proliferation, as well as release in the final stages. The injectability and the pH values decreased, as well the release in the first stage, due to its dependence on diffusion and where the viscosity provided by the sodium alginate interferes with the process.

The addition of tetracycline had less marked effects. In this case, all the magnitudes increased their value except the case of pH, results that agree with the state of the art of the subject. Certainly, when both substances coincided in the formulation, the values of the properties broke the trend, an unequivocal sign of materials science, where magnifying one property leads to sacrificing the values of another. However, the materials obtained proved to be a promising option in the restoration of bone tissue with the added functionality of a controlled drug release system.

References

[1] S. V. Dorozhkin, "Calcium Orthophosphate-Based Bioceramics," *Materials,* vol. 6, pp. 3840-3942, 2013, doi: https://doi.org/0.3390/ma6093840.

[2] M. Le Ferrec *et al.*, "In vivo resorption of injectable apatitic calcium phosphate cements: Critical role of the intergranular microstructure," *Journal of Biomedical Materials Research Part B: Applied Biomaterials,* 2019.

[3] D. K. Mills, "The Role of Polymer Additives in Enhancing the Response of Calcium Phosphate Cement," in *Orthopedic Biomaterials*: Springer, 2018, pp. 345-379.

[4] L. C. Chow and W. E. Brown, "Dental restorative cement pastes," USA Patent Appl. US 06/539,740, 1985.

[5] R. O'Neill *et al.*, "Critical Review: Injectability of Calcium Phosphate Pastes and Cements," *Acta Biomaterialia,* pp. 3-80, 2016. [Online]. Available: http://dx.doi.org/10.1016/j.actbio.2016.11.019.

[6] F. Tamimi, Z. Sheikh, and J. Barralet, "Dicalcium phosphate cements: Brushite and monetite," *Acta Biomaterialia,* vol. 8, no. 2, pp. 474-487, 2012, doi: 10.1016/j.actbio.2011.08.005.

[7] Z. Sheikh, "Mechanisms of in Vivo Degradation and Resorption of Calcium Phosphate Based Biomaterials," *Materials* vol. 8, pp. 7913-7925, 2015.

[8] C.-L. Ko, J.-C. Chen, C.-C. Hung, J.-C. Wang, Y.-C. Tien, and W.-C. Chen, "Biphasic products of dicalcium phosphate-rich cement with injectability and nondispersibility," *Materials Science and Engineering: C,* vol. 39, pp. 40-46, 2014, doi: 10.1016/j.msec.2014.02.033.

[9] D. L. Alge, W. S. Goebel, and T.-M. Gabriel Chu, "In vitro degradation and cytocompatibility of dicalcium phosphate dihydrate cements prepared using the monocalcium phosphate monohydrate/hydroxyapatite system reveals rapid conversion to HA as a key mechanism," *J Biomed Mater Res Part B: Appl Biomater,* vol. 100B, pp. 595-602, 2012, doi: 10.1002/jbm.b.31938/full.

[10] M.-P. Ginebra, C. Canal, M. Espanol, D. Pastorino, and E. B. Montufar, "Calcium phosphate cements as drug delivery materials," *Advanced Drug Delivery Reviews,* vol. 64, pp. 1090-1110, 2012.

[11] E. Vorndran, M. Geffers, A. Ewald, M. Lemm, B. Nies, and U. Gbureck, "Ready-to-use injectable calcium phosphate bone cement paste as drug carrier," *Acta Biomater,* vol. 9, no. 12, pp. 9558-9567, 12// 2013, doi: https://doi.org/10.1016/j.actbio.2013.08.009.

[12] K. Hurle, J. Oliveira, R. Reis, S. Pina, and F. Goetz-Neunhoeffer, "Ion-doped brushite cements for bone regeneration," *Acta Biomaterialia,* vol. 123, pp. 51-71, 2021.

[13] J. Luo, H. Engqvist, and C. Persson, "A ready-to-use acidic, brushite-forming calcium phosphate cement," *Acta biomaterialia,* vol. 81, pp. 304-314, 2018.

[14] X. Cui *et al.*, "Hyaluronic acid facilitates bone repair effects of calcium phosphate cement by accelerating osteogenic expression," *Bioactive Materials,* vol. 6, no. 11, pp. 3801-3811, 2021.

[15] M. Fathi, A. Kholtei, S. E. Youbi, and B. C. El Idrissi, "Setting properties of calcium phosphate bone cement," *Materials Today: Proceedings,* vol. 13, pp. 876-881, 2019.

[16] S. V. Dorozhkin, "Self-setting calcium orthophosphate formulations," *Journal of functional biomaterials,* vol. 4, no. 4, pp. 209-311, 2013.

[17] A. A. Mirtchi, J. Lemaitre, and N. Terao, "Calcium phosphate cements: study of the β-tricalcium phosphate - monocalcium phosphate system," *Biomaterials,* vol. 10, pp. 475-480, 1989, doi: 10.1016/0142-9612(89)90089-6.

[18] H. Guo, M. Pujari-Palmer, Y. Yu, B. Stevensson, H. Engqvist, and M. Eden, "Quantitative phase analyses of biomedical pyrophosphate-bearing monetite and brushite cements by solid-state NMR and powder XRD," *Ceramics International,* vol. 46, no. 8, pp. 11000-11012, 2020.

[19] J. Engstrand, C. Persson, and H. Engqvist, "The effect of composition on mechanical properties of brushite cements," *Journal of the Mechanical Behavior of Biomedical Materials,* vol. 29, pp. 81-90, 2014.

[20] S. Ucar, S. H. Bjørnøy, D. C. Bassett, B. L. Strand, P. Sikorski, and J.-P. Andreassen, "Nucleation and Growth of Brushite in the Presence of Alginate," *Crystal Growth & Design,* vol. 15, no. 11, pp. 5397-5405, 2015.

[21] S. H. Dabiri, A. Lagazzo, B. Aliakbarian, M. Mehrjoo, E. Finocchio, and L. Pastorino, "Fabrication of alginate modified brushite cement impregnated with antibiotic: Mechanical, thermal, and

biological characterizations," *Journal of Biomedical Materials Research Part A,* vol. 107, no. 9, pp. 2063-2075, 2019.
[22] S. M. H. Dabiri, A. Lagazzo, F. Barberis, M. Farokhi, E. Finochio, and L. Pastorino, "Characterization of alginate-brushite in-situ hydrogel composites," *Materials Science and Engineering: C,* vol. 67, pp. 502-510, 2016.
[23] H.-J. Lee, B. Kim, A. R. Padalhin, and B.-T. Lee, "Incorporation of chitosan-alginate complex into injectable calcium phosphate cement system as a bone graft material," *Materials Science and Engineering: C,* vol. 94, pp. 385-392, 2019.
[24] M. Rödel, J. Teßmar, J. Groll, and U. Gbureck, "Highly flexible and degradable dual setting systems based on PEG-hydrogels and brushite cement," *Acta biomaterialia,* vol. 79, pp. 182-201, 2018.
[25] M. Roy, K. DeVoe, A. Bandyopadhyay, and S. Bose, "Mechanical property and in vitro biocompatibility of brushite cement modified by polyethylene glycol," *Materials Science and Engineering: C,* vol. 32, no. 8, pp. 2145-2152, 2012.
[26] L. Schröter, F. Kaiser, S. Stein, U. Gbureck, and A. Ignatius, "Biological and mechanical performance and degradation characteristics of calcium phosphate cements in large animals and humans," *Acta Biomaterialia,* vol. 117, pp. 1-20, 2020.
[27] M. C. Bottino *et al.*, "Tetracycline-incorporated polymer nanofibers as a potential dental implant surface modifier," *Journal of Biomedical Materials Research Part B: Applied Biomaterials,* vol. 105, no. 7, pp. 2085-2092, 2017, doi: https://doi.org/10.1002/jbm.b.33743.
[28] R. G. Shahi, M. T. P. Albuquerque, E. A. Münchow, S. B. Blanchard, R. L. Gregory, and M. C. Bottino, "Novel bioactive tetracycline-containing electrospun polymer fibers as a potential antibacterial dental implant coating," *Odontology,* journal article vol. 105, no. 3, pp. 354-363, July 01 2017, doi: https://doi.org/10.1007/s10266-016-0268-z.
[29] R. G. Carrodeguas and S. De Aza, "α-Tricalcium phosphate: Synthesis, properties and biomedical applications," *Acta biomaterialia,* vol. 7, no. 10, pp. 3536-3546, 2011.
[30] J. Delgado, I. Harr, A. Almirall, S. del Valle, J. A. Planell, and M. Ginebra, "Injectability of a macroporous calcium phosphate cement," *Key engineering materials,* vol. 284, pp. 157-160, 2005.
[31] A. Almirall La Serna, "Desarrollo y caracterización de cementos óseos macroporosos de fosfatos de calcio," Doctor en Ciencias Técnicas, Centro de Biomateriales, Universidad de La Habana, La Habana, 2006.
[32] N. A. Peppas and B. Narasimhan, "Mathematical models in drug delivery: How modeling has shaped the way we design new drug delivery systems," *Journal of Controlled Release,* vol. 190, pp. 75-81, 2014/09/28/ 2014, doi: https://doi.org/10.1016/j.jconrel.2014.06.041.
[33] A. C. Tas and S. B. Bhaduri, "Preparation of brushite powders and their in vitro conversion to nanoapatites," in *Bioceramics: Materials and Applications V: Proceedings of the 106th Annual Meeting of the American Ceramic Society, Indianapolis, Indiana, USA*, 2004: American Chemical Society: Westerville, OH, USA, pp. 119-128.
[34] Y. M. L. Claudia Morilla, Gastón Fuentes, Amisel Almirall, "Synthesis and evaluation of a collagen–brushite cement as a drug delivery system," *International Journal of Materials Research,* vol. 110, 2019.
[35] N. A. Peppas and J. J. Sahlin, "A simple equation for the description of solute release. III. Coupling of diffusion and relaxation," *International journal of pharmaceutics,* vol. 57, no. 2, pp. 169-172, 1989.
[36] F. J. Richards, "A Flexible Growth Function for Empirical Use," *Journal of Experimental Botany,* vol. 10, no. 39, pp. 290-300, 1959, doi: 10.1093/jxb/10.2.290.
[37] E. Adams, "Non-linear mixed effects models for the evaluation of dissolution profiles," *International Journal of Pharmaceutics,* vol. 240, pp. 37-53, 2002, doi: 10.1016/S0378-5173(02)00127-8.
[38] S. El-Shatoury, R. Hassan, and A. Said, "Alginate Polyelectrolyte Ionotropic Gels-XVI. Kinetics and Chemical Equilibria Studies for Heterogeneous Ion Exchange of Polyvalent Metal Ions in Alginate Gel Complexes," *High Performance Polymers,* vol. 4, pp. 173-173, 1992.
[39] R. Hassan, M. T. Makhlouf, A. Summan, and A. Awad, "Influence of frequency on specific conductance of polyelectrolyte gels with special correlation between strength of chelation and stability of divalent metal alginate ionotropic gels," *European polymer journal,* vol. 25, no. 10, pp. 993-996, 1989.

