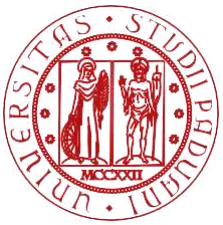

UNIVERSITÀ DEGLI STUDI DI PADOVA

DIPARTIMENTO DI INGEGNERIA DELL'INFORMAZIONE

DIPARTIMENTO DI INGEGNERIA DELL'INFORMAZIONE

CORSO DI LAUREA MAGISTRALE IN

BIOINGEGNERIA

Tesi di Laurea in

TECNOLOGIA DEI BIOMATERIALI

"ADDITIVE MANUFACTURING OF CALCIUM PHOSPHATE BASED BIOCERAMICS"

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ANNO ACCADEMICO 2023-2024

Data di Laurea 09/07/2024

Abstract

Additive manufacturing (AM) has emerged as a transformative technology in biomedical engineering, particularly in fabricating bioceramic materials. This thesis explores the application of AM techniques in the fabrication of calcium phosphate-based bioceramics, focusing on their potential in regenerative medicine and orthopaedic implant manufacturing.

The introduction provides an overview of the significance of AM in biomedical engineering, emphasizing the advantages it offers over traditional manufacturing methods. It highlights the precise control enabled by AM techniques in fabricating patient-specific structures and incorporating bioactive molecules, enhancing the regenerative potential of implants.

The subsequent chapters delve into the principles, techniques, and applications of AM in calcium phosphate-based bioceramics. Chapter one discusses the fundamentals of AM technology, providing insights into its evolution and impact on bioengineering. Chapter two explores the historical evolution, complex structure, and clinical applications of calcium phosphate bioceramics, with a focus on hydroxyapatite. Chapter three focuses on the analysis of the most significant biomedical studies on the use of calcium-based bioceramics, both in vivo and in vitro. The results of these studies are presented, and possible future directions for the development and application of these bioceramics are discussed in the field of bioengineering.

The thesis concludes by discussing the potential of AM in advancing personalized healthcare and addressing the demand for innovative solutions in bone repair and regeneration. It underscores the interdisciplinary nature of research in this field and the transformative impact AM could have on biomaterials science and biomedical device development.

Sommario

La produzione additiva (AM) è emersa come una tecnologia innovativa nell'ingegneria biomedica, in particolare nella fabbricazione di materiali bioceramici. Questa tesi esplora l'applicazione delle tecniche AM nella fabbricazione di bioceramiche a base di fosfato di calcio, concentrandosi sul loro potenziale nella medicina rigenerativa e nella produzione di impianti ortopedici.

L'introduzione fornisce una panoramica dell'importanza dell'AM nell'ingegneria biomedica, sottolineando i vantaggi che offre rispetto ai metodi di produzione tradizionali. Evidenzia il controllo preciso consentito dalle tecniche AM nella fabbricazione di strutture specifiche per il paziente e nell'incorporazione di molecole bioattive, migliorando il potenziale rigenerativo degli impianti.

I capitoli successivi approfondiscono i principi, le tecniche e le applicazioni dell'AM nella bioceramica a base di fosfato di calcio. Il primo capitolo discute i fondamenti della tecnologia AM, fornendo approfondimenti sulla sua evoluzione e sul suo impatto sulla bioingegneria. Il secondo capitolo esplora l'evoluzione storica, la struttura complessa e le applicazioni cliniche della bioceramica a base di fosfato di calcio, con particolare attenzione all'idrossiapatite. Il terzo capitolo si concentra sull'analisi degli studi biomedici più significativi sull'utilizzo di bioceramiche a base di calcio, sia in vivo che in vitro. Vengono presentati i risultati di questi studi e vengono discusse le possibili direzioni future per lo sviluppo e l'applicazione di queste bioceramiche nel campo della bioingegneria.

La tesi si conclude discutendo il potenziale dell'AM nel promuovere impianti bioceramici personalizzati e nel rispondere alla domanda di soluzioni innovative nella riparazione e rigenerazione ossea. Sottolinea la natura interdisciplinare della ricerca in questo campo e l'impatto trasformativo che l'AM potrebbe avere sulla scienza dei biomateriali e sullo sviluppo di dispositivi biomedici.

Index

Introduction

In recent years, additive manufacturing has emerged as a transformative technology in the field of biomedical engineering, particularly in the fabrication of bioceramic materials. Among these materials, calcium phosphate-based bioceramics have garnered significant attention due to their biocompatibility, osteoconductivity, and resemblance to the mineral phase of natural bone. The ability to precisely control the composition, structure, and architecture of calcium phosphate-based bioceramics through AM techniques has opened new avenues in regenerative medicine, tissue engineering, and orthopaedic implant manufacturing. [11]

Traditional manufacturing methods for bioceramics often involve complex processes, limited design freedom and difficulties in achieving patient-specific geometries, but with additive manufacturing techniques overcome these limitations by enabling the layer-by-layer deposition of materials based on digital designs. This approach not only allows for the creation of highly complex and patient-specific structures but also facilitates the incorporation of bioactive molecules and growth factors, enhancing the regenerative potential of the implants.

In this thesis, we provide an overview of the principles and techniques involved in the additive manufacturing of calcium phosphate-based bioceramics. We discuss the various AM methods utilized for bioceramic fabrication, highlighting their advantages and limitations.

Furthermore, we explore the applications of AM in the development of customized implants, scaffolds for tissue regeneration, and drug delivery systems, emphasizing the potential of this technology to revolutionize the field of orthopaedics and regenerative medicine. [1]

The first chapter provides a comprehensive overview of AM technology, outlining its basic principles and delving into the various types of both direct and indirect AM. Offering insights into the dynamic evolution of AM technology and its profound impact on the field of bioengineering.

The second chapter delves into the historical evolution, complex structure, various properties, and fundamental clinical applications of Ca-P bioceramics, with a focus on

hydroxyapatite. It highlights the biomedical applications of Ca-P, such as promoting bone regeneration and facilitating healing processes.

The third chapter focuses on the analysis of the most significant biomedical studies on the use of calcium-based bioceramics, both in vivo and in vitro. The results of these studies are presented, and possible future directions for the development and application of these bioceramics are discussed.

Overall, the additive manufacturing of calcium phosphate-based bioceramics holds great promise for advancing personalized healthcare and addressing the growing demand for innovative solutions in bone repair and regeneration. Through interdisciplinary research and technological advancements, AM has the potential to reshape the landscape of biomaterials science and contribute to the development of next-generation biomedical devices and therapies.

1. Additive Manufacturing (AM) Technologies for Bioceramics

Additive manufacturing (AM) is defined by the ASTM society as "a process of joining materials to make objects from 3D model data, usually layer upon layer, as opposed to subtractive manufacturing methodologies." The inception of AM technology can be well correlated with the rapid prototyping (RP) technique, which was first developed in the early 1980s. [1]

Additive Manufacturing represents a groundbreaking leap in digital manufacturing technology, that integrates machinery, computerized systems, numerical control, and a diverse range of materials within the global advanced manufacturing landscape over the last 30 years. Unlike conventional manufacturing methods, AM technology heralds a model shift, moving away from designs tailored to fit manufacturing constraints towards designs optimized for part performance, thus sparking a revolution within the contemporary manufacturing sector.

The more important innovations of AM technology are its forming equipment and the array of materials it employs. Currently, AM materials encompass a spectrum of options, including polymers, metals, ceramics, and composites, each offering unique properties and applications.

This chapter provides a comprehensive overview of the research and development initiatives shaping the landscape of AM technology worldwide. It offers insights into the dynamic evolution of AM technology and its profound impact on the bioengineering world. [2]

The focal point of AM technology resides primarily in Europe and the United States, with the United States serving as the birthplace of this transformative technology and leading the forefront of research in this field.

AM technology has revolutionized the established methodologies and principles of traditional manufacturing, effectively overturning its conventional model. The expansion of AM technology's application realm hinges on the continuous development of new materials.

Presently, the spectrum of AM materials encompasses polymers, metals, ceramics, and composites, constituting a crucial foundation for the advancement of AM technology. Frequently, the progress and versatility of materials determine the field of AM's

applicability. This thesis primarily focuses on the exploration of ceramic materials within the realm of AM.

Ceramic materials have great advantages such as exceptional heat resistance and robust strength, rendering them indispensable across various industries including industrial manufacturing, biomedicine and aerospace. However, despite their large utility, the field of ceramic materials compatible with Additive Manufacturing remains relatively limited, serving as a significant impediment to the advancement of AM ceramic technology.

Currently, the range of ceramic materials suitable for AM is predominantly represented by aluminium oxide (Al2O3), tricalcium phosphate (TCP), porous silicon nitride (Si3N4), and titanium silicide (Ti3SiC2). Additionally, ceramic materials integrated with organic precursors for AM primarily include silicon carbide (SiC), silicon nitride (Si3N4), silicon ox carbide (SiOC), and silicon carbonitride (SiNC). Despite these strides, the exploration and expansion of ceramic materials compatible with AM remain pivotal for unlocking the full potential of ceramic-based additive manufacturing processes. [2]

1.1 Basic principles of AM

Additive Manufacturing technologies are specifically aimed at producing complex geometric parts with near-net-shape functionality. Much like Rapid Prototyping (RP), AM involves layer-by-layer production of parts. In AM, each layer is formed by depositing material onto the preceding layer and then fusing it using various energy sources such as thermal, chemical or mechanical methods. Unlike traditional manufacturing processes, that involve material removal, deformation or solidification, AM builds components through the addition of material. Notably, AM systems do not necessitate tooling or extensive process and path planning.

AM technology presents numerous advantages over conventional manufacturing techniques, including enhanced design flexibility, the ability to incorporate internal cooling channels, the creation of functionally gradient structures and lattice formations. Additionally, many AM methods permit the utilization of materials like superalloys and amorphous metals, which are typically challenging to process using traditional methods. Studies have also shown that AM systems generate less material waste compared to traditional manufacturing methods, resulting in a higher "buy-to-fly" ratio, particularly prevalent in the aerospace industry.

However, it's important to acknowledge some drawbacks associated with AM methods.

These include longer processing times per part and higher costs of raw materials compared to those used in conventional manufacturing. Additionally, AM methods often struggle to match the material properties (such as density, porosity, and crystal structure) of parts produced through conventional routes like forgings or castings, nor do they achieve the same level of accuracy as the computerized numerical control machining, CNC. Nevertheless, considerable efforts and advancements are being made to address these faults in recent years.

AM methods find significant applications in manufacturing high-performance parts characterized by particular materials and complicate geometries. They are particularly valuable for producing parts with low production volumes, where the cost of tooling per part is prohibitively high, such as customized medical devices tailored to individual patients or replacement parts that would otherwise require expensive tooling recreation. These attributes have caught interest from researchers and industries, especially in the medical device sector. [1]

1.2 Types of Additive Manufacturing

Additive manufacturing technology can be divided into two main categories, as shown in Figure 1: direct and indirect Additive Manufacturing, and each one includes a series of different typologies with distinct processes, each with its own specific characteristics and applications.

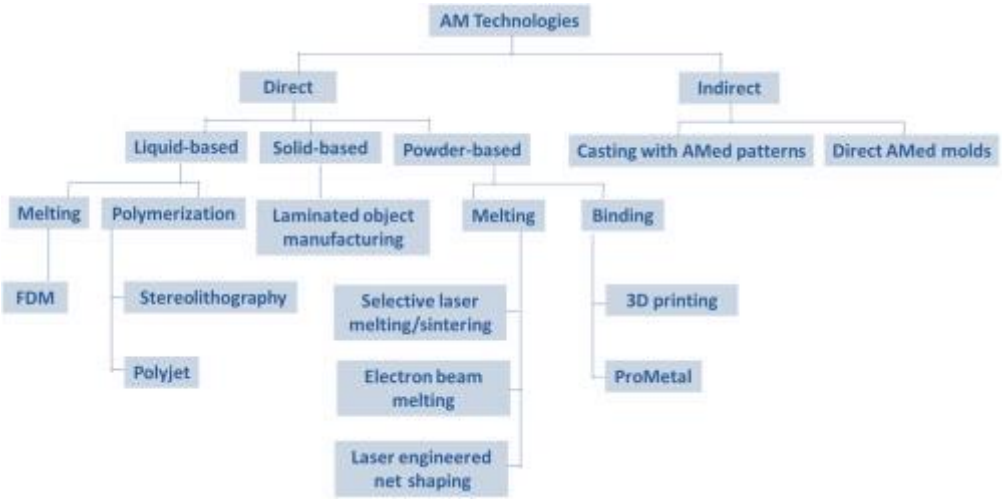

Figure 1: Different variants of additive manufacturing technologies.[1]

Direct Additive Manufacturing technology represents a technique wherein objects are fabricated directly by adding material layer by layer, eliminating the necessity for intermediate shapes or molds. This method provides unparalleled precision in material deposition, facilitating the production of components featuring intricate geometries and complex characteristics.

One of the distinguishing features of direct AM is its ability to create molds with markedly higher hardness and strength, owing to the rapid solidification characteristics inherent in techniques like Selective Laser Melting (SLM). However, this advantage also presents challenges in post-processing situations, particularly in finishing operations.

Despite these challenges, direct AM offers several advantages. Notably, it significantly reduces production times and associated costs. By eliminating the need for traditional manufacturing processes involving molds or tooling, direct AM streamlines production workflows and enhances overall efficiency. Moreover, its ability to fabricate intricate components with high precision contributes to improved product quality and functionality.

Indirect Additive Manufacturing technology represents a process that involves the creation of intermediary shapes or patterns utilized in the production of final objects. This technology boasts remarkable versatility in material selection and finds particular suitability in working with non-ferrous materials like aluminium and magnesium, especially in crafting cellular structures. These structures exhibit thin walls with an exceptional surface finish while upholding the isotropic behaviour of the overall structure.

A pivotal aspect of this technology resides in the strategic design of casting systems, crucial for ensuring the seamless flow of molten metal during the casting process of these intricate metal structures. Despite these complexities, one of the notable advantages of indirect AM lies in its ability to offer greater control over the properties of final materials. By utilizing intermediary models, it becomes feasible to execute heat treatments or surface finishing processes to achieve specific material properties that may prove challenging to achieve through direct production methods.

Moreover, indirect AM technology provides flexibility in tailor-made cellular design, offering opportunities to refine the productivity of metallic cellular and lattice structures. This aspect contributes significantly to their economic viability, thus broadening their applicability in various industries. [1]

So, both approaches, direct and indirect additive manufacturing, come with their sets of

advantages and disadvantages. The suitability and the choice between direct and indirect additive manufacturing of each method depends on various factors such as the specific requirements of the project, factors such as material selection, production volume, and desired part complexity. By carefully evaluating these factors, manufacturers can determine the most suitable approach for their production needs.