[40] L. J. Reed and J. Berkson, "The application of the logistic function to experimental data," *The Journal of Physical Chemistry,* vol. 33, no. 5, pp. 760-779, 2002.
[41] A. Van Staden, A. Brand, and L. Dicks, "Nisin F-loaded brushite bone cement prevented the growth of Staphylococcus aureus in vivo," *Journal of applied microbiology,* vol. 112, no. 4, pp. 831-840, 2012.
[42] X. Li, G. Li, K. Zhang, Z. Pei, S. Zhao, and J. Li, "Cu-loaded Brushite bone cements with good antibacterial activity and operability," *Journal of Biomedical Materials Research Part B: Applied Biomaterials,* vol. 109, no. 6, pp. 877-889, 2021.
[43] E. Şahin and M. Çiftçioğlu, "Compositional, microstructural and mechanical effects of NaCl porogens in brushite cement scaffolds," *journal of the mechanical behavior of biomedical materials,* vol. 116, p. 104363, 2021.
[44] L. Wang, R. Shelton, P. Cooper, M. Lawson, J. Triffitt, and J. Barralet, "Evaluation of sodium alginate for bone marrow cell tissue engineering," *Biomaterials,* vol. 24, no. 20, pp. 3475-3481, 2003.

Chapter 5: Compositional changes to brushite cements to improve mechanical and antibiotic-delivery properties.

This chapter is based on:

Morilla Espino, C., van der Weerd, L., de Geus-Oei, L. F., & van den Beucken, J. J. (2025). *Open Ceramics*, 100823.

Introduction

The global demand for dental, craniofacial, and orthopedic bone repair and regeneration has grown due to population aging and the increasing incidence of bone diseases and trauma-induced bone defects[1, 2]. For instance, bacterial infections of bone tissue in the form of osteomyelitis or orthopedic implant-related infections account for substantial clinical cases requiring therapeutic interventions annually [3-5]. Especially orthopedic implant-related infections are likely to become a burden in the near future due to the increasing popularity of orthopedic implants and complexity of the involved surgical procedures. In case these bone conditions result in bone defects that do not heal spontaneously, effective treatment strategies are required. For this, the tissue engineering paradigm has emerged as a 'toolbox' to select scaffolds, growth factors, stem cells, or a combination thereof to enhance bone regeneration [6]. Scaffolds, particularly calcium phosphate cements (CPCs), provide a robust scaffold component that can serve as a suitable environment for bone cell migration, proliferation, and differentiation while supporting defect reconstruction and promoting osteogenesis [2]. CPCs are biocompatible, bioactive, injectable, and resorbable, making them especially suitable for complex cranio- and maxillofacial applications where esthetics are critical. Conventional treatments for (local) bone infection extensive debridement combined with prolonged systemic antibiotic administration (via e.g. intravenous injection). However, these treatments often face challenges in terms of complete removal of bacteria and insufficient antibiotic levels at the infection site to effectively kill bacteria [7]. Emerging drug delivery systems utilizing CPCs offer sustained release of therapeutic agents while promoting osteointegration and reducing adverse side effects [7].

CPCs are mainly classified depending on the final product, i.e. apatitic CPCs (aCPCs) or brushite CPCs (bCPCs) [1]. Apatitic CPCs have α-tricalcium phosphate (α-TCP) as the main precursors, and the reaction normally occurs under basic conditions (pH > 4.2) resulting in the formation of apatite with high similarity to bone mineral [8]. However, the degradation of apatitic CPCs in physiological conditions is relatively slow [1, 9]. In contrast, bCPCs have monocalcium phosphate monohydrate (MCPM) and β-tricalcium phosphate (β-TCP) as their main precursors that form dicalcium phosphate dihydrate (DCPD; brushite) as their end-product [9, 10]. Both these types of CPCs have garnered attention for their use in orthopedic and craniofacial surgeries due to their distinctive properties. During their formation process, a CPC paste is obtained that solidifies in seconds to minutes [10]. bCPCs are known for their injectability, self-setting nature, high biocompatibility, osteoconductivity, and resorbability, which make them particularly

suitable for precise applications like craniofacial defect repairs [1, 11]. bCPCs normally form under acidic conditions (pH < 4.2) and at physiological temperature (37°C), making them thermodynamically metastable and appealing for applications requiring material resorption and replacement by new bone [1]. The rapid resorption of bCPCs within the first weeks post-implantation is driven by disintegration, dissolution, and macrophage activity, which facilitates the removal of bCPC crystals and promotes osteoblast-driven bone formation [11].

Despite their appealing premises, bCPCs face several limitations that hinder their broad application. One significant limitation is the short setting time, which limits the time available for surgeons to apply and manipulate the material for adequate bone defect filling during surgical procedures [12]. Furthermore, their injectability is hindered by issues like liquid phase separation during injection [13, 14]. Regarding mechanical properties, their compressive strength of ~1 MPa [10] is much weaker than cortical (~300 MPa; [15]) and even cancellous (~ 1–15 MPa; [16, 17]) bone; this mechanical mismatch limits their application in load-bearing scenarios [18]. To address these challenges, researchers have explored the incorporation of biopolymers such as chondroitin sulphate [19], chitosan [20], gelatine [21], and alginate [22, 23]. These additives, even in small amounts (e.g. less than 1 wt%), have demonstrated improvements in the mechanical properties and anti-washout characteristics of bCPCs [22]. Further advancements include the addition of natural polymers like silk fibroin, which has shown significant potential in enhancing the mechanical properties of bCPCs [24]. Silk fibroin's robust mechanical properties, biocompatibility, and adjustable bioresorbability make it an attractive additive for modifying mechanical properties of bCPCs [24]. Its unique β-sheet structure introduces surface potentials that promote hydroxyapatite (HA) crystallization, mimicking the natural bone environment and improving the molecular, structural, and biological compatibility of the material [24]. In previous work, it was observed that silk fibroin accelerates the transformation of DCPD into HA [25], which could in principle further improve mechanical properties and promote cell growth [26]. More straightforward, also the addition of α-TCP into a bCPC formulation would result in apatite formation and improvements of mechanical properties.

In view of bacterial bone infections, bCPCs are appealing due to their potential as delivery systems for drugs. Their intrinsic micro- and sub-microporous structure makes them appropriate carriers for active therapeutic agents. Consequently, previous work has explored bCPCs for the local delivery of drugs [27], antioxidants [28], growth factors [29], and antibiotics [27], offering a localized and controlled release of these agents [22]. Antibiotics have a pivotal role in the prevention of bacterial infections after any surgical intervention, particularly when applied locally. Notably, the conventional systemic administration of antibiotics has several side effects

and complications. For this reason, utilizing bCPCs as a drug delivery system for antibiotics presents a promising solution, allowing for minimal, localized dosing while reducing systemic exposure [27, 30].

We herein aim to improve the handling and mechanical properties of bCPC by adding α-tricalcium phosphate (α-TCP) and silk fibroin (SF) into bCPC formulations, and explore different bCPC formulations regarding their function as a drug delivery system for the broad-spectrum antibiotic tetracycline (TC).

Materials and Methods

bCPCs preparation

A basic bCPC formulation (monocalcium phosphate monohydrate, MCPM, Sigma-Aldrich, Germany; β-tricalcium phosphate, β-TCP, CaP Biomaterials LLC, United States) was modified by the addition of α-TCP (CaP Biomaterials LLC, United States) in the powder phase. SF was extracted from silk cocoons as described previously [31] and added into the liquid phase, when in liquid form. For the mechanical properties test the SF was also added as microparticles, obtained following the procedure described elsewhere [32], and fibers, obtained after lyophilization [31], and were added in the solid phase of the bCPC. These constituents were used to prepare a range of bCPC formulations (Table 1).

The formulations shown in Table 1 were designed as part of a full factorial experimental setup to systematically evaluate the individual and combined effects of adding α-TCP, SF, and TC on the performance of the bCPCs. Three α-TCP levels (0%, 10%, and 40%) were selected to study its influence on mechanical reinforcement and phase transformation. SF was incorporated at 1.5 wt% [24] based on literature demonstrating its ability to enhance matrix cohesion and strength. TC was added at 50 mg/cm^3 to assess the potential for local antibiotic delivery. The Mβ formulation (lacking α-TCP, SF, and TC) serves as the negative control, while the remaining formulations include all relevant single-variable and combination groups. This design ensures that the impact of each component can be evaluated independently and in synergy. Sample codes follow a consistent logic: "α" refers to α-TCP percentage, "S" to the presence (S1) or absence (S0) of SF, and "T" to the presence (T1) or absence (T0) of TC. For example, α10-S1-T1 represents a formulation with 10% α-TCP, 1.5% SF, and 50 mg/cm^3 TC.

Table 6. Composition of experimental bCPC formulations.

Formulations (L/P=0.35mg/ml of 8wt.% solution of Na_2HPO_4)	***SF proportions (wt.%; added in liquid phase, fibers and microparticles, added in solid phase)***	***Tetracycline proportions (mg/cm^3) (added in liquid phase)***	***Proportions of α-TCP (%) (added in solid phase)***
Mβ	0	0	0
Mβ-S0-T1	0	50	
Mβ-S1-T0	1.50	0	
Mβ-S1-T1	1.50	50	

α10	0	0	10
α10-S0-T1	0	50	
α10-S1-T0	1.50	0	
α10-S1-T1	1.50	50	
α40	0	0	40
α40-S0-T1	0	50	
α40-S1-T0	1.50	0	
α40-S1-T1	1.50	50	

Physico-chemical material characterization

Crystallographic phase characterization was carried out by means of X-ray diffraction, in a Philips X'pert Modular Powder Diffractometer (PANaytical), RU200B diffractometer with Cu-Kα radiation (1.54059 nm). The scans were made in a 2θ angular interval of 10 –40° and scanning speed of 0.02°/min. The results were interpreted using the X'Pert HighScore PANalytical program database, version 3.0 (PANalytical B. V. Almelo, The Netherlands).

Infrared spectra (FTIR) were obtained in a Fourier transform infrared spectrometer Shimadzu IR Tracer 100 (Japan), with a resolution of 4 cm^{-1} and 21 scans per sample in a range of 400-4000 cm^{-1}.

The total porosity of the bCPC formulations was determined by assessing the volume of water contained in the bCPCs pores relative to the total sample volume. The bCPCs were prepared in Teflon cylindric molds of 12 mm height by 6 mm diameter. Once the bCPCs were fully set, the wet samples were weighed. The samples were then completely dried by placing them under vacuum at room temperature for 24 hours. After drying, the samples were weighed again. Porosity was then determined as the ratio of the pore volume (the volume of water removed during drying) to the total sample volume. The pore volume was calculated as the difference between the wet and dry weights, assuming the density of water is 1 g/cm^3. The total volume of the sample was calculated using the formula $\pi r^2 h$. The porosity is then expressed as:

$$Porosity = \frac{Pore\ Volume}{Total\ Volume} = \frac{Wet\ Weight - Dry\ Weight}{Total\ Volume}$$

Handling properties

The setting time of bCPCs was determined using a Gillmore needle protocol according to a voluntary consensus technical international standard (American Society for Testing and Materials; ASTM C266-89). The lighter-weighted needle (100 g in weight and 2 mm in diameter) was used to determine the initial setting time, while the heavy-weighted needle (300 g in weight and 1 mm in diameter) for the final setting time [33]. After the homogeneous paste of bCPC was inserted into the mold, the setting time was quantified by measuring the timepoint when the needles did not make visible indentations anymore in the bCPCs surface.

The injectability of the bCPCs was determined by extruding a certain quantity of the paste placed in a commercial plastic syringe of 3 mL capacity and with an exit diameter in the nozzle of 2 mm. The extrusion was performed by placing the syringe in a universal testing machine (ESM 303, Mark 10, New York, USA) using a compression speed of 15 mm / min until reaching a maximum load of 100 N [34]:

$$Injectability = \frac{\text{mass of injected material}}{\text{total mass of material}} \cdot 100\%$$

Mechanical characterization

The compressive strength of bCPC formulations was assessed as described previously [35]. In brief, samples (12 mm height, 6 mm diameter, n=3) were immersed in water at 37°C and tested after 24h of sample preparation, immediately after being extracted in order to maintain hydration. The study was carried out in a universal testing machine (ESM 303, Mark 10, New York, USA) with a load cell of 200 N at 1 mm min^{-1} load application speed. The compressive strength (σ_c) in MPa was determined by the following formula:

$$\sigma_C = \frac{F}{A_0} = \frac{4P}{\pi d^2} \cdot 10^{-6}$$

where *P* is the maximum breaking load (N) and *d* the diameter of the specimen (m). Three samples were tested for each bCPCs formulation.

Drug release kinetics

bCPC samples (n=3) loaded with tetracycline were immersed in 2 mL of MES buffer (pH 4.6) at 37.0±0.5 °C throughout the study. Samples representing 1 mL of the supernatant were taken and replaced with an equal volume of fresh MES buffer to maintain submerged conditions; sampling was done at 24h, 72h, 120h, 168h and 336h. The concentration of released TC was measured

using a UV-Visible Spectrophotometer SpectraMax® iD3 Multi-Mode Microplate Reader (Molecular Devises, USA) at a wavelength of 276 nm. Results were calculated as cumulative release over time [36].

Antibacterial efficacy

For the evaluation of antibacterial efficacy [37], *Staphylococcus aureus* strain ATCC 29213 Agar Tripton was used at a strain concentration adjusted with a turbidimetric method employing as a reference a 0.5 McFarland standard (1×10^8 CFU mL^{-1}). Subsequently, 200 µL of a previously prepared culture medium of 90922-500G Mueller Hinton Broth 2 agar (Merck) was inoculated in Petri dishes. After a period of 1 hour, the bCPCs samples were placed on top of the plates containing the culture medium and the bacterial suspension and were incubated at 37 ± 1°C for a period of 168h. Three samples were tested for each formulation and the zone of inhibition was measured using APP Interscience scan 500 colony counter (diameters of inhibition zone less than 10 mm were recorded as non-active antibacterial activity [38]).

Statistical analysis

All data are presented as mean ± standard deviation (SD). Statistical analysis was performed using OriginPro 2018 (OriginLab, USA) and GraphPad Prism 8.0.1 (GraphPad Software, USA). Differences between groups were evaluated using one-way ANOVA followed by appropriate post-hoc tests. A p-value < 0.05 was considered statistically significant.

Results

Characterization of constituents, bCPC formulations, and handling properties

SF was used in different forms: dissolved in water, as fibers, and as particles (Figure 1a-c). The liquid SF (Figure 1a) appeared smooth and homogeneous, indicating its suitability for uniform mixing with the bCPCs components, while the SF fibers (Figure 1b) displayed an elongated structure, and the SF microparticles (Figure 1c) appeared irregular. X-ray diffraction (XRD) analysis (Figure 1d) confirmed DCPD (brushite, ICDD PDF 9-0077) as the dominant phase across all bCPC formulations (Mβ, α10, α40). bCPCs exhibited initial setting times between 4 and 7 minutes, and final setting times ranging from 7 to 30 minutes (Figure 1e). The setting time data shows that adding TC and/or SF to the baseline bCPC formulations (Mβ, α10, α40) tends to prolong both initial (Ti) and final (Tf) setting times. FTIR spectra of the bCPCs (Figure 1f) revealed the presence of characteristic phosphate bands $v_3(PO_4^{3-})$ and the double signal of $v_4(PO_4^{3-})$, across all groups, confirming the formation of calcium phosphate phases. Injectability of the bCPC formulations reached values up to 50% (Figure 1g), the α10 group showed significant differences upon addition of TC and SF. Comparisons between α10 and α40 groups also revealed formulation-dependent variability.

Porosity ranged from 39.6% to 49.9% (Figure 1h), and did not differ significantly across groups ($p > 0.05$), suggesting that the addition of SF or TC did not markedly alter the internal microstructure of the set bCPCs.

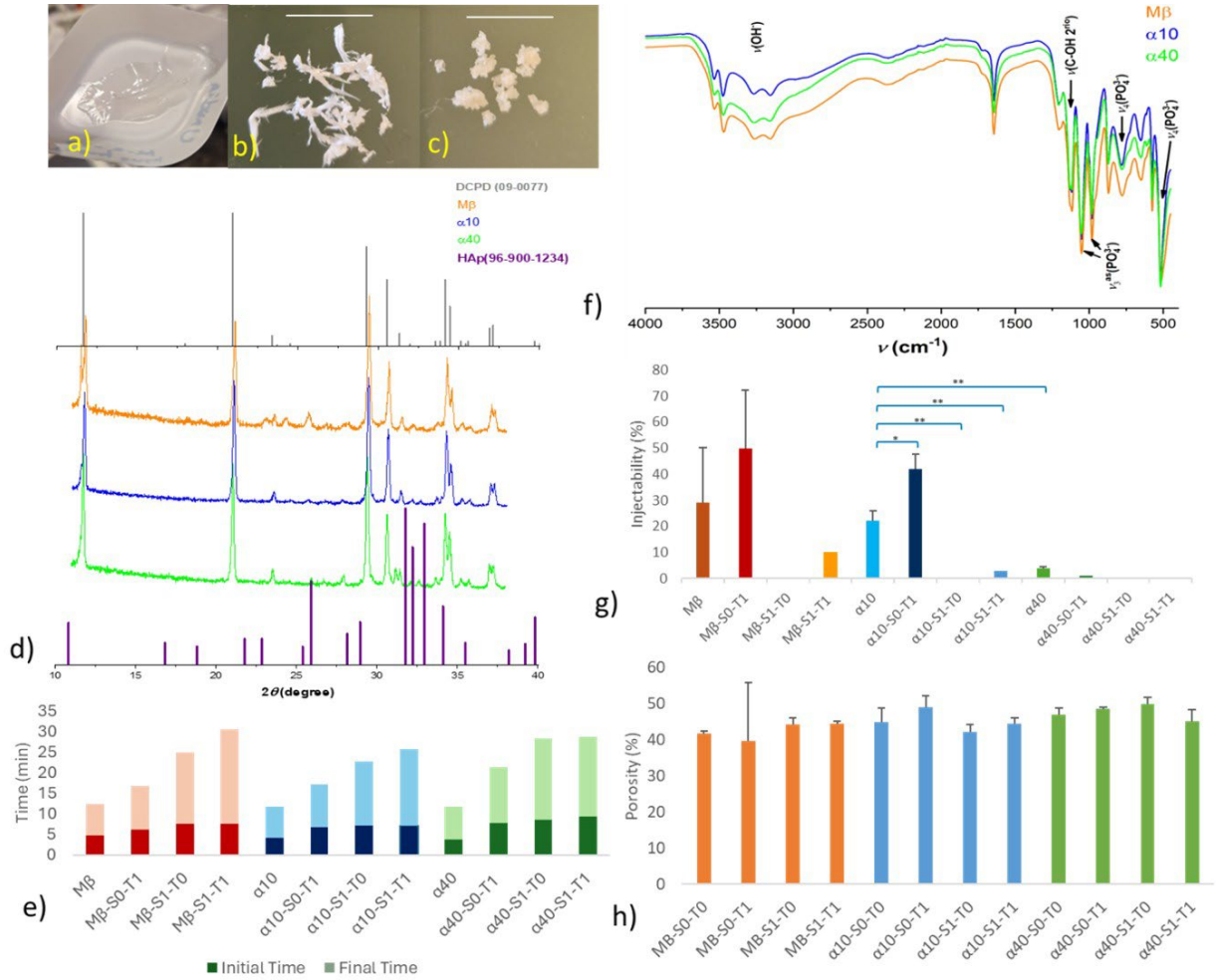


Figure 1. Characterization of constituents, bCPC formulations, and handling properties. *(a) SF liquid. (b) SF fibers (scale bar=1 cm). (c) SF microparticles (scale bar=1 cm). (d) XRD spectra of bCPC formulations and standards (DCPD, ICDD PDF 9-0077; HAp, 96- 900-1234). (e) Initial and final setting times of the bCPC formulations (f). FTIR spectra of the bCPCs (g) Injectability of bCPC formulations (* p<0.05; ** p< 0.01).h) Porosity of the bCPC formulation samples (p>0.05). Statistical significance was determined using one-way ANOVA followed by Tukey's post-hoc test ((* p<0.05; ** p< 0.01).*

Mechanical evaluation of bCPC formulations

Mechanical properties of the bCPC formulations were tested with an experimental set-up (Figure 2a). Macroscopic evaluation of fractured samples (after compressive strength testing) showed presence of SF as fibers or microparticles in the plane of fracture (Figure 2a). The compressive strength of the samples (Figure 2b) showed a range between 0.77 and 2.65 MPa, with statistically significant differences observed in several α10 formulations, were the addition of SF to the samples also containing TC decreased mechanical strength ($p < 0.01$). For the α40 formulations, all additives significantly decreased mechanical strength. Figure 2c displays the correlation between the α-TCP content and the compressive strength. Notably, the inclusion of α-TCP correlated with increased compressive strength. Comparisons between SF forms (Figure 2d)

indicated that SF microparticles reduce compressive strength compared to SF liquid and fibers for α40 formulations, while SF liquid and fibers showed comparable effects on mechanical strength ($p > 0.05$). For Mβ and α10 formulations, no clear effects on mechanical strength were detected upon addition of any type of SF. Overall, the results indicate that α40 formulations outperform Mβ and α10 in terms of compressive strength, and SF microparticles consistently decrease mechanical performance compared to SF liquid and fibers in these formulations.