1.2.1 Selective Laser Sintering, SLS

Selective laser sintering (SLS) is a layer-based additive manufacturing technique primarily utilized for rapid prototyping and rapid tooling purposes. In this process, laser beams, operating either continuously or in pulses, serve as the heat source for selectively scanning and fusing powders into predetermined shapes and sizes, layer by layer.

The configuration of the scanned layers mirrors the various cross-sections outlined in the computer-aided design (CAD) models or stereolithography (STL) files of the intended object. Following the scanning of the initial layer, a subsequent layer of loose powder is deposited atop it, and this sequence is iterated from the bottom to the top until the fabrication of the artifact is concluded.

When the chemical reaction of the mixture components is employed in conjunction with a laser beam, the process is termed selective laser reactive sintering. Alternatively, when complete melting of the powder predominates over solid-state sintering, it is referred to as selective laser melting (SLM), direct metal laser sintering (DMLS), or direct metal laser remelting.

When laser energy is absorbed by the materials, various mechanisms come into play to bind the powders. These mechanisms include viscous-flow binding, curvature effect, particle wetting, solid-state sintering, liquid-phase sintering, and true melting. Viscous-flow binding prevails in materials with temperature-dependent viscosity, while the curvature effect drives the binding process in nano-crystalline materials.

Laser sintering occurs rapidly, typically within milliseconds, which is insufficient for binding through solid-state diffusion. Therefore, powder fusion is achieved by either partially melting one of the low-melting-point components of the powder or completely melting the entire mass.

The most common approach involves sintering by melting a portion of the powders, achieved by using powder systems comprising a mix of low- and high-melting-point components. In this method, as shown in Figure 2, the laser beam selectively heats the

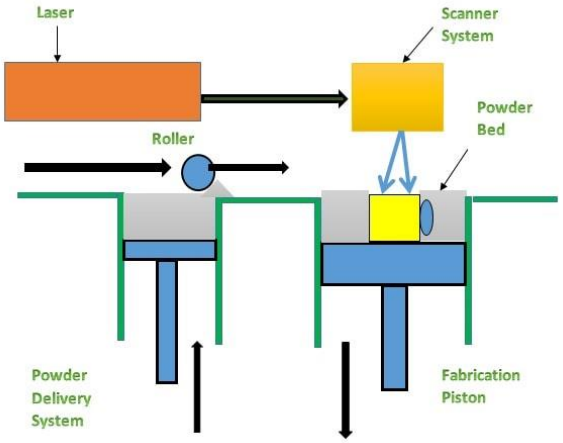

Figure 2: A Diagrammatic representation of the working of Selective Laser Sintering Technology. [47]

powder bed, causing only the low-melting-point solids to melt, which then bind with the high-melting-point components. Interparticle wetting, facilitated by this two-phase process, is essential to prevent "balling phenomena."

In cases of true melting, where the entire powder mass is melted, challenges such as wavy surfaces and dimensional inaccuracies in the finished part may arise.

The deposition and subsequent sintering of powders are influenced by several factors, including powder density, shape, size distribution, and flow rate. The manner in which powders are deposited significantly impacts the stresses and deflection of the final part. To enhance sintering, it is crucial to increase the density of metal powder layers. This can be achieved through optimization of particle shape and surface condition. Regular, equiaxed, and nonporous particles promote high layer density by ensuring an appropriate size ratio and composition. Additionally, the electrostatic technique has been employed to create dense layers.

Prior to the SLS process, it is beneficial to improve the sinter ability of powders through thermal pre-treatment, which may involve cleaning or degassing. This preparatory step helps enhance the performance and quality of the final product. [3]

1.2.2 Selective Laser Melting, SLM

Selective Laser Melting technology is distinguished by the complete melting and solidification of metal materials. This technology, as shown in Figure 3, involves the utilization of high-energy lasers to melt metal powders swiftly, followed by rapid cooling. This method unfolds through intricate interactions between the laser and the powdered metal, encompassing a cascade of physical and chemical phenomena. These encompass

energy transfer mechanisms and transformations in the material's physical state, in which the quality of metal powder materials significantly impacts the processing outcomes of SLM. Consequently, SLM technology imposes rigorous demands on various aspects of powder materials, including stacking characteristics, particle size distribution, particle shape, fluidity, oxygen content, and absorption rate. Within the SLM process, light energy undergoes conversion into thermal energy, leading to a material state transition. Depending on varying laser energies and residence times, the metal powder experiences corresponding shifts in state by absorbing different laser energies. When laser energy is low or residence time is short, the metal powder absorbs minimal energy, resulting in only a surface temperature rise of the metal particles. Consequently, the material softens and deforms while remaining in a solid state. As laser energy increases, the temperature of the metal powder surpasses its melting point, causing the metal particles to appear in a molten state. Upon instantaneous cessation of laser energy, the molten metal rapidly cools to form fine-grained parts. Excessive laser energy within the SLM process can lead to defects such as balling, thermal stress, and deformation of the processed parts, which should be minimized whenever possible. [4] [17]

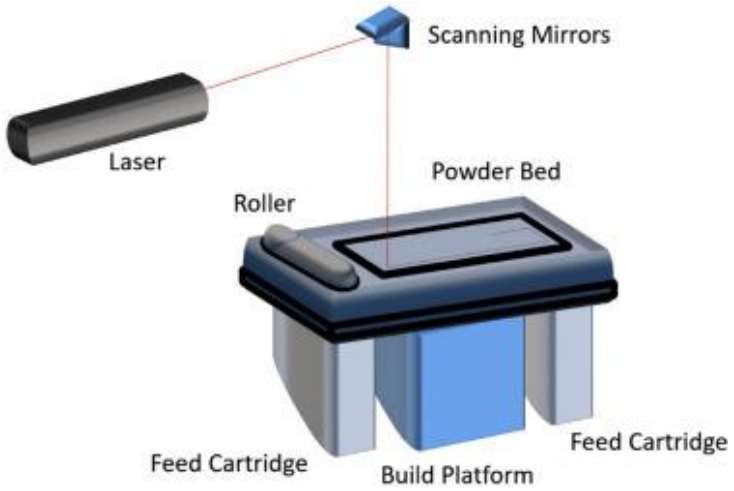

Figure 3: Selective Laser Melting Technology. [17]

1.2.3 Electron Beam Melting, EBM

Electron Beam Melting (EBM) represents a method of Additive Manufacturing akin to Selective Laser Melting (SLM). This process involves intricate interactions between a beam of electrons and powdered metal. In EBM, as shown in Figure 4, the powder bed is preheated, facilitating the transformation of melted particles and thereby enhancing the properties of the part. To enhance surface quality, increasing the size of the melt pool

becomes crucial to decrease the temperature gradient of the electron beam. With a reduced temperature gradient, surface tensions are minimized, resulting in the refinement of surface quality. [5] [17]

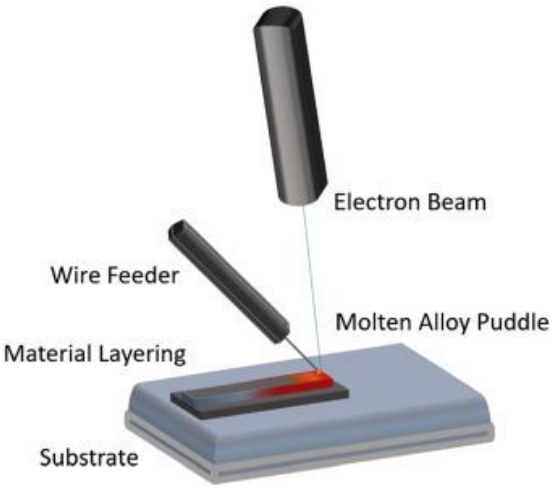

Figure 4: A schematic of an Electron Beam Melting device [17].

1.2.4 Fused Deposition Modelling, FDM

Fused Deposition Modelling (FDM) represents a method utilized in the realm of Solid Freeform Fabrication (SFF) for crafting three-dimensional scaffolds.

This technique, beginning with a three-dimensional computer-aided design (CAD) model, as shown in Figure 5, employs a layer-by-layer deposition process, wherein molten polymers or ceramics are extruded through a nozzle featuring a small orifice, which can vary or be arbitrarily defined for each deposited layer. This material is then fused with the material on the preceding layer.

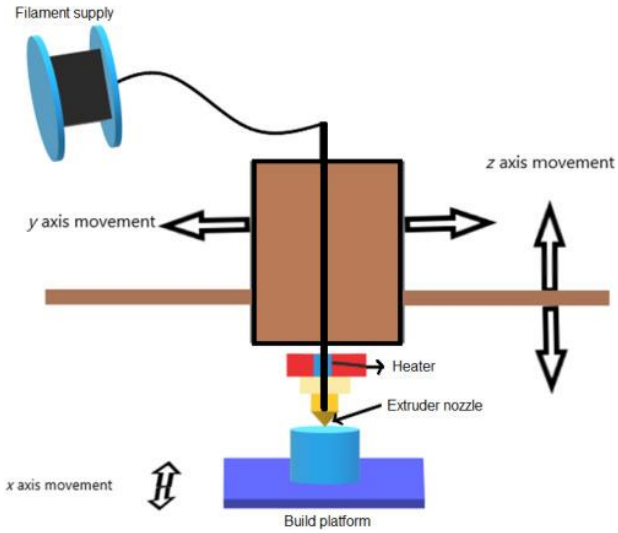

Figure 5: Schematic of the basic Fused Deposition Modelling process. [1]

Finally, the models are solidified through the focused application of heat from a laser beam. This process iterates continuously, layer by layer, until the fabrication of the part is achieved in its entirety. FDM has found application in the manufacturing of three-dimensional scaffolds utilizing polymers such as PCL and high-density polyethylene, as well as composites like PCL/hydroxyapatite. [6]

1.2.5 Stereolithography, SLA

Stereolithography stands as the oldest 3D printing technology, renowned for its ability to generate 3D structures with exceptionally high levels of resolution and accuracy compared to other methods. [**7**]

The basic principle of SLA involves fabricating three-dimensional constructs by irradiating light sources onto the light-sensitive resin, as shown in Figure 6.

When UV or visible light strikes the polymer resin, the monomers and oligomers combine to form a rigid structure through a layer-by-layer deposition method. The first step involves creating a digital model using computer-aided design (CAD) software in SLA printing. In the second step, an advanced digital scanner converts the complex structure into a 3D model. Finally, polymer resin is used to create the designed three-dimensional structure.

Within SLA, a 2D pattern undergoes solidification via photopolymerization, triggered by either single- or two-photon absorptions. 3D structures are then built through the layer-by-layer stacking of these two-dimensional patterns.

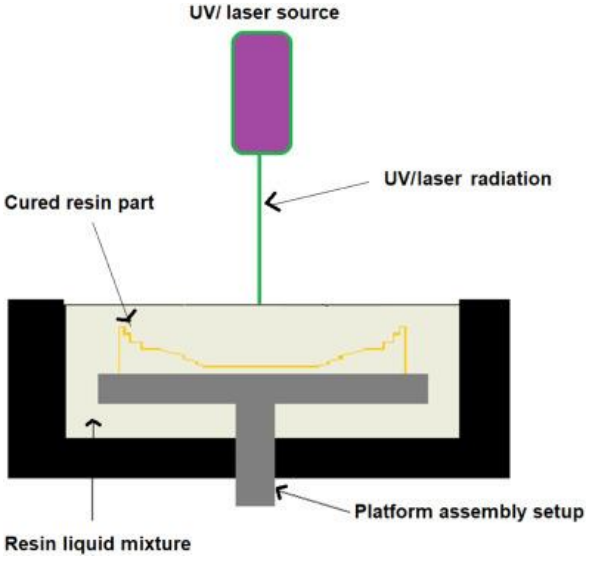

Figure 6: Schematic of Stereolithography process. [1]

Therefore, the advantages of SLA printing include high speed, strong printed structure, high resolution, and scalability.

Droplet-, extrusion-, and SLA-based bioprinting each possess unique characteristics enabling the creation of diverse structures. However, these methods also come with drawbacks that hinder the reproduction of complex tissue and organ architectures. Integrated 3D bioprinting systems offer a potential solution to this challenge by leveraging the distinct features of various bioprinting techniques. [8]

1.2.6 Inkjet Printing

The earliest iterations of bioprinting systems involved modifying conventional desktop inkjet printers. These printers are capable of depositing minute volumes (1–300 pL) of liquid onto a substrate via small nozzles. Bioprinters based on inkjet technology typically employ a bioink containing cells suspended in either culture medium or a cross-linkable hydrogel, or they can be acellular. Present inkjet-based bioprinters can achieve printing speeds ranging in the hundreds of millimetres per second and can deposit hundreds of thousands of droplets per second, with resolutions as fine as 50 µm. However, the resolution limit of inkjet printers depends on both the diameter of the nozzle and the viscosity of the bioink. Consequently, reducing the nozzle diameter alone increases the risk of clogging, restricting the types of materials suitable for printing to low-viscosity (~30 mPa s) or water-based materials.

As shown in Figure 7, there are currently three primary approaches to inkjet printing:

- *Thermal*: This method involves heating a small volume of the bioink (up to 300°C) for microseconds to vaporize the liquid and create an air bubble that expels the ink from the nozzle. Thermal inkjet printing is widely used due to its high cell viability post-printing, user-friendliness, and cost-effectiveness.
- *Piezoelectric*: Piezoelectric inkjet printing utilizes a mechanical pulse generated by a piezoelectric actuator. While there's no significant difference in cell viability between piezoelectric printed and unprinted fibroblasts, concerns exist regarding the frequency range employed by these printers (15–25 kHz), which may damage cell membranes and induce cell lysis.
- *Electromagnetic*: Electromagnetic inkjet printing employs miniature solenoid valves to dispense fluid. This method produces larger drop volumes compared to other inkjet printing techniques.

Although inkjet bioprinting initially generated considerable interest, progress toward fabricating large tissue constructs has been limited.

Challenges include difficulty in accurately layering low-viscosity liquid droplets on a solid surface and the high thermal or shear stress experienced by deposited cells, which can impact viability. Despite its affordability and flexibility, addressing issues related to bioink composition, resolution, layering, and printing speed is necessary to realize the construction of complex 3D models achievable by other 3D bioprinting methods. [9] [10]

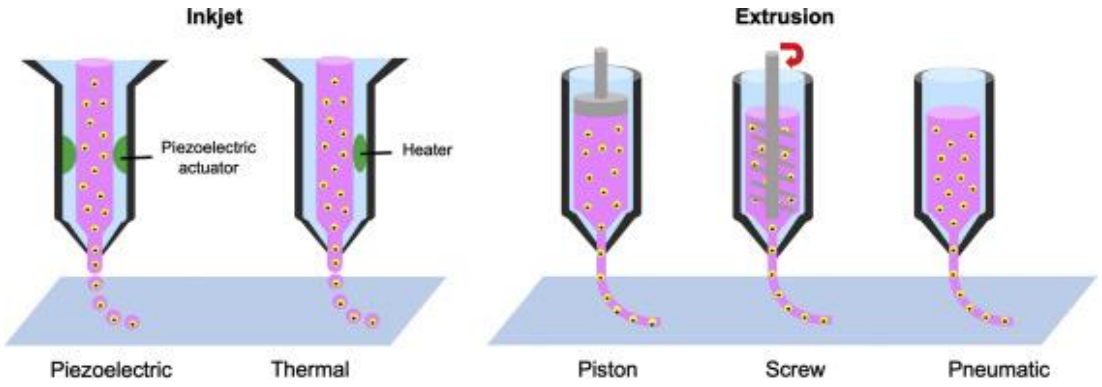

Figure 7: Schematic of different 3D bioprinting platforms.[9]

1.2.7 Light-Based Bioprinting

Light-assisted bioprinting operates by spatially controlling the solidification of a liquid photopolymerizable material using light. This method has garnered growing interest in tissue engineering due to its ability to encapsulate cells with high viability, along with its rapid fabrication speeds and high resolution. These features enable precise control over the mechanical, physical, and chemical properties of the printed models. Light-assisted bioprinting primarily exists in two forms: digital light processing (DLP) and two-photon polymerization (TPP) based bioprinting.

- *TPP-Based Bioprinting*: TPP-based bioprinting relies on a focused, near-infrared, femtosecond laser to polymerize a monomer solution. This technique operates by inducing polymerization solely within the peak intensity area of the laser focal spot. At this region, the energy is sufficiently intense to trigger nonlinear absorption of the femtosecond laser in the monomer solution, leading to photopolymerization for printing purposes. Consequently, this approach achieves resolution below the diffraction limit, enabling the creation of high-fidelity nanostructures with features smaller than 100 nm. The process also facilitates the

fabrication of 3D structures, including overhanging features, by rastering through the monomer solution.

However, the exceptional resolution of this printing method comes with trade-offs. There is a reduction in the fabrication size and speed due to the intricacies of the process, although it maintains good cell viability. As with inkjet- and extrusion-based printers, the noncontinuous nature of TPP-based bioprinting results in interfaces between photopolymerized parts, which can compromise the mechanical integrity of the fabricated structures.

- *DLP-Based Bioprinting*: DLP-based bioprinting platforms consist of essential components including a light source, a digital micromirror device (DMD) chip, a motorized computer-controlled stage and a probe. The light source, whether UV or visible, is directed onto the DMD chip, which contains millions of micromirrors that can be individually rotated to either reflect light ("on") or block it ("off") using a binary mask. Light reflected off the DMD chip is then transmitted through a series of optics into a solution of photopolymerizable biomaterials, enabling simultaneous printing of an entire plane of an optical pattern. By moving either the stage or the light focal plane along the z-direction, intricate 3D patterns can be fabricated.

  DLP-based bioprinters offer several advantages over inkjet- and extrusion-based counterparts, underscoring their versatility. Structures created using DLP-based bioprinting are notably smooth and exhibit greater mechanical integrity due to the absence of artificial interfaces between deposited materials, which are inherent in inkjet- and extrusion-based constructs. Additionally, this method boasts a significant time advantage, enabling the fabrication of structures within seconds to minutes. The resolution of DLP-based printers is determined by the focal size of the reflected light beam, typically on the scale of a few microns. Moreover, the stiffness of these structures can be tailored by adjusting the power from the light source or varying the duration of exposure, thereby influencing the degree of polymerization. This capability allows for the creation of single structures with multiple stiffness profiles, offering a more accurate representation of models associated with pathology or disease. When printing cells, DLP-based bioprinters exert no shear forces, resulting in higher cell viability compared to inkjet- or extrusion-based methods. In summary, DLP-based bioprinters are capable of

fabricating complex 3D structures with fine features rapidly and with precision, making them a valuable tool in tissue engineering and regenerative medicine.

Both DLP and TPP based bioprinters encounter certain limitations. Despite their advantages, these techniques are not without drawbacks. Unlike inkjet-based systems, which utilize nozzles to deposit materials precisely, both DLP and TPP methods often involve photopolymers residing in a reservoir from which objects are printed. This setup can lead to wastage of materials and increased costs, particularly as the unused portion of the reservoir may become unusable after a printing session.

Moreover, as shown in Figure 8, light-assisted printers rely on photopolymers that must undergo chemical modifications to enable 3D printing. This necessity restricts the range of materials that can be used and limits the types of structures that can be fabricated. Such constraints may hinder the versatility of these bioprinting techniques, especially when attempting to create complex or specialized constructs. [9] [10]

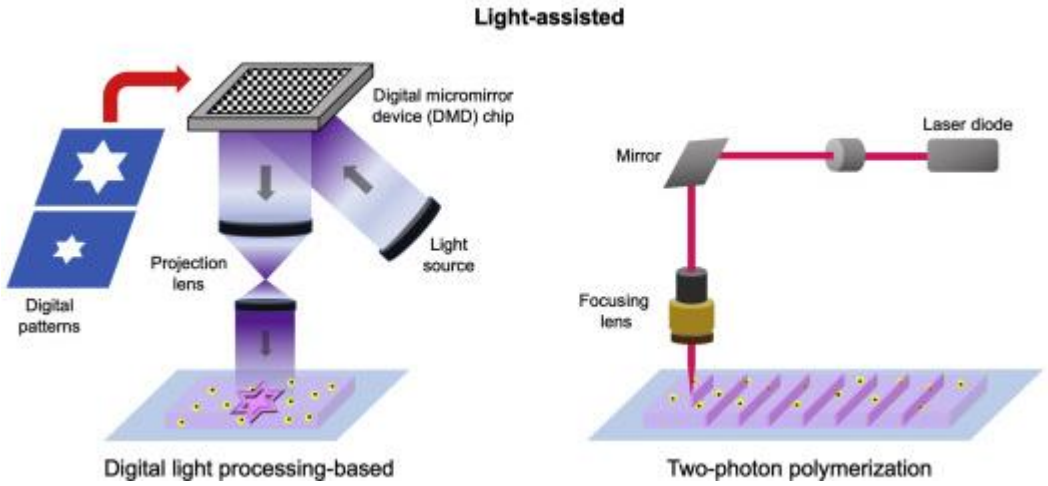

Figure 8: Schematic of different 3D bioprinting platforms.[9]

1.3 Recent Applications

Additive Manufacturing (AM) is a promising technology with the potential to revolutionize the healthcare sector and bring about significant social change, influencing aspects such as health, safety, social well-being, local employment, community engagement, social security, and overall quality of life for people. The key advantage of AM is its ability to deliver personalized results while streamlining the manufacturing process into a standardized framework. This customization allows for tailored solutions that can better address individual patients' medical conditions and incorporate elements that reflect their uniqueness across biological, social, and economic dimensions. Compared to traditional methods, as seen in this chapter, AM techniques offer time-saving benefits, require fewer tools during production, and often reduce or eliminate the need for storage, thereby cutting costs.

Beyond operational efficiencies, the increasing adoption of AM in healthcare also yields societal benefits, such as improvements in life expectancy, overall quality of life, and enhanced health and safety standards in the workplace. For instance, medical models produced through AM accurately replicate a patient's anatomy and structure, aiding in better understanding and visualization. This facilitates the creation of precise surgical models and implant dimensions, allowing doctors to plan surgeries with greater accuracy.

The evolution of this innovative production method is transforming the healthcare landscape, introducing applications like bioprinting of tissues and organs, medical models for educational purposes, personalized medicines, prosthetics, hearing aids, and surgical tools, among others. AM grants access to customized medical devices and prosthetics, enhancing quality and comfort for users while driving down costs. Moreover, it opens avenues for manufacturing body parts, such as internal organs, which are currently reliant on donors, which will be covered in subsequent chapters

Overall, AM's positive impact extends to aging societies, contributing to reduced healthcare costs and improved quality of life through the provision of customizable healthcare products tailored to individual needs. [13]

2. Calcium Phosphate-based Bioceramics

Calcium phosphate-based bioceramic materials are a vital class of biomaterials extensively utilized across various biomedical fields, including regenerative medicine, tissue engineering, and dentistry. These materials, composed primarily of calcium phosphate (Ca-P), hold a prominent position in bone regeneration, orthopaedics, and dentistry due to their remarkable biocompatibility, osseointegration, and osteoconduction properties. Indeed, the versatility and efficacy of Ca-P bioceramics have rendered them indispensable in these critical applications.

This chapter delves into the historical evolution, intricate structure, different properties, and pivotal clinical applications of Ca-P bioceramics. Whether in the form of bone cements, pastes, scaffolds, or coatings, Ca-P bioceramics play a significant role in promoting bone regeneration and facilitating healing processes. Through a comprehensive exploration of these materials, the objective is to shed light on their profound significance and multifaceted utility in advancing the field of bone tissue engineering and regenerative medicine. By elucidating the fundamental characteristics and practical applications of CaP bioceramics, we aim to contribute to the ongoing efforts aimed at improving patient outcomes and enhancing the quality of life through innovative biomaterials solutions. [11] [12]

2.1 Chemical Structure and Chemical Properties of Calcium Phosphate

Calcium phosphates ($\mathrm{Ca-P}$) are a privileged class of biomaterials due to their good biocompatibility, biodegradability and possible bioreactivity. [14]

Calcium phosphate, as shown in Figure 9, is a collective term referring to a group of minerals characterized by the presence of calcium cations (Ca^{2+}) combined with orthophosphate (PO_3^{-4}), metaphosphate (PO^{-3}), or pyrophosphate ($P_2O_4^{-7}$) anions, sometimes accompanied by hydrogen (H^+) or hydroxide (OH^-) ions.

The most common form is tribasic calcium phosphate, with the chemical formula $Ca_3(PO_4)_2$, so each molecule of tribasic calcium phosphate contains three calcium atoms (Ca) and two phosphate groups (PO_4).

The structure of tribasic calcium phosphate is composed of calcium ions (Ca^{2+}) and phosphate ions (PO_4^{3-}) arranged to form a crystalline network. Calcium ions are positively charged while phosphate ions are negatively charged. The crystalline structure of tribasic

calcium phosphate can vary slightly depending on the conditions of formation and the impurities present in the compound.

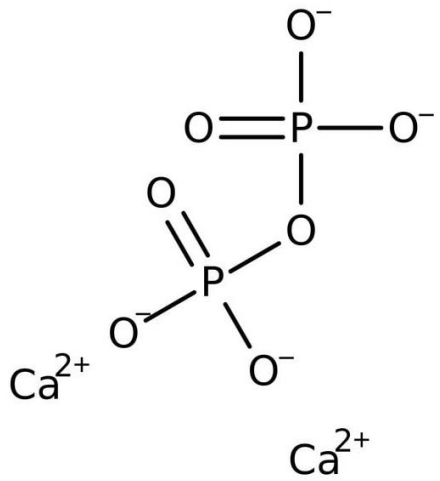

Figure 9: Chemical structure of Calcium phosphate. [49] [50]

All these calcium phosphate-based minerals have vital roles in various biological structures.

In bone tissue, calcium phosphate is the predominant inorganic component, constituting approximately 60% of bone by weight. Moreover, it serves as the primary constituent of tooth enamel, comprising about 90% of its composition. Calcium phosphates, possessing a Ca/P atomic ratio falling within the range of 1.5 to 1.67, are classified as apatite. Hydroxyapatite and fluorapatite are notable examples of apatite, distinguished by their respective compositions and properties. [11] [14]

2.2 Chemical Properties of Calcium Phosphate-based Bioceramics

Bioceramic materials are refractory inorganic materials that contain both metallic and non-metallic elements. Their composition and properties can vary depending on the bonds present, which can be predominantly ionic, predominantly covalent, constituting about 87%, or entirely covalent. The characteristics of bioceramics are influenced by their chemical composition of the starting powders and the presence of any impurities, the composition and distribution of phases, the phases present at the grain boundaries, and the size, shape, and distribution of the grains themselves are crucial and by factors related to their preparation process.

Among the prominent features of bioceramic materials there are good mechanical resistance, high hardness, high melting temperature, and very low thermal conductivity.

Bioceramics can be classified into four main categories:

- *Bioinert*: characterized by high chemical and physical resistance to the biological environment, such as alumina and zirconia.
- *Porous for tissue ingrowth*: these materials, such as metals coated with hydroxyapatite and alumina, promote tissue growth within their porous structure.
- *Bioactive*: capable of forming direct chemical bonds with bone tissue and with the soft tissues of living organisms. Examples include hydroxyapatite, bioactive glasses, and bioactive glass-ceramics.
- *Resorbable*: actively involved in metabolic processes, such as tricalcium phosphate, which can be gradually absorbed by the body. [15]

Bioceramics based on calcium phosphate are a type of ceramic material commonly used in biomedical applications due to their biocompatibility and similarity to the mineral component of bone. These ceramics are primarily composed of calcium and phosphate ions, resembling the mineral composition of natural bone tissue.

Calcium phosphate bioceramics can be engineered with various compositions and structures to mimic the properties of bone and promote tissue regeneration. They are often used in orthopaedic and dental applications, including bone graft substitutes, coatings for implants, and scaffolds for tissue engineering.

Various phases of calcium phosphate ceramics are utilized based on the desired characteristics, whether it's resorbable or bioactive. The stability of these phases heavily relies on temperature and the presence of water, either during processing or in the utilization environment. At body temperature, only two calcium phosphates remain stable in contact with aqueous media, like bodily fluids:

- under pH < 4.2, the stable phase is $CaHPO_4 \cdot 2H_2O$ (known as dicalcium phosphate or brushite, C_2P);
- under pH > 4.2, the stable phase shifts to $Ca_{10}(PO_4)_6(OH)_2$ (referred to as hydroxyapatite or HA).

At elevated temperatures, other phases such as $Ca_3(PO_4)_2$ (β-tricalcium phosphate or TCP) and $Ca_4P_2O_9$ (tetracalcium phosphate or C_4P) are present. These unhydrated, high-temperature calcium phosphate phases interact with water or bodily fluids at 37°C to convert into HA. Consequently, the solubility of a TCP surface approaches that of HA,

lowering the pH of the solution, which in turn further increases TCP solubility and enhances resorption. [15]

The sintering process of calcium phosphate ceramics typically takes place within a temperature range of 1000°C to 1500°C, following the compaction of the powder into the desired shape. The phases that form at high temperatures are contingent upon the temperature itself and the partial pressure of water (pH_2O) within the sintering atmosphere. In the presence of water, hydroxyapatite (HA) can be formed and remains a stable phase up to 1360°C, as depicted in the phase equilibrium diagram for calcium oxide (CaO) and phosphorus pentoxide (P_2O_5) with 500 mmHg (66 kPa) pH_2O. Conversely, in the absence of water, tetracalcium phosphate (C_4P) and β-tricalcium phosphate (C_3P) become the stable phases.