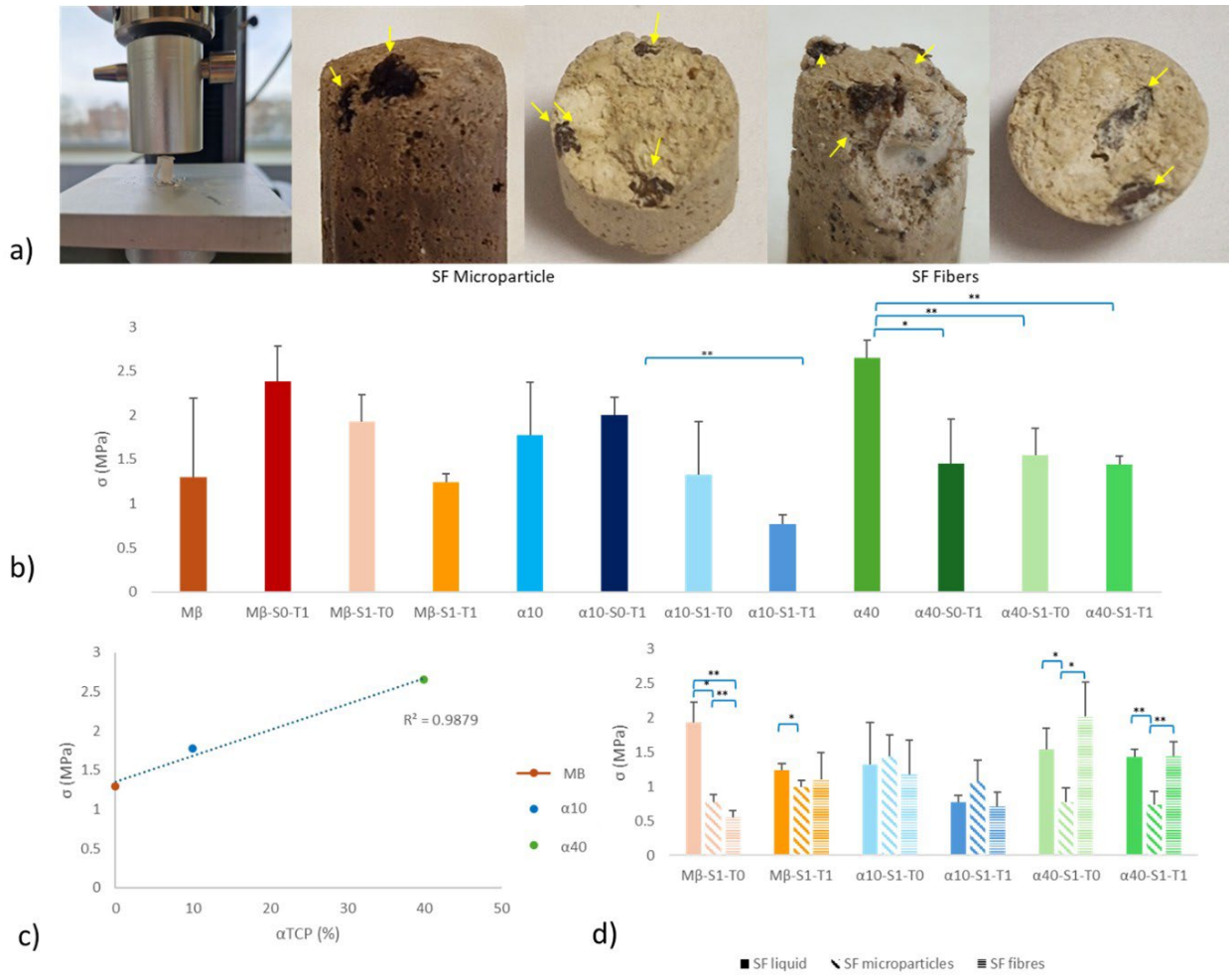


Figure 2. Mechanical properties of bCPC formulations. *(a) Experimental set-up of the test system used for compressive strength evaluation. SF microparticles and fibers were observed at the plane of fracture of samples after compressive strength (yellow arrows). (b) Compressive strength of bCPC formulations (SF in liquid form added in the liquid phase; * = p<0.05; ** = p< 0.01). (c) Compressive strength of bCPC formulations depending on αTCP addition. (d) Compressive strength depending on SF addition in different forms. Statistical significance was determined using one-way ANOVA followed by Tukey's post-hoc test (* p<0.05; ** p< 0.01).*

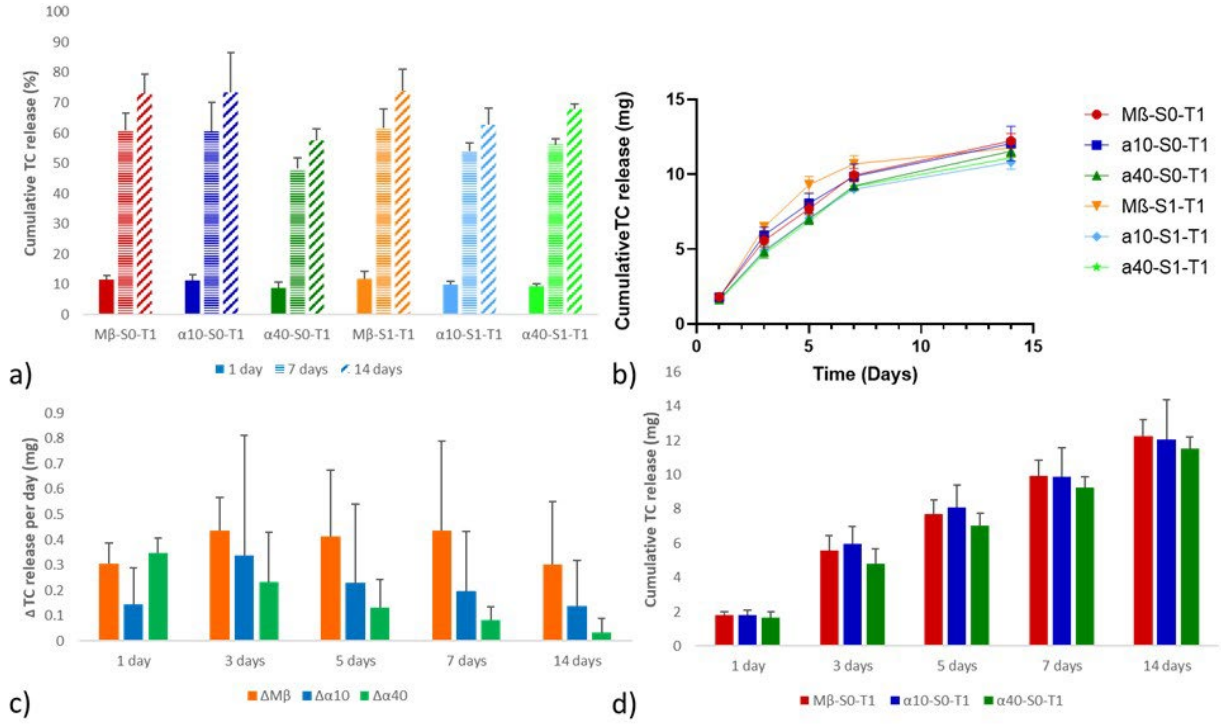


Figure 3. Evaluation of tetracycline release from bCPC formulations. (a) Cumulative TC release (%) after immersion of samples in MES buffer for 1, 7, and 14 days. (b) Cumulative TC release (mg) after immersion of samples in MES buffer for 1, 3, 5, 7, and 14 days. (c) Effects of SF addition on TC release (ΔTC release = release formulation with SF – release formulation without SF; p>0.05). (d) Effects of α-TCP addition on TC release (p>0.05). Statistical analyses were done using a one-way ANOVA followed by Tukey's post-hoc test.

Tetracycline release and antibacterial efficacy

Figure 3a show the cumulative TC release (%) after immersion of samples in MES buffer for 1, 7, and 14 days. On the first day, all samples showed a burst release of approximately 10%. Thereafter, TC release continued to reach approximately 56% in the first 7 days, increasing to approximately 77% cumulative release after 14 days. The release profiles (Figure 3b) show a steady and comparable release profile for all formulations over time. Examination of the influence of SF addition (Figure 3c) indicated no statistically significant differences between groups at any time point (p>0.05). Similarly, the addition of α-TCP (Figure 3d) did not affect TC release across groups (p>0.05).

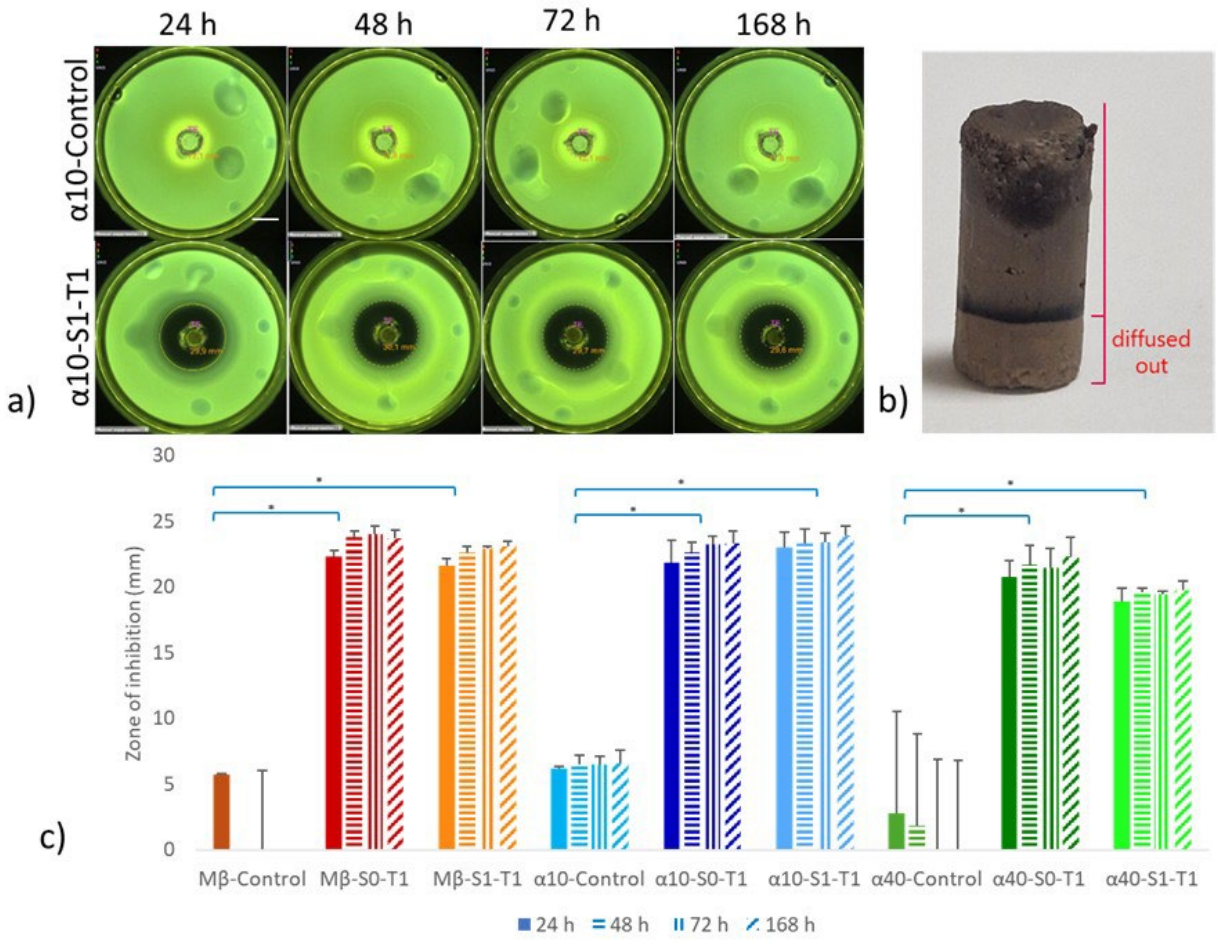


Figure 4. Antibacterial efficacy of bCPC formulations. (a) Representative visual aspect of Zone of Inhibition (ZOI) from α10-S1 samples (+/- TC) over a period of 168h (scale bar=10 mm). (b) Representative α10-S1-T1 sample after 168h showing colour differences along the height of the sample (high in the cylinder the TC remains undiffuse). (c) Quantitative ZOI values against Staphylococcus aureus at 24, 48, 72, and 168 h. Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test. Significant differences between T0 controls and most T1 groups are marked with brackets ($p < 0.05$) in the graph, confirming enhanced antibacterial activity due to TC addition.*

Figure 4 shows the evaluation of antibacterial efficacy of bCPC formulations against *Staphylococcus aureus*. In Figure 4a, the zone of inhibition (ZOI) for α10-S1 samples demonstrates significant antibacterial activity when TC is loaded, with consistent ZOI values observed over time. For α10-S1-T1 samples, the sample cylinder shows a clear line related to TC diffusion, with the top portion of the cylinder visibly darker, indicating undiffused TC (Figure 4b). The ZOI measurements for the bCPC formulations over 24, 48, 72, and 168 hours are displayed in Figure 4c. The controls show negligible ZOI values (<10 mm), confirming no inherent antibacterial effect without TC. In contrast, TC-loaded samples exhibit significantly larger ZOIs (20–25 mm) that remain consistent over time.