The temperature range within which HA remains stable expands with increasing pH_2O, as does the rate of phase transitions from C_3P or C_4P to HA. Due to kinetic barriers influencing the rates of formation of stable calcium phosphate phases, it proves challenging to predict the volume fraction of high-temperature phases formed during sintering and their relative stability upon cooling to room temperature. Starting powders can be synthesized by mixing the appropriate molar ratio of calcium nitrate ($Ca(NO_3)_2$ and ammonium phosphate ($(NH_4)_3PO_4$) in an aqueous solution, yielding a precipitate of stoichiometric HA. During processing or in physiological environments, the Ca^{2+}, PO_4^{3-}, and $OH-$ ions can be substituted by other ions, forming fluorapatite and carbonate apatite. Fluorapatite is commonly found in dental enamel, whereas hydroxyapatite is prevalent in bone. [15] The mechanical properties of calcium phosphate ceramics significantly influence their applications as implants. Tensile and compressive strength as well as fatigue resistance are contingent upon the total volume of porosity, as shown in Figure 10, and which can manifest as:

- micropores, with diameters <1mm due to incomplete sintering;
- macropores, with diameters >100mm created to facilitate bone growth.

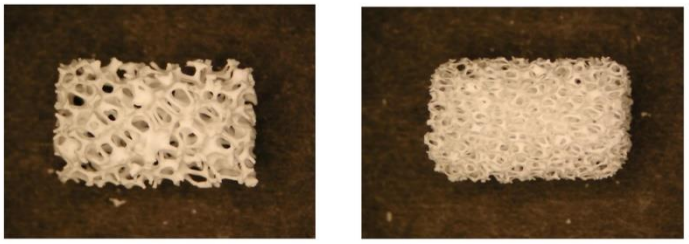

Figure 10: Example of calcium phosphate ceramics with different porosity. [12]

The Weibull modulus (m) of HA implants is low in physiological solutions ($m \leq 12$), indicating low reliability under tensile loads. Therefore, in clinical practice, calcium phosphate bioceramics should be utilized as powders, small-unloaded implants, dental implants, coatings on metal implants, low-loaded porous implants or as a bioactive phase within a polymer-bioactive ceramic composite. [15]

Another important characteristic that makes calcium phosphate-based bioceramics suitable for bone reconstruction and regeneration implants is the resorption, or biodegradation, of this material, which is caused by three factors:

- *Physic-chemical dissolution*, which depends on the material's solubility product and the local pH of its environment. New surface phases may form, such as amorphous calcium phosphate, dihydrate dicalcium phosphate, octa calcium phosphate, and hydroxyapatite substituted with anions.
- *Physical disintegration* into small particles due to preferential chemical attack at grain boundaries.
- *Biological factors*, such as phagocytosis, leading to a decrease in local pH concentrations.

Ideally, one might desire that the substitute material is slowly reabsorbed by the body once it has completed its task of serving as a scaffold for new bone. The degradation or resorption of calcium phosphates in vivo occurs through a combination of particle phagocytosis and acid production. However, when selecting a resorbable material for implantation, attention must be paid to matching the resorption rate with the anticipated tissue regeneration rate. Where the solubility of calcium phosphates exceeds tissue regeneration rates, they will not be useful for filling bone defects.

The dissolution rate increases with decreasing calcium-phosphorus ratio, and thus, tricalcium phosphate, with a $Ca{:}P$ ratio of 1.5, is reabsorbed more quickly than hydroxyapatite. Tricalcium phosphate has four polymorphs: α, β, γ and $super\alpha$. The γ polymorph is a high-pressure phase, while the superα polymorph is observed at temperatures above approximately 1500°C. Therefore, the most frequently observed polymorphs in bioceramics are a^{-} and $\beta - TCP$. X-ray diffraction studies indicate that the β polymorph transforms into the α polymorph at temperatures between 1120°C and 1290°C. [16]

All calcium phosphate ceramics biodegrade to various extents. The biodegradation rate increases as:

- Surface area increases (powders > porous solid > dense solid);
- Crystallinity decreases;
- Crystal perfection decreases;
- Crystal and grain size decrease;
- There are ionic substitutions of CO^{32-}, Mg^{2+} and Sr^{2+} in HA.

Factors that tend to decrease the biodegradation rate include:

- F substitution in HA;
- Mg^{2+} substitution in $\beta - TCP$;
- lower $\beta - {}^{TCP}/_{HA}$ ratios in biphasic calcium phosphates. [16]

2.2.1 Hydroxyapatite

Calcium hydroxyapatite (HAP), with a general formula of $Ca_{10}\,(PO_4)_6(OH)_2$, as shown in Figure 11, constitutes the main mineral component of bones, teeth, nails, and all "hard" parts of mammals, being responsible for their hardness and strength.

One of the properties that makes the structure of hydroxyapatite interesting is the ability to vary its composition without altering its structural properties. There are stoichiometric HPA_S, with the chemical formula $Ca_{10}\,(PO_4)_6(OH)_2$, where the Ca/P ratio is 1.67, and a whole series of non-stoichiometric HPA_S.

Hydroxyapatite is an environmentally friendly material, attractive for several reasons, including its non-toxicity and biocompatibility. The latter, combined with its other chemical and physicochemical properties, has led to the use of HAP in various processes in regenerative medicine.

Hydroxyapatite is virtually insoluble in water ($Ksp = 2.34 \times 10^{-59}$), and its dissociation equilibrium is represented by equation:

$$\boldsymbol{Ca_5(PO_4)_3(OH)_S \rightleftharpoons 5Ca^{2+} + 3PO_4^{3-} + OH^{-1}}$$

The dissociation equilibrium of HPA is influenced by pH, aqueous solutions at low pH remove OH^- ions from the equilibrium, shifting it to the right, resulting in increased solubility of HPA.

The incorporation of different ions into the structure of hydroxyapatite can also influence the properties of the crystal lattice, either improving or decreasing its thermal and chemical stability. Conversely, other substitutions may reduce the material's stability, increasing its solubility, such as substitutions of strontium, magnesium, manganese, and carbonate. [14]

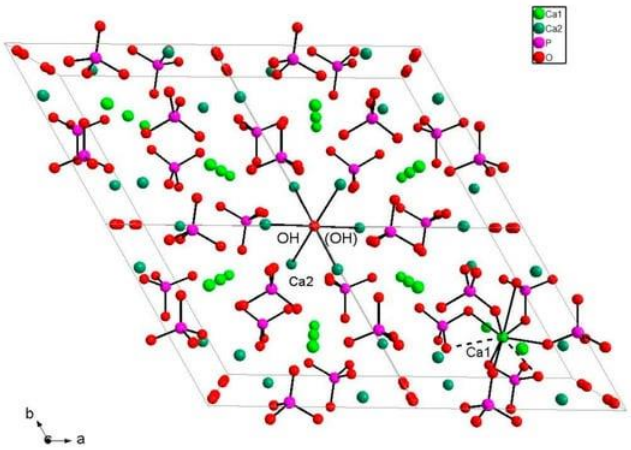

Figure 11: Chemical structure of Hydroxyapatite. [48]

2.2.2 β-TCP

β-TCP, or beta-tricalcium phosphate, is a brittle, white solid whose color can change depending on the presence of impurities. For instance, Mn-doped β-TCP appears intense pink, Cu-doped β-TCP can be blue or sometimes violet/purple, and Cr-doped β-TCP is green. [44]

The most relevant physical properties for bone substitution are the thermal stability range and the crystal structure.

Regarding its thermal stability, β-TCP is a high-temperature phase typically obtained by thermal conversion of amorphous calcium phosphate or calcium-deficient hydroxyapatite (CDHA) above 650-750°C. β-TCP transitions into two other allotropic phases at higher temperatures: α-TCP above 1115-1150°C and α'-TCP above 1430-1470°C. [44]

The exact temperatures for these phase transitions can vary widely depending on elemental impurities present in the material. For example, the β-α phase transition temperature can increase significantly with the addition of magnesium phosphate.

In terms of crystal structure, β-TCP crystallizes in the rhombohedral space group R3c and is typically described in the trigonal setting. The unit cell dimensions can vary, likely due to elemental impurities. Each β-TCP unit cell contains 63 calcium atoms and 42 phosphate groups, with calcium atoms distributed across five different sites. Notably, only 50% of the Ca(4) sites are occupied, exhibiting a specific repeating pattern along the c axis.

β-TCP can be synthesized through three main methods: solid-state reaction, thermal conversion, and precipitation. Each method has its specifics, and the resulting β-TCP can vary in terms of elemental impurities and surface characteristics, particularly due to phosphate species evaporation during sintering. These variations can significantly impact the material's suitability for bone substitution applications. [44]

2.2.3 Bioglass 45S5

Bioglass 45S5, also known as calcium sodium phosphosilicate, is a bioactive glass in which a three-dimensional SiO_2 network is modified by the incorporation of Na_2O, CaO and P_2O_5. It is primarily used in medical applications such as bone grafting, repair of periodontal defects, cranial and maxillofacial repair, wound care, blood loss control, vascular regeneration stimulation, and nerve repair.

Bioglass 45S5 is distinct due to its lower silica content and higher calcium and phosphorus levels compared to common soda-lime glass. The 45S5 name reflects its composition of 45 wt% SiO_2 and a 5:1 molar ratio of calcium to phosphorus, which promotes the formation of apatite crystals essential for bonding with bone. This composition closely resembles hydroxyapatite, the primary mineral in bone, allowing Bioglass 45S5 to integrate effectively with living bone.

Mechanically, Bioglass 45S5 is softer than other glasses and can be machined with diamond tools or ground to powder. As the first artificial material found to chemically bond with bone, Bioglass 45S5 avoids immune reactions and fibrous encapsulation, making it suitable for repairing large bone defects.

Borate-based bioactive glasses derived from Bioglass 45S5 can degrade at controlled rates matching natural bone formation, further enhancing bone repair capabilities. [45] [46]

2.3 Biomedical Applications

Calcium phosphate-based bioceramics have undergone extensive testing for clinical applications and are currently in use or under investigation in various areas of dentistry and orthopaedics, with many others still in development. For instance, loose materials, available in both dense and porous forms, are employed for alveolar ridge augmentation, immediate tooth replacement, and maxillofacial reconstruction. Additional applications include orbital implants, auditory ossicle augmentation, spinal fusion, and bone defect repair. To promote new bone tissue growth within defects, it is crucial to utilize

bioabsorbable materials capable of filling these spaces; otherwise, the growth of fibrous tissue may impede bone formation within the defects.

Calcium phosphate orthophosphate bioceramics are available in various physical forms, such as powders, particles, granules, dense blocks, porous scaffolds, injectable formulations, self-setting cements, coating implants, and composite components of various origins (natural, biological, or synthetic), often with specific shapes like implants, prostheses, or prosthetic devices. Additionally, bone grafts are also proposed in the form of non-setting pastes, typically composed of a mixture of calcium orthophosphate granules and a "glue," typically a highly viscous hydrogel. More specifically, custom-designed forms are also available, such as wedges for tibial opening osteotomy, cones for the spine and knee, and inserts for vertebral cage fusion. [12]

2.3.1 Bone Structure

Bone tissue is a type of specialized connective tissue characterized by the mineralization of the extracellular matrix, which gives it hardness and strength. It is a dynamic tissue, subject to continuous remodelling throughout life, maintained by a balance between the resorption of old tissue and the formation of new bone. Various local and hormonal factors influence this process, and their alterations can cause changes in the bone, such as osteoporosis. [35]

Bone tissue can be classified structurally into two main types: trabecular bone and cortical bone. These two types of bone tissue are found in variable proportions depending on the morphology of the bones and are organized in such a way as to provide the best possible structural configuration to ensure the necessary mechanical properties using the least amount of material. [43]

Both types of bone are present in all bone structures, with varying percentages:

- *Trabecular bone*, also known as spongy bone, is characterized by a dense network of trabeculae, beam-like structures, whose pores contain organic and liquid components. It is found mainly in the epiphyses of long bones and in the middle layer of flat bones, called the diploe. Trabecular tissue is a type of lamellar bone organized in a highly porous three-dimensional network structure. Its mechanical properties depend significantly on the structural density, which reflects the amount of bone tissue in each volume, minus the pore volume. [35] [43]

- *Cortical*, or compact, *bone* is made up of osteons aligned along a defined axis, formed

by concentric lamellae. This type of bone is found in the outer layer of short and flat bones, as well as in the diaphysis of long bones. The outer surface of the diaphysis is covered by the periosteum, a fibrous membrane that provides blood supply and nutrition to the bone. Internally, the periosteum contains an osteogenic layer that allows the bone to enlarge and remodel during growth and aids in healing if damaged. Cortical bone is composed of haversian systems or osteons, cylindrical structures with a central canal containing a blood vessel, surrounded by concentric lamellae. The osteons are connected to each other by Volkmann canals. The lamellae are made of collagen fibres embedded in a matrix of hydroxyapatite crystals. Osteocytes, bone cells, reside in spaces within the lamellae and are connected by canaliculi. [35] [43]

Two ossification processes can occur:

- *Endochondral*: Involves the bones of the spine, pelvis, and limbs, with bone forming from an embryonic cartilage model.
- *Intramembranous*: Occurs in the flat bones of the skull and parts of the mandible, where bone forms directly from connective tissue. [35]

Bones, as shown in the Figure 12, contain four types of cells: preosteoblasts, osteoblasts, osteocytes, and osteoclasts.