Discussion

In this study, we set out to develop a multifunctional bone graft based on brushite calcium phosphate cement (bCPC) that integrates α-tricalcium phosphate (α-TCP) and silk fibroin (SF) with the antibiotic tetracycline (TC) to achieve a balance of mechanical performance and sustained drug release. Our experimental set-up involved characterizing the different forms of SF (liquid, fibers, and microparticles), evaluating setting times, compressive strength, and assessing both drug release profiles and antibacterial efficacy. The main findings were that (i) adding SF and TC significantly prolonged setting times, (ii) adding α-TCP markedly increased compressive strength, and (iii) adding SF in liquid or fiber form reinforced the matrix. For all bCPC formulations, the TC release profile showed an initial burst followed by sustained release over 14 days, and TC-loaded formulations exhibited robust antibacterial efficacy against Staphylococcus aureus.

bCPC formulations clearly showed effects of additives on setting times. Our results indicated that the addition of TC and SF extends both the initial and final setting times of the bCPC formulations. This extended setting time is likely due to TC's ability to chelate calcium ions, thereby delaying brushite crystallization [30, 36]. From a clinical standpoint, these extended setting times can be beneficial, as they allow surgeons more flexibility during implantation [39]. However, overly extended setting times may be impractical in emergency surgical situations, such as cranioplasty [40]. Consequently, specific clinical applications might require individual optimization setting times to strike a balance between workability and efficiency.

Mechanical testing revealed that α-TCP plays a pivotal role in enhancing compressive strength, with bCPC formulations comprising 40 wt% αTCP achieving values up to 2.65 MPa, a 2.65-fold increase over the 1 MPa strength of the unmodified (bare) bCPC [10]. Notably, incorporation of SF in liquid or fiber form resulted in maintained or improved mechanical performance [41, 42], whereas SF microparticles consistently reduced compressive strength—most likely due to irregular shapes acting as stress concentrators [43]. Although prior studies have reported higher compressive strengths for pure α-TCP-based CPC systems [44], the multi-component nature of our bCPC formulations explains the lower absolute values. These observations corroborate recent literature. For instance, Roshanfar *et al.* [41] demonstrated that incorporating electrospun SF fibers into CPC increased compressive strength significantly by bridging microcracks and arresting crack propagation, while discrete polymer particles sometimes served as weak points in the matrix. Similarly, Cassel *et al.* [45] reported that even low concentrations of SF, when well-dispersed, can refine the crystallization of the CPC, resulting in a denser microstructure with enhanced load-bearing capability. These studies support our findings and underscore the

importance of not only the presence but more importantly the form of SF in determining mechanical performance.

TC release profiles demonstrated a predictable burst-sustained release pattern, with approximately 10% release on the first day and up to 77% cumulative release at 14 days. This release profile suggests that the initial burst release is related to diffusion of TC through the intrinsic porosity of the bCPC formulations [27], and corresponds to the Higuchi model [46]. Importantly, neither SF addition nor α-TCP significantly altered TC release kinetics ($p > 0.05$). Antibacterial tests against *Staphylococcus aureus* confirmed the efficacy of the TC-loaded bCPCs, with inhibition zones sustained for up to 168 hours, demonstrating prolonged diffusion of TC out of the cylindrical samples. Comparisons with recent literature further elucidate these findings and bCPCs are known to exhibit burst release due to their high solubility. In studies by Fosca *et al*. [47], pure bCPCs systems were shown to release a majority of their drug payload in a rapid initial phase, whereas incorporating a secondary phase such as HA (or partially converted α-TCP) moderated the burst, shifting the release profile toward a more sustained pattern. Similarly, Rödel *et al*. [21] incorporated gelatin into bCPCs, which transformed the typical burst release into a more prolonged, controlled delivery, following a near-linear release over several days. In work by Dong *et al*. [48], the integration of SF as a hydrogel network moderated the initial burst, allowing for sustained release without compromising mechanical integrity. Our findings align with literature reporting that while bCPC composition impacts initial burst magnitude, the microstructural porosity ultimately controls sustained drug diffusion [23, 30].

Collectively, these results indicate that SF (particularly in liquid or fiber form) and α-TCP can be effectively combined within bCPC matrices to improve both mechanical integrity and therapeutic functionality without negatively affecting drug release behaviour. The observed improvements in compressive strength as well as the predictable, sustained drug release provide a robust foundation for further optimization of bCPC-based drug delivery systems. However, several challenges remain. First, our experiments were conducted exclusively in vitro, which does not fully capture the complexities of the in vivo environment, such as variations in fluid dynamics, local tissue interactions, long-term biodegradation behaviour, and effects of proteins in bodily fluids. In addition, although our data demonstrate clear effects of SF morphology on mechanical properties, the current study does not fully elucidate the optimal size, shape, or distribution of SF microparticles to minimize stress concentration without sacrificing their bioactive benefits. Finally, additional drug-loading strategies should be explored to further enhance and tailor the antibiotic release profile for different clinical scenarios. By integrating these strategies and

leveraging recent advances in multifunctional bCPCs, future research can pave the way for clinically effective, economically viable materials for bone regeneration.

Conclusions

Our study demonstrates that bCPC systems are promising multifunctional platforms for bone regeneration, effectively balancing mechanical strength, injectability, and sustained antibiotic delivery. Incorporating SF (in liquid or fiber form) and α-TCP allows for tuneable setting times and improved compressive strength without compromising porosity or drug diffusion. These effects of integrating these additives in bCPC provide a robust foundation for optimizing bCPC formulations for clinical applications in bone repair. Future studies should focus on in vivo validation, long-term degradation, and advanced drug-loading strategies to expand the therapeutic reach of these biomaterials.

References

[1] K. Hurle, J. Oliveira, R. Reis, S. Pina, and F. Goetz-Neunhoeffer, "Ion-doped brushite cements for bone regeneration," *Acta Biomaterialia,* vol. 123, pp. 51-71, 2021.

[2] Y. Lin *et al.*, "Calcium phosphate cement scaffold with stem cell co-culture and prevascularization for dental and craniofacial bone tissue engineering," *Dental Materials,* vol. 35, no. 7, pp. 1031-1041, 2019.

[3] H. M. Kremers, M. E. Nwojo, J. E. Ransom, C. M. Wood-Wentz, L. J. Melton III, and P. M. Huddleston III, "Trends in the epidemiology of osteomyelitis: a population-based study, 1969 to 2009," *JBJS,* vol. 97, no. 10, pp. 837-845, 2015.

[4] E. Witso, "The rate of prosthetic joint infection is underestimated in the arthroplasty registers," vol. 86, ed: Taylor & Francis, 2015, pp. 277-278.

[5] J. Schimmel, P. Horsting, M. De Kleuver, G. Wonders, and J. Van Limbeek, "Risk factors for deep surgical site infections after spinal fusion," *European Spine Journal,* vol. 19, pp. 1711-1719, 2010.

[6] R. Langer, "I articles," *Science,* vol. 260, p. 5110, 1993.

[7] M. Gallarate, D. Chirio, G. Chindamo, E. Peira, and S. Sapino, "Osteomyelitis: focus on conventional treatments and innovative drug delivery systems," *Current Drug Delivery,* vol. 18, no. 5, pp. 532-545, 2021.

[8] P. Arokiasamy *et al.*, "Synthesis methods of hydroxyapatite from natural sources: A review," *Ceramics International,* vol. 48, no. 11, pp. 14959-14979, 2022.

[9] C. M. Espino, G. F. Estévez, L. van der Weerd, L.-F. de Geus-Oei, and J. J. van den Beucken, "Innovations for Brushite Cements toward applications in Bone Regeneration and Drug Delivery," *Ceramics International,* 2024.

[10] A. A. Mirtchi, J. Lemaitre, and N. Terao, "Calcium phosphate cements: study of the b-tricalcium phosphate - monocalcium phosphate system," *Biomaterials,* vol. 10, pp. 475-480, 1989, doi: 10.1016/0142-9612(89)90089-6.

[11] R. Jayasree, T. S. Kumar, R. Venkateswari, R. P. Nankar, and M. Doble, "Eggshell derived brushite bone cement with minimal inflammatory response and higher osteoconductive potential," *Journal of Materials Science: Materials in Medicine,* vol. 30, pp. 1-14, 2019.

[12] A. C. de Franca Silva Azevedo *et al.*, "Brushite bone cement containing polyethylene glycol for bone regeneration," *Bio-Medical Materials and Engineering,* vol. 33, no. 3, pp. 221-233, 2022.

[13] S. V. Dorozhkin, "Self-Setting Calcium Orthophosphate Formulations," *J. Funct. Biomater,* vol. 4, no. 4, pp. 209-311, 2013, doi: https://doi.org/10.3390/jfb4040209.

[14] A. T. Saleh, L. S. Ling, and R. Hussain, "Injectable magnesium-doped brushite cement for controlled drug release application," *Journal of materials science,* vol. 51, no. 16, pp. 7427-7439, 2016.

[15] T. Weerasooriya, B. Sanborn, C. A. Gunnarsson, and M. Foster, "Orientation dependent compressive response of human femoral cortical bone as a function of strain rate," *Journal of Dynamic Behavior of Materials,* vol. 2, pp. 74-90, 2016.

[16] C. E. Dunham, S. E. Takaki, J. A. Johnson, and C. E. Dunning, "Mechanical properties of cancellous bone of the distal humerus," *Clinical Biomechanics,* vol. 20, no. 8, pp. 834-838, 2005.

[17] L.-C. Gerhardt and A. R. Boccaccini, "Bioactive glass and glass-ceramic scaffolds for bone tissue engineering," *Materials,* vol. 3, no. 7, pp. 3867-3910, 2010.

[18] Q. Liu, W. F. Lu, and W. Zhai, "Toward stronger robocast calcium phosphate scaffolds for bone tissue engineering: A mini-review and meta-analysis," *Biomaterials Advances,* vol. 134, p. 112578, 2022.

[19] F. Tamimi-Mariño, J. Mastio, C. Rueda, L. Blanco, and E. López-Cabarcos, "Increase of the final setting time of brushite cements by using chondroitin 4-sulfate and silica gel," *Journal of materials Science: Materials in medicine,* vol. 18, pp. 1195-1201, 2007.

[20] S. Rattanachan, C. Lorprayoon, and P. Boonphayak, "Synthesis of chitosan/brushite powders for bone cement composites," *Journal of the Ceramic Society of Japan,* vol. 116, no. 1349, pp. 36-41, 2008.

[21] M. Rödel, J. Teßmar, J. Groll, and U. Gbureck, "Dual setting brushite—gelatin cement with increased ductility and sustained drug release," *Journal of Biomaterials Applications,* vol. 36, no. 10, pp. 1882-1898, 2022.