- *Preosteoblasts***:** Derived from mesenchymal cells, they are relatively undifferentiated and proliferative, giving rise to osteoblasts during bone growth and remodelling. They form the lining cells that cover trabeculae, preventing access to osteoclasts. [35]
- *Osteoblasts***:** Cuboidal or cylindrical cells that synthesize the organic intercellular substance, primarily composed of type I collagen and glycoproteins, contributing to calcification through osteocalcin. [35]
- *Osteocytes***:** These are osteoblasts in a quiescent state, trapped in the calcified matrix of lacunae. They are characterized by numerous extensions in bone canaliculi, a flattened cell body, and a reduced capacity for protein synthesis. [35]
- *Osteoclasts***:** Giant multinucleated cells derived from the monocyte-macrophage line, responsible for bone resorption. They have a ruffled border with microvilli that secrete protons, creating an acidic environment for the degradation of the bone matrix through enzymes like acid phosphatase and metalloproteins. [35]

Lastly there are two intercellular substances:

- *Organic Matrix*: Composed of over 90% type I collagen, it provides tensile and compressive strength. It includes proteoglycans and glycoproteins such as osteonectin and osteocalcin. Collagen is a triple helix with cross-links involving lysine or hydroxylysine residues. [27][35]
- *Inorganic Matrix*: Composed of calcium phosphate and carbonate in the form of hydroxyapatite, which provides hardness and rigidity. Hydroxyapatite crystals are arranged between collagen fibrils during calcification. Deficiencies in these minerals cause rickets and osteomalacia.[35]

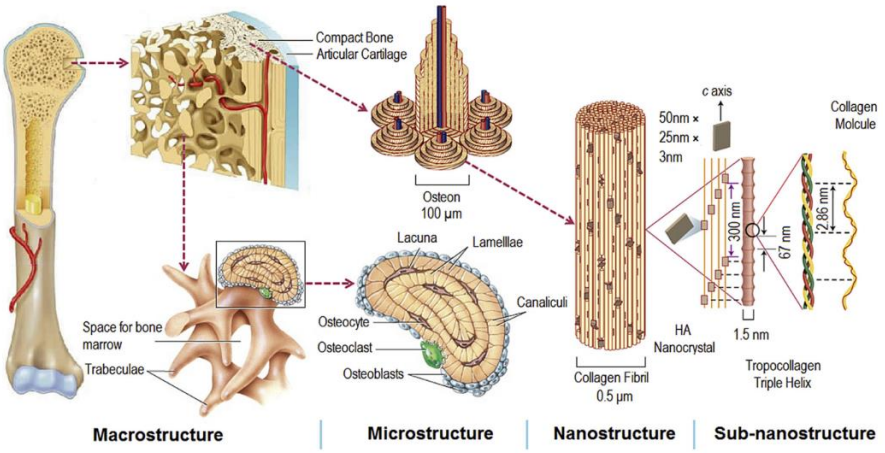

Figure 12: Hierarchical structural organization of bone.[22]

Mechanical Properties

- Cortical bone is a composite material, the main components of which are hydroxyapatite and collagen. Apatite crystals provide strength and stiffness, with an elastic modulus of about 165 GPa, while collagen contributes to ductility and toughness, with an elastic modulus of about 2 GPa. Overall, the elastic modulus of cortical bone, for example in the human femur, is about 18 GPa. The spatial orientation of the osteons determines the anisotropy of the tissue, with mechanical properties that vary depending on the direction of the load. [43]
- Trabecular bone, being highly porous, has mechanical properties that are strongly influenced by the structural density. Stiffness and strength vary as a function of density and strain rate. The reticular structure of the trabecular tissue is optimized to withstand the applied mechanical conditions, maintaining the lowest possible weight. However, with increasing age, the ultimate stress decreases, and the risk

of fractures increases, especially in the elderly or in osteoporotic subjects, due to the accumulation of damage from cyclic loading. [43]

In summary, cortical bone mainly provides structural support and protection, while trabecular bone is more involved in mineral homeostasis and, to a lesser extent, structural function. The different mechanical and microstructural properties of these two types of bone tissue allow bones to effectively adapt to the various functional and biomechanical demands of the human body. [43]

Mechanical Behaviour

The mechanical behaviour of bone tissue is highly complex and influenced by its unique structural properties. One of the key characteristics of bone tissue is its anisotropy, meaning that its mechanical properties vary depending on the direction of the load applied. This anisotropy is a result of the hierarchical and heterogeneous structure of bone. [43]

Longitudinal Direction: Bone exhibits higher stiffness, strength, and resistance to fracture in the longitudinal direction due to the alignment of osteons in cortical bone and the orientation of trabeculae in trabecular bone.

Transverse and Radial Directions: Bone is less stiff and strong in these directions. The arrangement of collagen fibres and the microstructure of the bone matrix play a significant role in this reduced mechanical performance. [43]

- The mechanical properties of cortical bone are highly anisotropic due to the orientation of osteons. The highest strength and stiffness are typically observed along the longitudinal axis (parallel to the osteons) compared to the transverse direction.

  The elastic modulus of cortical bone in the longitudinal direction is about 18 GPa, while in the transverse direction it is significantly lower.

  Cortical bone exhibits higher strength in compression compared to tension. The orientation of the collagen fibres and the mineralized matrix contributes to this difference.

  The presence of collagen fibres increases the toughness of cortical bone, providing resistance to crack propagation. [43]

- The anisotropic nature of trabecular bone is due to the orientation and density of trabeculae, which are aligned along the principal stress directions. This alignment

optimizes the bone's mechanical efficiency and load-bearing capacity.

The mechanical properties of trabecular bone, such as elastic modulus and strength, are highly dependent on its apparent density. Denser regions of trabecular bone exhibit higher stiffness and strength.

Trabecular bone's high porosity reduces its weight while maintaining structural integrity, which is crucial for functions such as shock absorption and mineral storage. [43]

Bone modelling and remodelling

Bone modelling and remodelling are processes of mechanical and functional adaptation, involving changes in the shape of the bone and the properties of bone tissue in response to changing environmental conditions (not only mechanical). Every living system can respond to changes in its surrounding environment, continuously adapting to new functional conditions. Adaptation is a biological process aimed, among other things, at optimizing bone structure in terms of stiffness, strength, and weight. [43]

Bone Modelling:

- *Location***:** Zones of bone resorption, where only osteoclasts (cells that break down bone tissue) act, and zones of bone deposition, where only osteoblasts (cells that form new bone tissue) act, can be identified.
- *Effect***:** Influences the dimensions and shape of the entire bone (e.g., widening of the diaphysis, growth of the proximal area).
- *Duration***:** Requires a relatively long period; skeletal maturation takes about 18 years, while fracture healing takes about one year.
- *Timing***:** Stops at the end of skeletal growth, unless fractures occur. [43]

Bone Remodelling:

- *Location***:** Bone resorption (osteoclasts) and new bone deposition (osteoblasts) occur in the same region through a combined action.
- *Effect***:** Influences the density, mineralization, and structural organization of bone tissue.
- *Duration***:** Resorption takes about 3 weeks, while new bone formation takes about 13 weeks.
- *Timing***:** Lasts throughout the entire life cycle, with a reduction in activity intensity as age progresses. [43]

In summary, bone modelling is predominant during growth and healing, shaping the bone's dimensions and form, while bone remodelling is a continuous process that maintains the quality and structure of bone tissue, adapting to functional needs and environmental conditions throughout life. [43]

The repair and healing process of bone tissue is a complex series of stages including inflammation, hematoma formation, stem cell recruitment and proliferation, blood vessel formation, mesenchymal stem cell differentiation and tissue remodelling. The repair process is shown in the Figure 13. Fractures disrupt the local vascular network, leading to hematoma formation and subsequent inflammation. Immune cells are recruited to the hematoma site within 24 hours to initiate an inflammatory response. After one week, the hematoma and inflammation are cleared, replaced by granulation tissue. Following this phase, various cells including osteoblasts and endothelial cells become activated at the defect site.

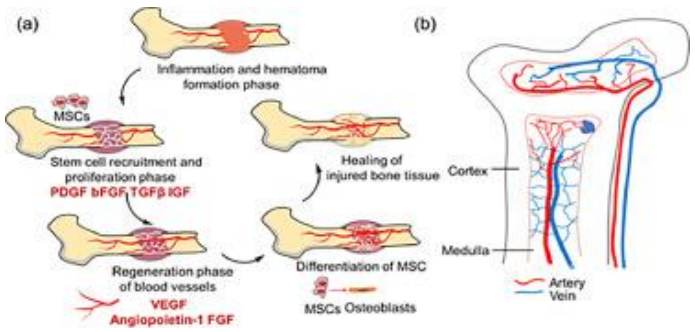

Figure 13: (a) The process of bone tissue repair. (b) The distribution of blood vessels in the bone. [18]

Angiogenesis accompanies stem cell proliferation in bone tissue repair, as shown in the Figure 14, and the abundance of blood vessels is crucial for bone regeneration. The final stage involves bone remodelling, where the interaction between osteoblasts and osteoclasts is significant. It is essential to integrate the characteristics of each process in bone tissue repair with scaffolds, modifying them to enhance efficiency in treating bone defects.[18]

The bone tissue engineering theory aims to cultivate seed cells in vitro, place them on scaffolds to create tissue engineering repair materials, and then implant them into bone defect sites for repair. Key factors in tissue repair engineering include scaffolds, seed cells, and bioactive growth factors, which synergize to offer innovative possibilities for regenerative medicine. Scaffolds play a central role, providing a temporary site for cell

adhesion, proliferation, and differentiation, while also supporting tissue generation and interacting with cells and bioactive molecules to regulate tissue remodelling.

However, current scaffold materials often have limitations, necessitating the rational design of materials with enhanced osteogenic properties. Popular scaffold materials are classified into organic and inorganic categories. [18]

Bioceramics are crucial in bone tissue engineering, utilized in hybrid constructs with synthetic scaffolds and cells for complex skeletal injuries lacking osteogenic potential. Therefore, development focuses on scaffolds supporting cell growth and guiding cell behaviour effectively. [19]

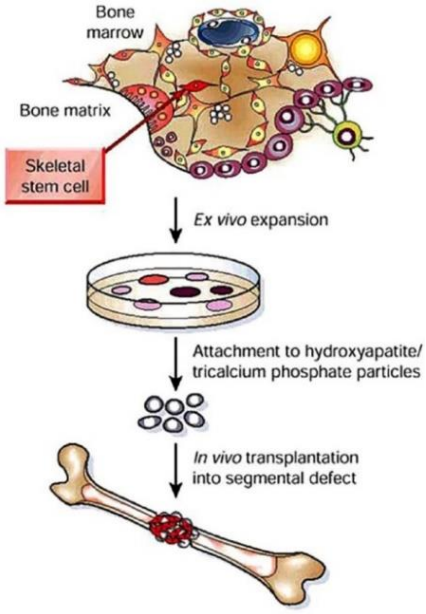

Figure 14: Bone tissue engineering requires ex vivo expansion of marrow-derived skeletal stem cells and their attachment to 3D scaffolds, such as calcium phosphate ceramic particles. This hybrid construct can be transplanted into segmental defects and will subsequently regenerate an appropriate 3D structure in vivo. [19]

2.3.2 Injectable materials

Injectable materials offer numerous advantages in surgical practice. The design of synthetic bone grafts that combine osteogenic and antimicrobial properties represents a significant advancement. The biomimetic Ca-P provides an excellent platform for incorporating antibiotics, allowing control over the release kinetics by adjusting the structural properties of the biomaterial while preserving drug activity. Similarly, other active agents, such as anti-inflammatory or anti-tumour drugs, and growth factors like BMPs, can be incorporated to enhance the osteogenic potential of the bone graft.

In addition to antibiotic release, bioceramics can be used for the local delivery of active

ions that trigger specific biological responses. Ions such as copper, strontium, zinc, cobalt, silicon, and boron can stimulate osteogenesis and angiogenesis, while copper, zinc, and silver also have anti-inflammatory and antibiotic properties. This approach offers advantages such as lower costs and better stability.

Injectable porous biomaterials offer many advantages in surgical practice. An injectable biomaterial that combines multiscale porosity with the ability to harden in situ would significantly benefit clinical practice by being compatible with minimally invasive techniques, geometrically adapting to defects, providing superior reactivity, drug release capability through nano-/micropores, and allowing tissue colonization due to macropores, as shown in the Figure 15. Additionally, using these materials as drug delivery matrices is promising, as their multiscale porosity improves osteoconduction, material resorption, and local drug delivery regulation. [19]

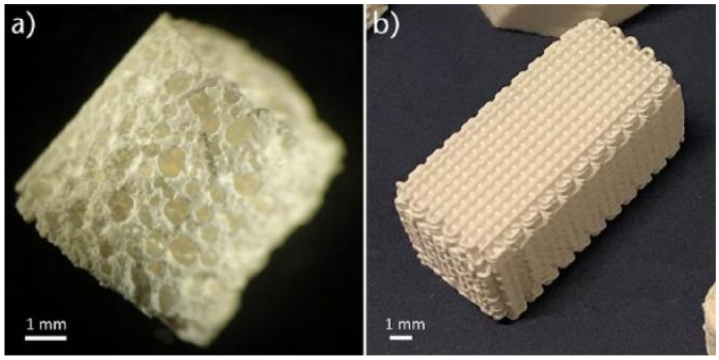

Figure 15: Images of macro porous scaffolds obtained with biomimetic hydroxyapatite: a) injectable self-setting hydroxyapatite foam; b) structure obtained by 3D micro-extrusion of a self-setting hydroxyapatite ink.[19]

2.3.3 Personalized Bone Grafts

The latest 3D printing technologies are ideal for creating personalized bone grafts due to their precise control over geometry. Designs based on X-ray computed tomography data ensure a perfect fit between the graft and the anatomical defect, as shown in the Figure 16. Significant advancements have been made in printing calcium phosphate structures using technologies such as powder bed fusion, binder jetting, fused deposition modelling, evaporative-assisted printing, and micro extrusion. The ability to work at low temperatures broadens the technique's applicability, potentially allowing in-hospital production of patient-specific bone grafts. This enables surgeons to quickly obtain ready-to-use grafts, revolutionizing emergency treatments and influencing surgical planning for non-urgent cases.

An important aspect is the ability to adjust the scaffold's geometry and microstructure to optimize its mechanical properties. Additionally, using in silico models that combine biomechanical requirements with predictions of cellular outcomes can further aid in designing patient-specific 3D functional scaffolds at reduced costs. [19]

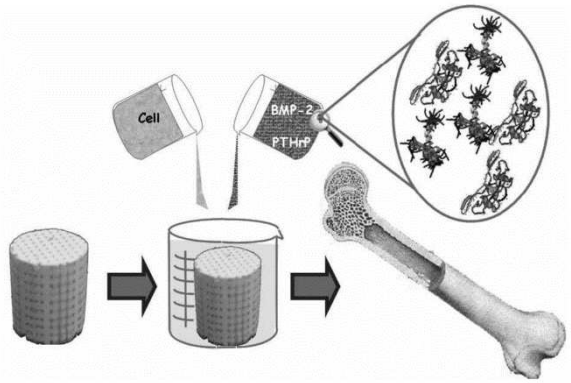

Figure 16: A schematic view of a third-generation biomaterial, in which porous calcium orthophosphate bioceramic acts as a scaffold or template for cells, growth factors, etc. [12]

2.4 Requirements for TE Scaffolds

In the last years bone scaffolds were examined both those made either entirely with hydroxyapatite and both those combinate of HA and other materials such as tricalcium phosphate, chitin, and $PLA-PEG$. Currently, HA is used in bone cement to repair craniofacial defects and as a coating for human hip prostheses. [20]

The architecture of scaffolds plays a fundamental role in promoting bone growth.

There are some key parameters to consider:

- *Macrostructure*: a three-dimensional scaffold that mimics the physiological functions of native ECM is vital for maintaining cells' ability to express their native differentiated phenotypes. An optimal scaffold design will promote cell proliferation and the production of cell-specific matrix, which will eventually assume the role of supporting the degrading scaffold. [21]
- *Porosity* and *pore interconnectivity*: scaffolds must possess an open-pore geometry with a highly porous surface and microstructure that allow for cellular growth and reorganization in vitro and provide the necessary space for neovascularization from surrounding tissues in vivo. Highly porous microstructure with interconnected pore networks is essential to ensure spatially uniform cell distribution, cell survival, proliferation, and migration in vitro. [21]

Macro porosity promotes osteogenesis by facilitating the transport of cells and ions, while *microporosity* enhances protein adsorption, increases ionic solubility, and provides attachment sites for osteoblast. [20]

Pore interconnectivity positively influences bone deposition and infiltration depth, providing space for the vascular system necessary for nourishment and waste removal. [20]

- *Pore size*: the different nature of tissue architectures requires different microenvironments for their regeneration, including the use of scaffolds with optimal pore sizes. Scaffold pore sizes between 20 and 125 µm have been used to regenerate adult mammalian skin and 45-150 µm to regenerate hepatic tissues. When pore sizes are too small, cell pore occlusion by cells will hinder cell penetration and matrix elaboration within the scaffolds. [21]
- *Surface area* and *surface chemistry*: high ratios of internal surface area to volume are essential to accommodate the large number of cells needed to replace or restore tissue or organ functions, and they are linearly correlated. In addition to maximizing surface area, the morphology and physicochemical (material-dependent) properties of the scaffold surface are important factors influencing cell attachment, migration, and intracellular signalling in vitro and cell recruitment and healing at the scaffold-tissue interface in vivo. [21]
- *Mechanical properties*: scaffolds should have sufficient mechanical strength during in vitro culture to maintain the spaces required for cell growth and matrix elaboration. To allow for early mobilization of the treated site, degradable scaffolds should maintain sufficient mechanical strength to withstand any stresses in vivo and physiological loads imposed on the engineered structure. Scaffold degradation should be appropriately regulated to maintain sufficient structural integrity until newly grown tissue has replaced the support function of the scaffolds. [21]

2.5 Solid Freeform Fabrication Techniques: Capabilities and Limitations

Solid Freeform Fabrication techniques (SFF techniques) are computerized fabrication methods discussed in the previous chapter capable of quickly producing highly intricate three-dimensional physical objects using data generated by CAD systems, computer-based medical

imaging modalities, digitizers and other data sources. Unlike conventional computerized machining processes that involve material removal from a stock, SFF techniques operate on the principle of layered manufacturing. This means that three-dimensional objects are built layer by layer through the processing of solid sheets, liquids or powdered material stocks.

One of the most used is the technique known as three-dimensional printing, 3D-P, that utilizes inkjet printing technology for processing powdered materials. The versatility and simplicity of 3D-P enable the processing of a wide range of powder materials, including polymers, metals and ceramics.

During fabrication, a printer head deposits a liquid binder onto thin layers of powder according to the object's profile generated by the computer system. Subsequently, material layers are stacked and printed on top of the previously deposited layers to recreate the full structure of the desired object. Once fabrication is complete, the object is embedded in a cake of unprocessed powders and can be extracted by brushing away the loose powders, as shown in the Figure 17. [21]

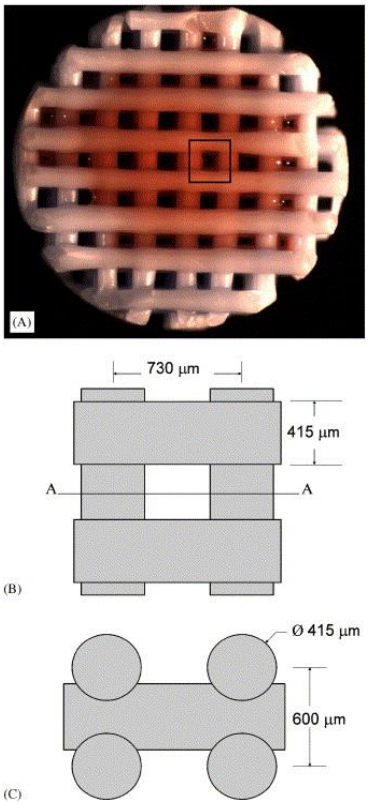

Figure 17: (A) Optical micrograph of microporous scaffold containing 15 µL of a gelatine solution that was dyed red and that localized within the central macropores of the scaffold.

(B) Schematic of the scaffold of the box in (A), viewed from the top. The rod diameter was 415 µm and the centre-to-centre rod spacing was 730 µm in-plane.

(C) Cross-section from (B). [20]

Among the most used free-form solid manufacturing techniques are:

- *Selective Laser Sintering (SLS)* is an additive manufacturing technique that uses high-power laser sources to fuse small plastic, ceramic, and glass particles to print the desired structure. During the printing process, the SLS printer uses CAD software to control the laser. Information from the CAD can be converted into 3D objects by the printer using a laser source and powdered material. The powder bed assists the laser beam by preheating the powdered material; it heats the powder just below its melting point, allowing the particles to sinter into a 3D object. [8]

  This can produce scaffolds with high porosity and interconnected pores by adjusting parameters such as laser power, scan speed, and bed temperature. These parameters influence the thermal energy delivered to the powders, affecting the sintering quality.

  SLS can process polymers, ceramics, and metal powders producing scaffolds with irregular shapes, not requiring solvents or secondary binding systems, minimizing the risk of material contamination.

  Among the limitations of SLS is the size of the pores in the fabricated scaffolds which depends on the particle size of the powder and the compaction pressure during layer deposition. Typical pore sizes are limited to smaller ranges (<50 μm) and are also distributed over a wide range. [21]

- *Stereolithography (SLA)* uses a light source, such as UV or visible light, and a photopolymer resin to create 3D structures. The process begins with the creation of a digital model using CAD software. A digital scanner converts this model into a 3D format, and then the photosensitive resin is irradiated by the light, forming a rigid structure layer by layer. SLA is known for its high resolution, allowing the production of complex details and geometries with smooth, refined surfaces, ideal for the production of detailed prototypes, electronic components, medical devices and model making. SLA can utilize a wide range of photopolymer resins, including biocompatible, heat-resistant, flexible and high-strength materials, which tend to have isotropic mechanical properties.

  Although SLA can produce highly detailed parts, build times can be longer than other 3D printing technologies, especially for large objects. Photopolymer resins used in SLA are generally more expensive than materials used in other 3D printing technologies and may be brittle or susceptible to breakage under high mechanical loads and sensitive to light and moisture. [8]

- *Digital Light Processing (DLP)* technology stands out as a promising method for 3D printing high-performance ceramics, thanks to its ability to uniformly distribute ceramic particles and reduce internal stress. DLP-produced structures boast high density and mechanical properties, [24]

  This technology uses a projector to expose the entire layer of photopolymer resin allowing you to obtain very fine, detailed details and smooth surfaces very quickly.

  DLP printers are often limited in build area size due to the need for high-quality projectors that can maintain high resolution over larger areas, and by the use of specific photopolymer resins, which can be very expensive, and they may have lower mechanical strength than those produced with other techniques such as selective laser sintering (SLS) or material deposition melting (FDM). [10]

- *Fused Deposition Modelling (FDM)* uses works through extrusion melted material to deposit a series of parallel paths to form layers, creating structures with uniform, honeycomb-like internal geometry and controllable pore morphology. In FDM, filament material (usually thermoplastic) is fed and melted within a heated liquefaction head before being extruded through a nozzle with a small orifice. For each deposited layer, the direction of material deposition can be modified.

  Using Fused Deposition Modelling pore morphology can be varied by altering the deposition pattern, including the deposition angle, the width of the deposited material paths, and the spacing between them. Different deposition patterns can produce scaffolds with multi-layer designs and localized pore morphologies, suitable for regenerating various tissue types or multi-tissue interfaces. FDM scaffolds have good structural integrity and mechanical properties due to stable designs and proper fusion between material layers.

  However, FDM has limitations. Pore openings are not consistent in all three dimensions. Pore openings in the z-direction form at intersections of material paths. In the x and y directions, pore openings are created by stacking material layers, limited by the material layer thickness. Thus, FDM allows limited pore morphology variation within the same scaffold volume, except in the z-direction, however the continuous material deposition partially occludes pore openings in the x and y directions. [21]

3. In Vitro and In Vivo Case Studies

In this chapter, we delve into the realm of in vitro and in vivo case studies, exploring the practical applications and outcomes of various techniques, materials, and approaches in the field of bone tissue engineering and regenerative medicine. Through a combination of laboratory experiments and clinical investigations, these case studies provide valuable insights into the efficacy, safety, and potential of different bone repair strategies.

From in vitro studies conducted in controlled laboratory settings to in vivo trials involving animal models or human patients, each case study offers a unique perspective on the advancements in bone regeneration. By examining the outcomes of these case studies, we gain a deeper understanding of the factors influencing tissue regeneration, including biomaterial properties, scaffold design, cellular interactions and environmental cues. Furthermore, we explore how these findings translate into clinical practice, informing the development of novel therapies and personalized treatment approaches for bone defects and injuries. Through the synthesis of in vitro and in vivo data, this chapter aims to provide a comprehensive overview of the current landscape of bone tissue engineering research. [23]

3.1 The most relevant AM Technologies for BTE

The most used Additive Manufacturing technologies in the production of prototypes and fixture structures are S*tereolithography (SLA), Selective Laser Sintering (SLS), Digital Light Processing (DLP),* and *Fused Deposition Modelling (FDM)*. As reported in the first chapter, each technology has its pros and cons, and indeed SLA, SLS and DLP are characterized by:

- *Precision*: SLA, SLS and DLP utilize the polymerization of photosensitive resins through exposure to UV light, allowing for the creation of components with very precise details, complex geometries and smooth surfaces.
- *Production speed*: SLA, SLS and DLP technologies are capable of rapidly producing moving parts, thanks to their ability to polymerize large areas of resin simultaneously.
- *Wide range of materials*: SLA, SLS and DLP printers support a wide range of materials, including thermoplastic, thermosetting, and composite resins, enabling the production of components with specific properties such as temperature resistance, rigidity, and transparency. [3][8][9]

While FDM is used for its:

- *Design flexibility*: FDM, on the other hand, uses fused thermoplastic filament to create objects layer by layer, allowing for greater design freedom and the possibility of integrating complex internal parts.
- *Accessibility*: FDM printers are often more affordable and accessible than SLA and DLP printers, making them a popular choice for hobbyists, small businesses, and education. [6]

In summary, each AM technology has its specific advantages that make it suitable for various applications and specific industrial sectors. The Figure 18 shows some examples of scaffolds.

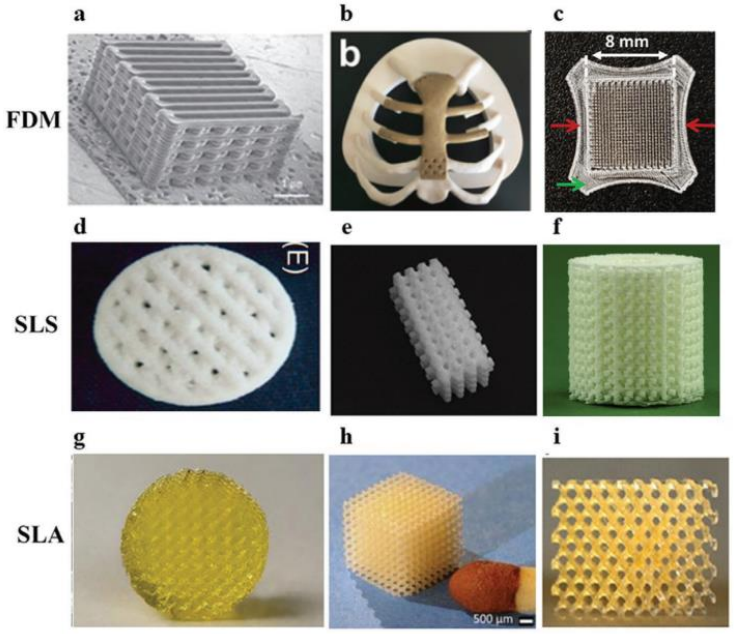

Figure 18: The polymeric materials used for bone regeneration fabricated by three commonly used printing systems.

- *FDM: a) PCL/PLGA, b) PEEK, and c) PLA;*
- *SLS: d) PCL/TCP, e) PCL and f) PCL/TCP;*
- *SLA: g) PDLLA-PEG-PDLLA. h) PDLLA and i) PCL. [23]*

3.2 The Synergy of β-TCP Composites

β-Tricalcium phosphate serves as a valuable material in bone regeneration due to its balanced absorption rate within scaffolds and its ability to provide essential ions like calcium and sulphate. However, its lack of osteoinductivity and osteogenicity limits its efficacy. To overcome this challenge, researchers have turned to β-TCP composite materials in orthopaedics.

These composites leverage the strengths of other bone repairing materials, including biodegradability, osteoinductivity, osteogenicity, and osteoconductivity. By combining β-TCP with these materials, the deficiencies of standalone β-TCP are addressed, enhancing its overall biological and physical properties. This approach broadens the applications of β-TCP in bone regeneration, offering more effective solutions in orthopaedic practices. [26]

Among the combinations that are still being studied are the following:

- *The combination of β-TCP with hydroxyapatite (HA) in biphasic calcium phosphate (BCP)*: this composite addresses the limitations of each material individually. While β-TCP offers rapid absorption and high hydrophilicity, HA presents poor absorption and brittleness. BCP enhances mechanical strength, improves degradation of β-TCP, and promotes new bone formation more effectively than either β-TCP or HA alone. Adjusting the β-TCP/HA ratio allows for maintaining the balance between ceramic material absorption and new bone formation, overcoming the drawbacks of rapid absorption or hard absorption of standalone β-TCP or HA. [26]

- *The combination of β-tricalcium phosphate with Mesenchymal Stem Cells (MSC):* MSCs derived from bone marrow, muscles, and periosteum, can differentiate into various types of cells and promote bone regeneration through the secretion of growth factors.