[22] S. H. Dabiri, A. Lagazzo, B. Aliakbarian, M. Mehrjoo, E. Finocchio, and L. Pastorino, "Fabrication of alginate modified brushite cement impregnated with antibiotic: Mechanical, thermal, and biological characterizations," *Journal of Biomedical Materials Research Part A,* vol. 107, no. 9, pp. 2063-2075, 2019.
[23] C. Morilla *et al.*, "Effect of the addition of alginate and/or tetracycline on brushite cement properties," *Molecules,* vol. 26, no. 11, p. 3272, 2021.
[24] J. B. Cassel, M. C. Tronco, T. C. Paim, M. R. Wink, and L. A. dos Santos, "Reinforcement of injectable premixed α-tricalcium phosphate cements with silk fibroin solutions," *Materials Today Communications,* vol. 38, p. 108440, 2024.
[25] X.-D. Sun, Y.-L. Zhou, J.-Y. Ren, and F.-Z. Cui, "Effect of pH on the fibroin regulated mineralization of calcium phosphate," *Current Applied Physics,* vol. 7, pp. e75-e79, 2007.
[26] R. Shi *et al.*, "Extrusion printed silk fibroin scaffolds with post-mineralized calcium phosphate as a bone structural material," *International Journal of Bioprinting,* vol. 8, no. 4, 2022.
[27] M.-P. Ginebra, C. Canal, M. Espanol, D. Pastorino, and E. B. Montufar, "Calcium phosphate cements as drug delivery materials," *Advanced Drug Delivery Reviews,* vol. 64, pp. 1090-1110, 2012.
[28] S. Pujari-Palmer, X. Lu, V. P. Singh, L. Engman, M. Pujari-Palmer, and M. K. Ott, "Incorporation and delivery of an organoselenium antioxidant from a brushite cement," *Materials Letters,* vol. 197, pp. 115-119, 2017.
[29] R. Reyes, B. De la Riva, A. Delgado, A. Hernandez, E. Sanchez, and C. Evora, "Effect of triple growth factor controlled delivery by a brushite–PLGA system on a bone defect," *Injury,* vol. 43, no. 3, pp. 334-342, 2012.
[30] Y. M. L. Claudia Morilla, Gastón Fuentes, Amisel Almirall, "Synthesis and evaluation of a collagen–brushite cement as a drug delivery system," *International Journal of Materials Research,* vol. 110, 2019.
[31] D. N. Rockwood, R. C. Preda, T. Yücel, X. Wang, M. L. Lovett, and D. L. Kaplan, "Materials fabrication from Bombyx mori silk fibroin," *Nature protocols,* vol. 6, no. 10, pp. 1612-1631, 2011.
[32] A. Nisal *et al.*, "Silk fibroin micro-particle scaffolds with superior compression modulus and slow bioresorption for effective bone regeneration," *Scientific reports,* vol. 8, no. 1, p. 7235, 2018.
[33] Z. Wang *et al.*, "Dual-functional porous and cisplatin-loaded polymethylmethacrylate cement for reconstruction of load-bearing bone defect kills bone tumor cells," *Bioactive Materials,* vol. 15, pp. 120-130, 2022.
[34] C. Persson and S. Berg, "Strategies towards injectable, load-bearing materials for the intervertebral disc: a review and outlook," *Journal of Materials Science: Materials in Medicine,* vol. 24, pp. 1-10, 2013.
[35] S. Gallinetti, C. Canal, and M. P. Ginebra, "Development and characterization of biphasic hydroxyapatite/β-TCP cements," *Journal of the American Ceramic Society,* vol. 97, no. 4, pp. 1065-1073, 2014.
[36] F. Tamimi *et al.*, "Doxycycline sustained release from brushite cements for the treatment of periodontal diseases," *Journal of Biomedical Materials Research Part A: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials and the Korean Society for Biomaterials,* vol. 85, no. 3, pp. 707-714, 2008.
[37] R. Salomoni, P. Léo, A. Montemor, B. Rinaldi, and M. Rodrigues, "Antibacterial effect of silver nanoparticles in Pseudomonas aeruginosa," *Nanotechnology, science and applications,* pp. 115-121, 2017.
[38] E. M. Tekwu, A. C. Pieme, and V. P. Beng, "Investigations of antimicrobial activity of some Cameroonian medicinal plant extracts against bacteria and yeast with gastrointestinal relevance," *Journal of ethnopharmacology,* vol. 142, no. 1, pp. 265-273, 2012.
[39] B. van Oirschot, A. G. Mikos, Q. Liu, J. J. van den Beucken, and J. A. Jansen, "Fast Degradable Calcium Phosphate Cement for Maxillofacial Bone Regeneration," *Tissue Engineering Part A,* vol. 29, no. 5-6, pp. 161-171, 2023.
[40] P. D. Costantino *et al.*, "Applications of fast-setting hydroxyapatite cement: cranioplasty," *Otolaryngology—Head and Neck Surgery,* vol. 123, no. 4, pp. 409-412, 2000.
[41] F. Roshanfar *et al.*, "Reinforcement of calcium phosphate cement with hybrid silk fibroin/kappa-carrageenan nanofibers," *Biomedicines,* vol. 11, no. 3, p. 850, 2023.
[42] B. Wang, R. J. Xie, Q. Wan, Y. Wang, and Y. Y. Huang, "Effect of silk fibroin on the properties of calcium phosphate cement," *Advanced Materials Research,* vol. 175, pp. 100-104, 2011.

[43] A. Ayyar and N. Chawla, "Microstructure-based modeling of crack growth in particle reinforced composites," *Composites Science and technology,* vol. 66, no. 13, pp. 1980-1994, 2006.
[44] I. Lodoso-Torrecilla *et al.*, "Multimodal porogen platforms for calcium phosphate cement degradation," *Journal of Biomedical Materials Research Part A,* vol. 107, no. 8, pp. 1713-1722, 2019.
[45] J. B. Cassel, M. C. Tronco, B. A. de Melo, F. d. S. de Oliveira, and L. A. Dos Santos, "α-Tricalcium phosphate cement reinforced with silk fibroin: A high strength biomimetic bone cement with chloride-substituted hydroxyapatite," *Journal of the Mechanical Behavior of Biomedical Materials,* vol. 143, p. 105936, 2023.
[46] D. Paul, "Elaborations on the Higuchi model for drug delivery," *International journal of pharmaceutics,* vol. 418, no. 1, pp. 13-17, 2011.
[47] M. Fosca, J. V. Rau, and V. Uskoković, "Factors influencing the drug release from calcium phosphate cements," *Bioactive Materials,* vol. 7, pp. 341-363, 2022.
[48] Z. Dong, D. Wu, H. Engqvist, J. Luo, and C. Persson, "Silk fibroin hydrogels induced and reinforced by acidic calcium phosphate–A simple way of producing bioactive and drug-loadable composites for biomedical applications," *International Journal of Biological Macromolecules,* vol. 193, pp. 433-440, 2021.

Chapter 6: SUMMARY AND DISCUSSION

Summary

Bone regeneration remains a critical challenge in clinical orthopaedics and dentistry, requiring the development of biomaterials that combine structural support with bioactivity and drug delivery capabilities to expedite the clinical availability of synthetic bone graft materials. Brushite calcium phosphate cements (bCPCs) have emerged as promising bone graft candidates due to their biocompatibility, bioresorbability, and ability to serve as localized drug delivery systems. bCPCs are prepared from an acidic calcium phosphate and a basic one, which upon mixing with a liquid phase form a mouldable paste that easily adapt to the bone defect shape. Furthermore, bCPCs can be injected, which facilitates the use of minimally-invasive surgical techniques. However, optimizing the mechanical properties, handling characteristics, and controlled release mechanisms of bCPCs is essential to enhance their clinical applicability and efficacy.

To address these limitations, this thesis aimed to enhance the mechanical and handling properties of bCPCs, and evaluate the effectiveness of bCPCs as a local antibiotic delivery system, focusing on their potential for improved clinical applications in bone regeneration therapies.

Chapter 1 offered an overview of the current state-of-the-art for the different limitations of bCPCs and the possible strategies for their improvement. The subsequent chapters each addressed distinct research questions and the obtained results to answer these. In brief, these research questions, the experimental results toward answering these, and the conclusions are summarized below:

1) How can the synergy between bCPCs and drug delivery mechanisms be optimized to advance bone regeneration therapies and improve clinical outcomes? *(Chapter 2)*

The synergy between bCPCs and drug delivery mechanisms can be optimized to advance bone regeneration therapies and improve clinical outcomes by addressing key challenges, while leveraging their unique advantages. bCPCs are highly valued for their biocompatibility, bioactivity, and rapid resorption rates, which facilitate integration with host tissue and enhance long-term (complete) regeneration. However, challenges such as rapid setting times, suboptimal mechanical properties, and limited handling characteristics must be mitigated to fully unlock their clinical potential. Strategies like the use of setting retarders to modulate hardening and the integration of polymers to improve mechanical strength and injectability have shown promise in overcoming these limitations.

Additionally, bCPCs' capability as localized drug delivery systems offers a significant advantage in addressing post-surgical complications, particularly bacterial infections. By

enabling targeted antibiotic release directly at the surgical site, bCPCs reduce the need for systemic antibiotic therapy, minimizing adverse side effects and contributing to the global effort to combat antibiotic resistance. Optimizing this synergy involves fine-tuning the formulation of bCPCs to maintain bCPC handling properties, control drug release kinetics, enhance porosity for improved drug loading, and ensure sustained therapeutic action. Incorporating materials such as silk fibroin or other polymers could further improve the mechanical and handling properties, while supporting controlled drug release.

In summary, by optimizing the composition, mechanical performance, and drug delivery mechanisms of bCPCs, their role in bone regeneration therapies can be significantly enhanced. This not only improves immediate post-surgical outcomes by preventing complications but also ensures long-term defect regeneration and tissue integration, addressing critical clinical needs for effective and reliable bone repair solutions.

2) What is the effect of varying precursor proportions and the addition of collagen on the formulation, biocompatibility, and bioactivity of bCPCs? *(Chapter 3)*

Varying precursor proportions and incorporating collagen significantly influence the formulation, mechanical performance, and bioactivity of bCPCs. Six formulations of a dicalcium phosphate dehydrate (DCPD) were prepared, in which a malleable paste that sets within about 2 or 3 min was obtained. The addition of collagen emerged as a key factor in improving the mechanical properties of the bCPCs, particularly increasing compressive strength to a range of 0.8–1.7 MPa. A higher proportion of β-tricalcium phosphate (β-TCP) enhanced the release of tetracycline, highlighting the role of precursor proportions in modulating drug delivery kinetics. This understanding enables better prediction of drug release behaviour within the complex bCPC matrix, particularly when reinforced with polymers like collagen. From a bioactivity perspective, the bCPC formulations demonstrated effective antimicrobial activity against *Escherichia coli* strains over a period of 24 to 72 hours, further underlining their suitability as drug delivery systems with antimicrobial properties.

3) How does the incorporation of sodium alginate in bCPC formulations influence tetracycline release and cell viability? *(Chapter 4)*

The incorporation of sodium alginate in bCPC formulations influenced tetracycline release and cell viability in several ways. This combination of sodium alginate and tetracycline led to a decrease in compressive strength, primarily due to the antibiotic's effect on setting times and porosity. Tetracycline forms chelates with calcium ions (Ca^{2+}), delaying primary crystal

nucleation, which increases porosity and setting time, negatively impacting the bCPCs mechanical properties. However, this effect also improves injectability, as the delayed setting time allows the material to remain fluid(ic) for longer periods, facilitating easier handling and application. Tetracycline release from the bCPC matrices followed a diffusion mechanism in the initial hours, driven by drug solubility and proximity to the matrix surface. Exploring pH fluctuations highlighted a decrease in pH due to the hydroxyl groups from sodium alginate, which can impact cell survival in *in vitro* conditions. Despite this, sodium alginate, as a biocompatible polymer, supported cell growth and improved cell viability by providing a favourable substrate. Tetracycline, on the other hand, evoked a bacteria-free environment, promoting safer conditions for cell growth within the implant. Generally, sodium alginate enhanced the controlled release of tetracycline and improved biocompatibility, while its combination with tetracycline positively influenced cell viability.