  In β-TCP/MSC composite materials, MSCs address the limitations of β-TCP in osteogenicity while β-TCP provides a scaffold for MSC differentiation and proliferation. Studies have shown promising results, with β-TCP/MSC composites demonstrating higher fusion rates and increased new bone mass compared to autografts or single β-TCP implants. MSCs adhere to the inner surface of β-TCP scaffolds, enabling osteogenesis throughout the scaffold and effectively preventing scaffold corrosion or dissolution. [26]

- *The combination β-TCP with bone marrow*: Bone marrow, a rich source of MSCs, when combined with β-TCP, improves fusion rates and promotes lamellar bone formation, surpassing the effects of single bone marrow or β-TCP alone. [26]
- *The combination β-TCP with Platelet-Rich Plasma (PRP*): β-TCP with its growth factors promote neovascularization and tissue repair. When combined with β-TCP, PRP enhances cortical bone thickness and new bone mass. [26]
- *The incorporation of Plasma transglutaminase (F XIII) into β-TCP*: this promotes new bone formation by inducing fibrin clot formation and enhancing osteoblast activity. Similarly, the addition of Poly-Caprolactone (PCL) improves the mechanical properties of β-TCP, enhancing scaffold stability while promoting new bone formation. [26]

3.3 The Importance of the Morphology and Porosity of the Structure

The study conducted by L. Shen, Y. Yang and colleagues, particular attention was paid to the morphology and porosity of the structures produced. Using Digital Light Processing technology, structures with high density and high mechanical properties were created.

However, the main challenge remains the replication of artificial bone scaffolds that faithfully mimic human bones. Natural bones are structurally complex and anisotropic, where porous lattice structures can regulate mechanical properties but often fail to achieve specific strength in all directions, which can lead to potential joint failures.

To address this problem, the study proposes a bionic approach to the design of irregular porous structures, aimed at effectively mimicking the natural trabeculae of bone. Morphology and porosity emerge as key parameters in bone repair, as they directly influence the strength and functionality of the produced structures.

Specifically, the material used in the study was a composite made up of 20% by weight of zirconia doped with hydroxyapatite, chosen for its properties compatible with bone repair. After printing, the green bodies were cleaned and sintered at various temperatures (1400, 1450, and 1500 °C). This approach allowed for optimal morphology and porosity, essential for the success of the bone integration process and to ensure the necessary mechanical strength of the produced structures. [24]

Voronoi Tessellation

An innovative method based on probability spheres using Voronoi tessellation has been

employed to generate random seeds for constructing irregular porous structures. Using a computer-aided design (CAD) software, as shown in the Figure 19, it was possible to model porous scaffolds with varying porosities and irregularities. The porosity and irregularity of the scaffolds were adjusted, keeping in mind that as irregularity approaches zero, the corresponding scaffold adopts a type of lattice structure. [25]

The method for generating irregular porous scaffolds begins with the arrangement of points on a plane, to create a regular grid of points within a limited space. Subsequently, each point is randomly redistributed within an imaginary spherical area, where the radius defines the degree of irregularity. Next, Voronoi cells are generated based on these random points, with the point density and edge diameter representing porosity. Finally, the initial porous structure is refined and smoothed at the nodes. [24]

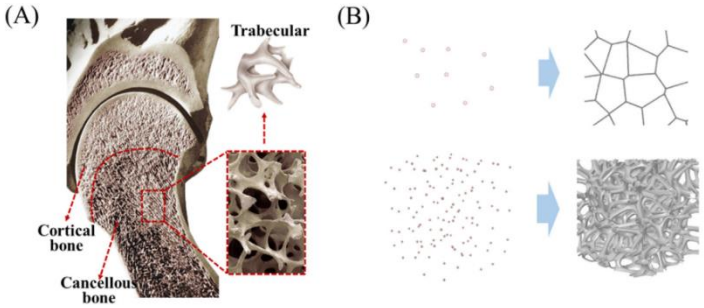

Figure 19: Design method of bio-inspired porous support:

(A) Porous structure in human bone; (B) 2D and 3D Voronoi methods. [24]

Different distinct structures can be generated by defining with different levels of irregularity and levels of porosity, as shown in the Figure 20. [24]

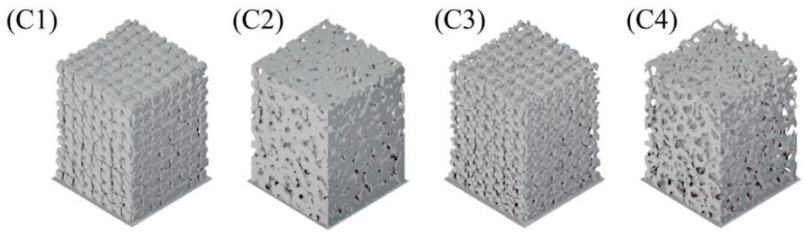

Figure 20: (C1-C4) Por1Ir1, Por1Ir5, Por3Ir1 and Por3Ir5 scaffolds respectively

In this case Ir1-Ir5 denote various degrees of irregularity, with Ir5 representing the most irregular scaffold, while Por1-Por3 indicate increasing levels of porosity, with Por3 the highest level of porosity. [24]

The zirconia exhibited a tetragonal phase at lower temperatures, providing energy absorption and superior fracture toughness, while higher temperatures led to a cubic phase that enhanced

wear resistance. However, excessive temperatures caused a transition to a monoclinic phase, altering the zirconia's structure.

Hydroxyapatite decomposed at 1200°C during sintering, forming tricalcium phosphate (TCP) and biphasic calcium phosphate (BCP), which are known for their mechanical properties and biocompatibility. BCP filled the spaces between zirconia particles, improving the overall structure. The sintering process induced shrinkage while maintaining the shape of the scaffolds due to the layer-by-layer formation process. A higher porosity correlated with greater shrinkage, and scaffolds with higher specific surface areas exhibited easier shrinkage due to increased sample density.

Compression tests revealed that all scaffolds initially showed elastic deformation, with more porous scaffolds displaying greater fluctuations due to smaller strut sizes and higher defect proportions. As compression progressed, destructive cracks appeared, but the scaffolds withstood subsequent compression loads after initial yielding. Mechanical properties, such as compressive strength and elastic modulus, varied significantly with porosity and irregularity, mimicking the characteristics of spongy bone.

Biocompatibility tests showed that adding hydroxyapatite enhanced cell proliferation and differentiation, with moderate irregularity and porosity promoting better cell viability, adherence, and mineralization. This combination of mechanical performance and biocompatibility is critical for effective bone repair.

This study concluded that scaffolds with different porosities and irregularities were analysed regarding shrinkage rate, compression characteristics, and cell-promoting effects. Results suggested that moderate irregularity improves compressive strength by avoiding stress concentration and altering stress transfer pathways. Structural changes significantly influenced parameters such as cell viability, particularly in the early stages, aligning roughness with changes in mechanical properties to enhance biocompatibility. [24]

3.4 In Vitro Applications

Bioceramics, such as bioactive glass, glass-ceramics, and calcium phosphates (Ca-P), as mentioned so far, are widely studied due to their good osteoconductive abilities. It is believed that the bioactivity of bioceramics depends on their ability to induce the formation of hydroxyapatite (HA) in physiological environments. However, this association is not without controversy, as other phases of calcium phosphate, such as biphasic calcium phosphate (BCP), octa calcium phosphate (OCP), and dicalcium phosphate dihydrate (DCPD), can form

in the physiological environment and sometimes be mistakenly identified as HA.
To deepen the understanding, the following are some in vitro and in vivo studies in animal models. [30]

PCL/β-TCP Composite Material Cultured from Mesenchymal Stem Cells

In a study conducted by Zhiyong Ma, Qifan Wang and colleagues, a PCL/β-TCP composite material was prepared using a hybrid sol-gel process, combining Fused Deposition Modelling (FDM) and Meltelectrowriting (MEW) techniques. The result is a cross-scale scaffold with thick fibres (500 μm) providing structural stability and thin fibres (10 μm) filling the pores, facilitating cell growth. The addition of β-TCP to the scaffold provides calcium (Ca^{2+}) and phosphate (PO_4^{3-}), further enhancing bone formation capability.
During in vitro experiments, bone marrow mesenchymal stem cells (BMSCs) were cultured on these scaffolds to evaluate their biocompatibility and osteogenic properties. The results showed that cross-scale scaffolds promote greater cell survival and proliferation compared to traditional scaffolds. Additionally, analysis of osteogenic differentiation revealed significant alkaline phosphatase (ALP) activity and increased calcium deposition, indicating improved bone formation. [32]
In conclusion, cross-scale scaffolds in PCL/β-TCP, prepared using the combination of MEW and FDM printing techniques, provide a favourable microenvironment for bone cell growth and differentiation, demonstrating significant potential for accelerating bone defect repair. This innovative combination of technologies offers promising prospects in the field of bone tissue engineering. [32]

Calcium Phosphate Formation on HA/TCP and α-TCP Bioceramics

The study conducted by L. Yang, X. Renlong, and collaborators examined the formation of Ca-P on various bioceramics through in vitro experiments in simulated body fluid (SBF) and in vivo experiments on animal models, rabbit muscles. The results showed that Ca-P precipitation occurs on all bioceramics immersed in SBF, except for β-TCP. In vivo, Ca-P formation was more complex and variable among the different types of bioceramics, with HA/TCP and α-TCP bioceramics showing the best precipitation capacity.
The most formed Ca-P phase on all bioceramics, except β-TCP, was found to be the octa calcium phosphate (OCP), both in vitro and in vivo. HA was identified only on some bioceramics, with the OCP formation that is prevalent, while HA does not always form. Additionally, Ca-P formation in vivo is more difficult than in vitro, and the differences

between bioceramics are less pronounced in vitro compared to in vivo. It is interesting to note that β-TCP bioceramics, despite demonstrating good osteointegration capabilities, show poor Ca-P formation capacity both in vitro and in vivo.
In conclusion, these results suggest that the bioactivity of bioceramics does not depend on HA formation, but on Ca-P steps through OCP formation. [30]

PCL/β-TCP Membranes for Guided Bone Regeneration

The study conducted by Jung-Bo Huh and colleagues focuses on improving the properties of the resorbable membrane using different manufacturing methods, including 3D printing. In particular, PCL/PLGA/β-TCP membrane was fabricated via 3D printing and demonstrated superior bone regeneration capabilities in animal models compared to other previously recreated membranes. Mechanical tests showed that PCL/β-TCP membranes had similar elastic modulus to collagen membranes when wet, indicating good mechanical properties under humid conditions.
In vitro studies have shown increased cell proliferation and osteogenic differentiation on PCL/β-TCP membranes, suggesting their potential for guided bone regeneration.
In vivo results showed successful healing without complications in all groups. In fact, micro-CT imaging indicated greater bone regeneration in the PCL and PCL/β-TCP groups compared to the collagen group, confirming the efficacy and osteogenic potential of PCL/β-TCP membranes for guided bone regeneration. [33]

HA/β-TCP scaffold for oral and maxillofacial bone regeneration

The study conducted by Di Pietro Natalia and colleagues, had as its primary objective to evaluate the efficacy of an HA/β-TCP scaffold in promoting angiogenesis and bone regeneration in oral and maxillofacial tissue engineering. The HA/β-TCP scaffold was prepared with defined channel and pore sizes to support angiogenesis. Primary cultures of human osteoblasts (hOBs), osteoclasts (hOCs), and human umbilical vein endothelial cells (HUVECs) were used. The cells were co-cultured with the HA/β-TCP scaffold under normoxic conditions (20% oxygen tension) for 14 days. Cell adhesion and morphology were assessed using scanning electron microscopy (SEM) and confocal microscopy, and cell viability was assessed using the Calcein AM/Propidium Iodide assay. All cell types showed viability and strong adhesion to the scaffold surface. Formation of bone matrix and vessel-like structures was observed, indicating successful differentiation and functionality of cells within the scaffold. Integration of differentiated cells with the HA/β-TCP scaffold prior to

implantation shows significant potential as a strategy for bone regeneration. [39]

3.5 In Vivo Applications

Clinical treatment of massive bone defects due to traumatic injury or tumour resection is still a significant challenge. Alternatives to autologous bone grafts, such as bone substitute materials, have been a focus of biomaterial engineering. Ideal bone substitute materials promote cell migration, proliferation, and differentiation, supporting bone tissue growth. [38]
As anticipated in paragraph 3.2, the combination of β-tricalcium phosphate (β-TCP) with hydroxyapatite (HA) in biphasic calcium phosphate (BCP) combines the strengths of both HA and β-TCP for better biomechanical properties and greater osteoinductive potential, and they are key materials in bone tissue engineering due to their biocompatibility and bioactivity. They are widely studied in in vivo studies for their ability to adsorb bone-related proteins such as bone morphogenetic proteins (BMPs), which are crucial for bone regeneration and the good outcomes that can be achieved. [36] [37]

In fact, their main properties are:

- Hydroxyapatite (HA): Highly biocompatible, stable, and has high osteogenic potential.
- Tricalcium Phosphate (TCP): More degradable than HA but with lower mechanical strength.
- Biphasic Calcium Phosphate (BCP): Combines HA and TCP, offering better mechanical properties and osteoinductive potential.
- Bone Morphogenetic Proteins (BMPs): BMPs, especially BMP-2, are vital for bone formation and differentiation of osteoblasts. They enhance bone repair when combined with CaP materials but require controlled release due to rapid diffusion.
  The adsorption of BMP-2 on CaP ceramics depends on the surface properties and composition of the ceramic, as we will see in subsequent studies. [37]

Regenerative Repair of a Goat Bone Defect

The study by W. Jianxin, X. Zhao, Z. Xiangdong, and colleagues focuses on developing biphasic calcium phosphate (BCP) bioceramic scaffolds for effectively regenerating large segmental load-bearing bone defects. These defects pose a significant challenge due to the lack of synthetic biomaterials with both osteoinductive and osteoconductive properties. The researchers aimed to address this gap by creating BCP scaffolds with optimized porosity, interconnectivity, and compressive strength, utilizing a HA/β-TCP composition (30/70).
The BCP precursor powder was prepared using the wet precipitation method, and porous

ceramics were produced using the H_2O_2 foaming method, followed by microwave foaming, drying, and sintering.

In vitro tests showed enhanced cell proliferation due to the scaffold's porous structure, with significant upregulation of osteogenic markers.

In vivo tests in goats demonstrated new bone growth and scaffold integration, with gradual bone remodelling over 18 months. Mechanical testing revealed a progressive improvement in bending load, reaching near-normal levels by the end of the study. Bone volume (BV) and bone mineral density (BMD) increased over time, with homogeneous bone distribution observed by the 18-month mark.