4) What strategies can improve the handling characteristics, mechanical strength, and antimicrobial efficacy of bCPCs, and how does the addition of α-tricalcium phosphate (α-TCP) and silk fibroin (SF) impact these properties along with tetracycline (TC) release dynamics? *(Chapter 5)*

The incorporation of α-TCP and SF into bCPCs demonstrated to be an effective strategy to enhance bCPC performance. α-TCP improved compressive strength, particularly at higher concentrations (40wt%), without compromising injectability or porosity. SF, especially in liquid or fiber form, contributed to structural reinforcement, while microparticles reduced mechanical performance due to their irregular morphology. Handling characteristics were influenced by the presence of TC and SF, with extended setting times observed for dual-loaded formulations. This effect, linked to calcium chelation by TC and polymer interactions, highlights the importance of balancing additive concentrations for optimal clinical usability. Antimicrobial efficacy was consistently achieved across all TC-loaded formulations, with sustained inhibition of *Staphylococcus aureus* growth for up to 1 week. The TC release profile followed a typical burst/sustained pattern (~77% release over 14 days), and was not significantly affected by SF or α-TCP content—suggesting diffusion was primarily driven by intrinsic porosity. Together, these results demonstrate that α-TCP and SF can be used to tune the mechanical and handling properties of bCPCs without negatively impacting drug release or antibacterial performance, offering a promising approach for multifunctional, resorbable bone graft materials.

General Discussion

The development of bCPCs represents a significant stride in the pursuit of effective, resorbable bone graft substitutes with multifunctional properties. This thesis explored several modification strategies to address bCPCs' inherent limitations, such as poor mechanical strength, (too) rapid setting times, and limited control over drug delivery, with the ultimate goal of enhancing their clinical applicability and efficacy. This general discussion provides a critical reflection on the broader scientific and clinical implications of the findings and evaluates how these results align with previous research within the context of current literature.

A key takeaway message from the presented studies is that material composition and additive choice exert a profound influence on the handling properties and physicochemical characteristics of bCPCs. For instance, the inclusion of collagen and sodium alginate showed a clear trade-off between mechanical performance and biological functionality. Collagen improved compressive strength, but introduced variability in injectability, while sodium alginate enhanced biocompatibility and sustained drug release, but reduced mechanical strength due to increased porosity. These findings are consistent with prior research, which suggests that the integration of biopolymers apparently requires a careful balance to preserve critical handling properties and clinical needs [1].

Furthermore, the incorporation of tetracycline into bCPCs reaffirmed its dual role in antimicrobial defence and modulation of cement setting behaviour. The observed chelation of calcium ions and resultant delay in brushite crystallization are well-documented phenomena [2, 3], but the practical implications, such as enhanced injectability or the risk of compromised mechanical strength, require further exploration. The sustained release profile of tetracycline in this thesis (~77% over 14 days) is in line with other calcium phosphate drug delivery systems [2], reinforcing the viability of bCPCs for localized, controlled antibiotic administration.

Another important insight relates to the role of precursor ratio and phase selection, particularly the use of α-TCP. α-TCP not only improved compressive strength, but also contributed to a more stable material matrix that tolerated the integration of silk fibroin with minimal disruption to the setting process. However, the morphology of the polymer additive—liquid vs. fibers vs. microparticles—proved to be crucial. While liquid silk fibroin and fibers reinforced the cement structure, microparticles led to mechanical degradation, likely due to heterogeneity-induced stress concentration. This highlights the critical role of not only chemical composition but also the physical form of additives during formulation development [4].

From a translational perspective, these findings together suggest that bCPCs can be engineered to suit specific clinical scenarios. For example, formulations prioritizing injectability and antimicrobial function could be used in maxillofacial surgeries or minimally-invasive orthopedic procedures, while those enhanced for mechanical integrity might find application in non-load-bearing defects. Nevertheless, many of these conclusions are drawn from in vitro data. While these systems showed promise in antimicrobial testing and preliminary cytocompatibility assessments, in vivo studies will be essential prior to potential clinical applications to validate degradation kinetics, immunogenic response, and long-term therapeutic performance in conditions with physiological resemblance to clinical conditions.

In alignment with the overarching aim of this thesis—to develop clinically relevant, multifunctional bCPCs—this body of work offers foundational insights into formulation optimization and translational potential.

Closing remarks and future perspectives

The research presented in this thesis reinforces the concept that bCPCs offer a promising platform for the development of multifunctional synthetic bone graft materials. By systematically evaluating different strategies—including the use of α-TCP, silk fibroin, collagen, sodium alginate, and the antibiotic tetracycline—the work has expanded our understanding of how material design can influence both structural performance and biological function.

One of the central contributions of this thesis lies in demonstrating that multifunctionality in biomaterials does not necessarily come at the expense of mechanical or handling properties. Through careful selection of precursor ratios and additives, it is possible to simultaneously enhance mechanical strength, injectability, and bioactivity. This nuanced control over multiple parameters supports the vision of adaptable bCPC systems that can be tailored for specific clinical applications, such as bone defect fillers with antimicrobial protection or scaffolds for dental bone regeneration in guided tissue regeneration treatments.

Nevertheless, the work also underscores critical trade-offs. For instance, the improved injectability associated with tetracycline and sodium alginate coincides with reductions in compressive strength. These opposing trends highlight the importance of formulation balance and the need for integrated testing approaches that consider mechanical, biological, and practical requirements in tandem. In this context, the adoption of machine learning and high-throughput screening may help predict optimal compositions more efficiently in future studies.

Looking ahead, several avenues are poised for further exploration. First, (long-term) in vivo studies are essential to confirm the biocompatibility, degradation behaviour, and therapeutic efficacy of the developed bCPC formulations. While in vitro antimicrobial testing demonstrated sustained tetracycline release and effective bacterial inhibition, the impact on host immune response and bone remodelling dynamics remains unknown. Incorporating advanced in vivo imaging techniques and longitudinal histological analyses will be crucial in validating the translational potential of these materials. Second, the field may benefit from exploring multifunctional additives that offer both mechanical and biological enhancements. Polymers such as gelatin methacryloyl (GelMA) [5], hyaluronic acid derivatives [6], or bioinspired peptides [7] could offer new mechanisms for promoting osteogenesis while maintaining desirable physical properties. The emergence of "smart" and multifunctional biomaterials is another key trend. Studies have shown that integrating controlled drug delivery into scaffold systems can significantly modulate local inflammation and promote tissue regeneration [8, 9]. For example, stimuli-responsive scaffolds have been engineered to release therapeutic agents on demand—triggered by environmental cues such as pH changes common in injured or infected tissues [10]. Moreover, integrating additive manufacturing and 3D bioprinting with bCPC formulations can unlock the potential for personalized implants with anatomically precise geometries [11, 12]. Custom 3D-printed scaffolds, for example, can be made to precisely fit a bone defect while incorporating porous networks that promote cell infiltration and nutrient flow. Researchers are already leveraging biofabrication techniques to integrate cells and growth factors into printed scaffolds, essentially building living implants that encourage tissue integration from the moment of implantation. Such personalized, cell-seeded constructs blur the line between an implant and a tissue graft, and they hold promise for treating critical-size bone defects that cannot heal on their own. These emerging technologies (from 3D bioprinting to smart surface chemistry) are highlighted as promising strategies for next-generation implants that meet complex clinical needs and push the boundaries of regenerative medicine [13].

Translating these advanced biomaterials from lab to clinic, however, presents significant challenges that the field is actively working to overcome. One issue is scalability and manufacturing consistency: many of the most advanced materials (especially those combining living cells, growth factors, or intricate architectures) are difficult to produce in large quantities under the strict quality controls required for medical products. Automated, standardized fabrication methods will be crucial so that implants can be made reliably [14]. Alongside manufacturing, regulatory approval is a major hurdle. Regulatory agencies have well-defined pathways for drugs and conventional devices, but a multi-component bone-regenerating

scaffold with cells or novel biomolecules does not fit neatly into existing medical class categories. New biomaterials must be rigorously tested for biocompatibility, long-term stability, and safety (i.e. absence of toxicity or adverse immune reactions). For example, a complex scaffold that degrades in the body must prove that its degradation products are harmless and that it degrades at the appropriate rate to allow natural bone to replace it [15].

Data-driven approaches are also beginning to accelerate medical device development [16, 17]. The rise of artificial intelligence (AI) and machine learning provides powerful methods to design and test biomaterials in silico before they ever enter the stage of animal experimentation or clinical trials. Researchers can now train models on large datasets of material properties and biological outcomes, enabling predictive modelling, for example, predicting which material composition or scaffold structure will best induce bone formation [18]. These techniques can dramatically cut down on trial-and-error, optimizing material properties and design parameters much faster than traditional R&D.

On a personal note, I believe that the real breakthrough in this field will emerge from a tighter integration of interdisciplinary collaboration and a willingness to embrace novel regulatory standards. In my view, researchers must actively engage with clinical practitioners earlier in the development process to ensure that the materials we design address tangible, unmet clinical needs. Equally, I feel that fostering partnerships with industry and regulators from the outset will be essential to navigate the complexities of product approval and commercialization. This proactive approach, combined with the exciting technological advances we are witnessing, such as AI-driven material optimization and 3D bioprinting, will accelerate the translation of laboratory innovations into real-world therapies.

In addition to the impressive technical advances in multifunctional biomaterials, I consider it vital to address their affordability and accessibility, particularly for low-resource settings. Coming from a third-world country, I have witnessed firsthand that many of the techniques and materials we discuss in high-end research are often out of reach for nations facing economic challenges or recovering from conflict. Therefore, while continuing to push the envelope in terms of innovation, we must also invest in developing cost-effective, scalable technologies and simplified production methods that can be adapted to meet the urgent needs of poor and war-devastated regions, where the prevalence of bone defects is alarmingly high. Addressing these challenges will not only broaden the clinical impact of advanced biomaterials but also contribute to more equitable global healthcare outcomes.

References.

[1] Ö. Demir-Oğuz, A. R. Boccaccini, and D. Loca, "Injectable bone cements: What benefits the combination of calcium phosphates and bioactive glasses could bring?," *Bioactive Materials,* vol. 19, pp. 217-236, 2023.

[2] M.-P. Ginebra, C. Canal, M. Espanol, D. Pastorino, and E. B. Montufar, "Calcium phosphate cements as drug delivery materials," *Advanced Drug Delivery Reviews,* vol. 64, pp. 1090-1110, 2012.

[3] F. Tamimi *et al.*, "Doxycycline sustained release from brushite cements for the treatment of periodontal diseases," *Journal of Biomedical Materials Research Part A: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials and the Korean Society for Biomaterials,* vol. 85, no. 3, pp. 707-714, 2008.

[4] R. A. Perez, H.-W. Kim, and M.-P. Ginebra, "Polymeric additives to enhance the functional properties of calcium phosphate cements," *Journal of tissue engineering,* vol. 3, no. 1, p. 2041731412439555, 2012.

[5] N. Mamidi, F. Ijadi, and M. H. Norahan, "Leveraging the recent advancements in GelMA scaffolds for bone tissue engineering: an assessment of challenges and opportunities," *Biomacromolecules,* vol. 25, no. 4, pp. 2075-2113, 2023.

[6] O. Mishchenko *et al.*, "Synthetic calcium–phosphate materials for bone grafting," *Polymers,* vol. 15, no. 18, p. 3822, 2023.

[7] Y. Chen *et al.*, "Recent advances in seafood bioactive peptides and their potential for managing osteoporosis," *Critical reviews in food science and nutrition,* vol. 62, no. 5, pp. 1187-1203, 2022.

[8] M. Zhang, W. Hu, C. Cai, Y. Wu, J. Li, and S. Dong, "Advanced application of stimuli-responsive drug delivery system for inflammatory arthritis treatment," *Materials Today Bio,* vol. 14, p. 100223, 2022.

[9] P. Lavrador, V. M. Gaspar, and J. F. Mano, "Stimuli-responsive nanocarriers for delivery of bone therapeutics–Barriers and progresses," *Journal of Controlled Release,* vol. 273, pp. 51-67, 2018.

[10] P. Pan *et al.*, "Smart cargo delivery system based on mesoporous nanoparticles for bone disease diagnosis and treatment," *Advanced Science,* vol. 8, no. 12, p. 2004586, 2021.

[11] N. Wu *et al.*, "3D printed biopolymer/black phosphorus nanoscaffolds for bone implants: A review," *International Journal of Biological Macromolecules,* p. 135227, 2024.

[12] R. Trombetta, J. A. Inzana, E. M. Schwarz, S. L. Kates, and H. A. Awad, "3D printing of calcium phosphate ceramics for bone tissue engineering and drug delivery," *Annals of biomedical engineering,* vol. 45, pp. 23-44, 2017.

[13] J. W. Łuczak *et al.*, "The future of bone repair: emerging technologies and biomaterials in bone regeneration," *International Journal of Molecular Sciences,* vol. 25, no. 23, p. 12766, 2024.

[14] M. E. F. Agnieszka Piegat. "Challenges in Biomaterials' Clinical Translation: Navigating Innovation and Complexity." BIOMATDB. https://www.biomatdb.eu/2023/08/09/challenges-in-biomaterials-clinical-translation-navigating-innovation-and-complexity/#:~:text=biological%20complexities%20of%20physiological%20and,various%20clinical%20disciplines%20through%20the (accessed.

[15] H. Wei, J. Cui, K. Lin, J. Xie, and X. Wang, "Recent advances in smart stimuli-responsive biomaterials for bone therapeutics and regeneration," *Bone research,* vol. 10, no. 1, p. 17, 2022.

[16] S. T. Nuka, "The Role of AI Driven Clinical Research in Medical Device Development: A Data Driven Approach to Regulatory Compliance and Quality Assurance," *Global Journal of Medical Case Reports,* vol. 2, no. 1, p. 1275, 2022.

[17] A. Arnould, R. Hendricusdottir, and J. Bergmann, "The complexity of medical device regulations has increased, as assessed through data-driven techniques," *Prosthesis,* vol. 3, no. 4, pp. 314-330, 2021.

[18] Y. Gokcekuyu, F. Ekinci, M. S. Guzel, K. Acici, S. Aydin, and T. Asuroglu, "Artificial Intelligence in Biomaterials: A Comprehensive Review," *Applied Sciences,* vol. 14, no. 15, p. 6590, 2024.

Hoofdstuk 6: SAMENVATTING

Samenvatting

Botregeneratie blijft een belangrijke uitdaging binnen de klinische orthopedie en tandheelkunde. Het vereist de ontwikkeling van biomaterialen die structurele ondersteuning combineren met bioactiviteit en geneesmiddelafgifte, om zo de klinische beschikbaarheid van synthetische bottransplantatiematerialen te versnellen. Brushiet-calciumfosfaatcementen (bCPC's) worden beschouwd als veelbelovende materialen voor bottransplantatie vanwege hun biocompatibiliteit, bioresorbeerbaarheid en vermogen om te functioneren als lokale geneesmiddeltoedieningssystemen. bCPC's worden bereid uit een zure en een basische calciumfosfaatcomponent, die bij vermenging met een vloeibare fase een kneedbare pasta vormen die zich gemakkelijk aanpast aan de vorm van het botdefect. Bovendien zijn bCPC's injecteerbaar, wat het gebruik van minimaal invasieve chirurgische technieken vergemakkelijkt. Het optimaliseren van de mechanische eigenschappen, hanteringseigenschappen en gecontroleerde afgiftemechanismen van bCPC's is echter essentieel om hun klinische toepasbaarheid en effectiviteit te verbeteren.

Dit proefschrift richtte zich op het verbeteren van de mechanische en hanteringseigenschappen van bCPC's, en op het evalueren van hun effectiviteit als lokaal afgiftesysteem voor antibiotica, met bijzondere aandacht voor hun potentieel in botregeneratieve therapieën.

Hoofdstuk 1 gaf een overzicht van de huidige stand van zaken met betrekking tot de beperkingen van bCPC's en mogelijke strategieën voor verbetering. De daaropvolgende hoofdstukken behandelden elk afzonderlijke onderzoeksvragen en rapporteerden de resultaten ter beantwoording daarvan. In het kort worden deze onderzoeksvragen, de experimentele resultaten en de conclusies hieronder samengevat:

1. **Hoe kan de synergie tussen bCPC's en geneesmiddelafgifte worden geoptimaliseerd om botregeneratie te bevorderen en klinische uitkomsten te verbeteren?** (hoofdstuk 2)

   De synergie tussen bCPC's en geneesmiddelafgifte kan worden geoptimaliseerd door belangrijke uitdagingen aan te pakken en tegelijkertijd hun unieke voordelen te benutten. Hoewel bCPC's gewaardeerd worden om hun biocompatibiliteit, bioactiviteit en snelle resorptie, vormen korte uithardingstijden, beperkte mechanische sterkte en matige

hantering belangrijke obstakels. Strategieën zoals het gebruik van uithardingsvertragers en polymeren kunnen deze beperkingen helpen overwinnen.

Daarnaast biedt het vermogen van bCPC's om lokaal antibiotica af te geven voordelen in het voorkomen van postoperatieve infecties. Dit vermindert de noodzaak voor systemische antibiotica en draagt bij aan het bestrijden van antibioticaresistentie. Het optimaliseren van deze synergie vereist verfijning van de formulering om hanteringseigenschappen te behouden, afgiftekinetiek te controleren, porositeit te verbeteren voor geneesmiddelbelading en een langdurig therapeutisch effect te garanderen. Materialen zoals zijdefibroïne kunnen bijdragen aan verbeterde mechanische eigenschappen én gecontroleerde afgifte.

Samenvattend kan de rol van bCPC's in botregeneratie aanzienlijk worden versterkt door samenstelling, mechanische prestaties en afgiftemechanismen te optimaliseren.

2. **Wat is het effect van variërende hoeveelheden precursors en toevoeging van collageen op de formulering, biocompatibiliteit en bioactiviteit van bCPC's?** (hoofdstuk 3)

   Variatie in precursorverhoudingen en toevoeging van collageen hadden duidelijke invloed op de formulering, mechanische prestaties en bioactiviteit. Zes formuleringen met dicalciumfosfaatdihydraat (DCPD) werden bereid, waarbij een kneedbare pasta met een uithardingstijd van 2–3 minuten werd verkregen. Collageen verbeterde de druksterkte (0,8–1,7 MPa). Hogere hoeveelheden β-tricalciumfosfaat (β-TCP) verhoogden de tetracycline-afgifte, wat het belang onderstreept van precursorverhoudingen in het sturen van afgiftekinetiek. Bioactiviteit bleek uit antimicrobiële werking tegen *Escherichia coli*, 24 tot 72 uur na toepassing.

3. **Hoe beïnvloedt toevoeging van natriumalginaat in bCPC's de afgifte van tetracycline en de celviabiliteit?** (hoofdstuk 4)

   Toevoeging van natriumalginaat beïnvloedde tetracycline-afgifte en celviabiliteit. De combinatie met tetracycline verminderde de druksterkte door vertraagde kristalvorming en toegenomen porositeit (chelatie van calciumionen door tetracycline). Dit verbeterde echter de injecteerbaarheid. Tetracycline-afgifte volgde een diffusiemechanisme. Veranderingen in pH door alginaat beïnvloedden celoverleving, maar als biocompatibel polymeer ondersteunde alginaat celgroei. Tetracycline creëerde een bacterievrije omgeving, wat celgroei ten goede kwam.

4. **Welke strategieën verbeteren hantering, sterkte en antimicrobiële werking van bCPC's, en hoe beïnvloeden α-TCP en zijdefibroïne (SF) deze eigenschappen en de afgifte van tetracycline (TC)?** (hoofdstuk 5)

   Toevoeging van α-TCP en SF bleek effectief. α-TCP verhoogde druksterkte (vooral bij 40 gew.%), zonder porositeit of injecteerbaarheid aan te tasten. SF in vloeibare of vezelvorm verstevigde de structuur; micropartikels verzwakten juist. Uithardingstijd werd verlengd bij TC en SF, door calciumchelatie en polymereninteractie. Alle TC-bevattende cementen remden de groei van *Staphylococcus aureus* tot 1 week. De afgifte (~77% in 14 dagen) werd niet beïnvloed door α-TCP of SF, wat wijst op porositeitsgestuurde diffusie. Samengevat bieden α-TCP en SF mogelijkheden om bCPC's multifunctioneel te maken zonder de afgifte- of antimicrobiële werking te schaden.

Curriculum Vitae

Claudia Morilla Espino was born on January 13, 1994, in Havana, Cuba. She completed her pre-university education in 2012 at Preuniversitario “José Martí”, where she followed a science-focused curriculum. In 2013, she enrolled at the Technological University of Havana to study Biomedical Engineering, earning her Bachelor of Science degree in April 2017.

She then pursued a Master of Science degree in Materials Science and Technology at the University of Havana. Her master’s research focused on the development of composite biomaterials for biomedical applications, and she successfully defended her thesis in January 2020.

In November 2020, Claudia commenced her Ph.D. research in Medicine at Leiden University Medical Center (LUMC) in the Netherlands, under the supervision of Prof. Dr. Lioe-Fee de Geus-Oei, Dr. Ir. Jeroen van den Beucken, and Prof. Dr. Ir. Louise van der Weerd. Her doctoral research focused on brushite calcium-phosphate cement formulations for bone regeneration and controlled drug delivery.

Alongside her academic path, Claudia served as an Assistant Professor at the Technological University of Havana between September 2017 and April 2021. In this role, she was involved in teaching courses related to biomaterials and supervising interdisciplinary student projects. She has presented her work at international conferences and published in peer-reviewed scientific journals.

Acknowledgements

I would like to express my sincere gratitude to my promotor, Professor Dr. Lioe-Fee de Geus-Oei, for her invaluable guidance, scientific insight, and continuous support throughout the course of my PhD. Your expertise and constructive feedback were essential in shaping this research and driving it forward.

I am also deeply thankful to my co-promotors, Dr. Ir. Jeroen van den Beucken and Prof. Dr. Ir. Louise van der Weerd, for their patient supervision, critical input, and encouragement during all phases of this project. Your thoughtful advice and practical perspectives greatly enriched both the scientific content and my development as a researcher.

I wish to acknowledge the members of my research group and laboratory colleagues at the Radiology Department, especially Sana and Somayet, for the helpful discussions, technical assistance, and collaborative spirit. Your contributions made a significant difference in both the quality of the work and the daily experience in the lab.

My appreciation also goes to the Department of Dentistry – Regenerative Biomaterials at Radboudumc for their professional support and assistance during the experimental phase of the final part of this journey.

I would like to thank Prof. Dr. Gastón Fuentes Estévez, who supported me during the early days of this PhD. Your effort and attention to detail are sincerely appreciated.

On a personal note, I am profoundly grateful to my family. To my parents and sister, for instilling in me the values of perseverance and curiosity. To my partner, thank you for your unwavering support, patience, and encouragement throughout these demanding years. Your presence has been my grounding force.

To my close friends, both near and far, thank you for your understanding, moral support, and for always reminding me of life beyond the PhD. A special thanks to Maite and Ariel, for taking me under their wing and showing me how to live in a foreign country.

To all those who contributed to this work in seen and unseen ways, my heartfelt thanks.