The study concluded that BCP ceramic scaffolds demonstrated effective bone regeneration, integration, and mechanical function restoration. The success of the scaffolds was attributed to their optimal porosity, degradation rate, and fixation techniques, suggesting significant potential for clinical applications in treating large segmental bone defects. [28]

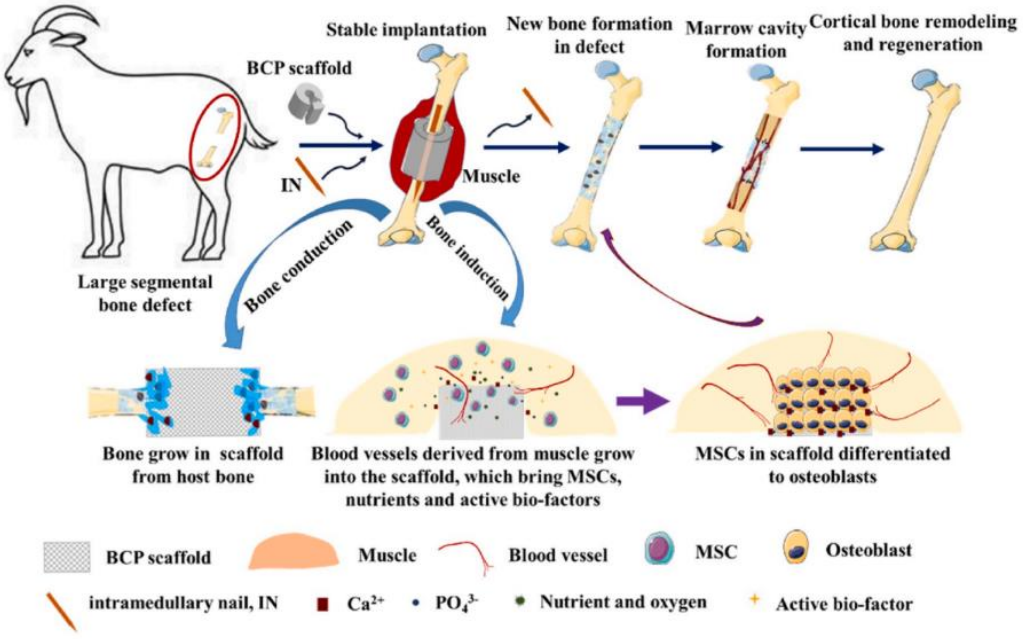

Figure 21: Schematic diagram of combining osteoinductive biphasic calcium phosphate (BCP) bioceramic scaffolds with intramedullary nail fixation for creating stable osteogenic microenvironment was applied to repair large segmental bone defects (3.0 cm in length) in goat femur model.

Regenerative Repair of a Rabbit Calvarial Defect

The study conducted by W. Chao, Z. Hongmei and colleagues aims to demonstrate that bioceramic scaffolds, fabricated using digital light processing (DLP) technology, possess the

necessary biomaterial properties for bone growth. The objective is to compare the osteogenic properties of four biomimetic scaffolds through in vivo experiments, without using cellular components or growth factors.

The experiment was conducted on rabbits, where the scaffolds were implanted in a calvarial bone augmentation model, as shown in the Figure 22, to evaluate their osteogenic and biomechanical properties. After 4- and 8-weeks post-implantation, rabbit calvarial samples were collected to assess bone formation capabilities using Micro-CT scanning.

3D-reconstructed Micro-CT images showed significant new bone formation adjacent to the calvaria in each of the four different scaffold microstructures.

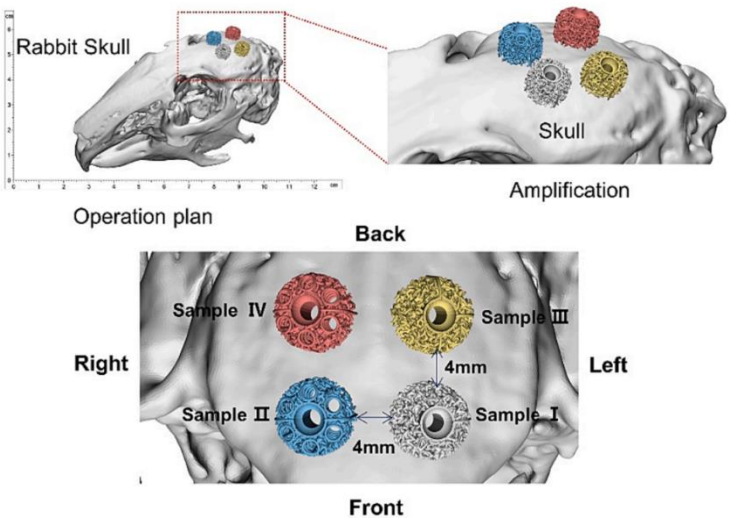

Figure 22: Surgical planning diagram on a Rabbit Calvarial Defect. [31]

A greater volume of newly formed bone was observed after 8 weeks compared to 4 weeks. The new bone tissue mainly grew in the lower half of the scaffolds, with the highest osteogenic height at the interface between the titanium retention pins and the surrounding bioceramic scaffold, indicating that the structural characteristics of the scaffolds facilitate nutrient and oxygen transport and promote angiogenesis. No significant structural collapse or fracture was observed in the scaffolds after 8 weeks, demonstrating their stability.

In conclusion, the additively manufactured bioceramic scaffolds have demonstrated effective bone-forming capabilities, with significant bone integration and structural stability observed at 4 and 8 weeks. Their structural design facilitated nutrient and oxygen transport, thereby promoting the formation and maturation of new bone. These results support the potential of bioceramic scaffolds for applications in bone surgery and regeneration, providing an innovative solution for future clinical applications in bone growth. [31]

Adsorption of proteins on BCP

As anticipated in paragraph 3.2, the combination of β-tricalcium phosphate (β-TCP) with hydroxyapatite (HA) in biphasic calcium phosphate (BCP) combines the strengths of both HA and β-TCP for better biomechanical properties and greater osteoinductive potential.

Bone morphogenetic proteins (BMPs), particularly BMP-2, are critical for bone formation and osteoblast differentiation. CaP biomaterials loaded with BMP-2, in the study conducted by Yanying Wang and colleagues, showed improved bone repair capabilities. However, the efficiency of BMP-2 depends on its controlled release, which is influenced by the properties of the CaP carrier.

The study focuses on the adsorption behaviours of BMP-2 on HA and BCP ceramics. It was found that BCP, with a larger specific surface area (SSA), had a higher adsorption capacity for BMP-2 than HA. The adsorption process was analysed using various isothermal models, with the Langmuir model fitting the data best. Kinetic studies revealed that the adsorption followed a pseudo-first-order model, indicating that physisorption, including electrostatic attractions and hydrogen bonds, was the main mechanism.

The study shows that BCP, with its higher specific surface area (SSA), has a greater adsorption capacity for BMP-2 compared to HA. The interaction between BMP-2 and CaP surfaces involves electrostatic attractions and hydrogen bonding, essential for efficient protein adsorption and subsequent bone regeneration. This study of BMP-2 adsorption on CaP ceramics highlights the superior performance of BCP over HA due to its higher SSA and more abundant adsorption sites. These findings support the potential of BCP as a carrier for BMP-2 in bone regeneration applications. [36]

Regenerative Repair of Dogs Femur Defect

The study conducted by Xiao Yang and colleagues, aims to fabricate micro/nano hybrid BCP bioceramics for segmental bone defect regeneration. Beagle dogs were used as the experimental model, as mouse models do not accurately reflect human cortical bone. During implantation, autologous bone marrow was applied to the implant surface to accelerate healing.

In this study, biphasic calcium phosphate (BCP) bioceramics composed of micro-whiskers and nanoparticles hybrid-structured surface (hBCP) were fabricated via a hydrothermal reaction.

Twelve Beagle dogs, divided into control and experimental groups, were used in the in vivo study. The dogs underwent surgery to create a segmental defect in the femur, which was

stabilized with stainless steel plates. After implantation, dogs were monitored with X-rays and implants were harvested after 12 weeks for analysis.

hBCP bioceramics were then characterized using techniques such as continuous stiffness measurement (CSM), X-ray diffraction (XRD), and micro-CT to assess structural changes and phase compositions.

In vitro studies such as protein adsorption assay and cell proliferation were performed using fetal bovine serum and mouse bone marrow-derived mesenchymal stem cells (MSCs) to assess biocompatibility and cellular responses.

MSCs cultured on hBCP and control bioceramics were subjected to microarray analysis to identify differentially expressed genes (DEGs) associated with bone regeneration and inflammation.

hBCP bioceramics demonstrated a complex structure with increased bone integration capacity, by quantification of micro-CT analysis, which revealed that the bone/material ratio was 9.1% higher in the hBCP group compared to that of the BCP control group.

Macro- and nano-level mechanical tests indicated that hBCP implants had elastic modulus values closer to natural bone, potentially due to higher collagen content.

Finally, MSCs cultured on hBCP showed significantly lower expression of genes associated with inflammatory response pathways compared to bioceramics of

The results suggest that hBCP could be a promising alternative for the treatment of massive bone defects, warranting further long-term studies for clinical translation, being a potential alternative to autologous grafts. [38]

3.6 Purpose of the Studies

The studies conducted so far, some of which are reported in this thesis, concern the development and characterization of porous ceramic structures of beta-tricalcium phosphate (β-TCP) using 3D printing technology. In all these studies, porous ceramic structures of β-TCP have been designed for bone repair applications and have been evaluated in terms of morphology, phase composition, mechanical strength, and biocompatibility. In particular, the DLP technique has proven to be promising compared to other techniques for manufacturing these particular structures, which have to exhibit a good balance between mechanical properties and adequate porosity to promote bone growth. In vitro and in vivo studies have confirmed the cell adhesion capability and biodegradability of porous β-TCP structures, suggesting their potential for bone repair applications, but highlighting a fundamental

problem in bone regeneration, as well as the rapid absorption of the treated material, discussed in the next paragraph. [34]

3.7 Limits of Calcium Phosphate-based Bioceramics

Calcium phosphate ceramics, as discussed so far, are widely used in bone tissue repair due to their biocompatibility and osteoconductivity. Among these, β-tricalcium phosphate (β-TCP) is notable for bone regeneration and bone-engineering applications thanks of its similarity to human bone. However, β-TCP has some limitations, such as low mechanical strength and high resorption rate, which restrict its broader use. [29]

Recently, akermanite ($Ca_2MgSi_2O_7$), a bioceramic containing calcium, magnesium, and silicon, has gained attention for its controllable mechanical properties and degradation rate. Studies have shown that stem cells and osteoblasts exhibit better proliferation and osteogenesis on akermanite compared to β-TCP, suggesting akermanite might be a superior bone graft material. The bioactivity of akermanite is likely influenced by the release of ions (Si, Ca, Mg) that regulate cell growth and gene expression.

Research, conducted by Yan Huang, Xiaogang Jin and colleagues, involved using human bone marrow-derived stromal cells (hBMSC) to assess the effects of akermanite and β-TCP extracts on cell proliferation, differentiation, and osteogenic marker gene expression. Results indicated that hBMSC proliferation and ALP activity were higher with akermanite extract than with β-TCP extract. In vivo experiments with rabbit femoral condyle models demonstrated greater new bone formation and material degradation in akermanite implants compared to β-TCP. [29]

Histological analyses confirmed more substantial new bone penetration and degradation for akermanite after 16 weeks. This improved performance is attributed to the beneficial effects of Si and Mg ions on bone metabolism and mineralization.

Therefore, akermanite has showed superior bioactivity, promoting better hBMSC proliferation, differentiation, and bone regeneration compared to β-TCP. Its appropriate degradation rate and biocompatibility make it a promising bioceramic for bone tissue engineering and regeneration. [29]

A recent study, conducted by Xiang Li and colleagues, aimed to improve the mechanical performance and biocompatibility of β-TCP by incorporating 15% Akermanite (AK) into the structure. The fabrication process utilized Digital Light Processing (DLP) technology to create green ceramic bodies, followed by debinding and high-temperature sintering to obtain

the ceramic scaffold. Subsequently, employing an in-situ growth technique to generate micro/nano surface topography on the scaffolds promoted the synergistic regulation of cellular behaviour and controlled release of bioactive agents. Scanning electron microscopy (SEM) revealed that the addition of AK increased the grain size and reduced the number of micropores in the composite scaffold. Furthermore, a significant enhancement of micro/nano surface features was observed after the in-situ growth treatment. Mechanical tests showed that incorporation of AK improved the mechanical properties of the composite scaffold, increasing the strength by about 20%. Cell experiments indicated that AK integration further improved the cellular compatibility of the material, and the presence of micro/nanostructures effectively promoted cell adhesion, proliferation, and expression of osteogenic genes. [40]

These results suggest the potential of Akermanite as a promising bioceramic material for bone tissue engineering applications due to its favourable degradation rate and biocompatibility. [29]

This could be a strategy to fabricate porous bioceramics that stimulate bone growth, offering high strength and improving the degradation microenvironment, thus opening new perspectives for future applications in bone tissue engineering. [40]

Conclusions

Bone diseases and injuries are becoming increasingly common in society, particularly as conditions like diabetes significantly impair the bones' ability to self-repair. This rising prevalence has fueled a growing demand for effective therapies, continually driving research toward new techniques for improved treatments. Current strategies for enhancing bone regeneration focus on incorporating drugs, growth factors, or genes to support the natural healing process. Scaffold-based delivery systems, given their suitability for such applications, are likely to become more prevalent in the coming years. [42]

Calcium phosphates, especially β-tricalcium phosphate (β-TCP), are considered the gold standard for bone tissue regeneration owing to their osteogenic, bioresorbable, and antibacterial properties. Recent studies have focused on developing and characterizing porous ceramic structures of β-TCP using 3D printing technology. Particularly, the Digital Light Processing (DLP) technique has shown promise in creating structures that balance mechanical properties and porosity, essential for promoting bone growth. These studies have confirmed the cell adhesion capability and biodegradability of porous β-TCP structures, highlighting their potential for bone repair applications despite issues with rapid absorption. However, studies have revealed that β-TCP has limitations such as low mechanical strength and rapid absorption, which can outpace new bone formation, undermining its effectiveness in long-term bone repair and restricting its broader application. [41]

To address these limitations, researchers have explored various composites and combinations. Based on osteoinductivity, good results are achieved using β-tricalcium phosphate (β-TCP) composites, such as β-TCP/HA (hydroxyapatite) and β-TCP/MSC (mesenchymal stem cells). However, even better results have been obtained by combining β-TCP with akermanite. Due to its favorable degradation rate and biocompatibility, akermanite can be used to fabricate porous bioceramics that stimulate bone growth by offering high strength and improving the degradation microenvironment. This combination of materials opens new perspectives for future applications in bone tissue engineering, providing advanced solutions for bone regeneration and repair. [29] [40]

Combining bioactive chemical composition, nanostructure, and bone-like mechanical performance is a challenge for ceramics due to the need for sintering and the difficulty in creating complex 3D structures that mimic natural bone. Traditional methods developed over

the last decades, such as partial sintering, replication method, sacrificial template, direct foaming, and various 3D printing technologies are promising. Recent innovative approaches focus on low-temperature processes, such as chemically induced consolidation of CaP pastes or biomorphic transformation processes. These methods enable the creation of scaffolds that maintain nanocrystallinity, ion-doped bioactive compositions, and hierarchically organized multiscale architectures derived from natural sources, but often fail to produce bioactive and effective bone scaffolds. Therefore, future progress depends on the development of new approaches to create scaffolds with bone-mimicking characteristics and effective regenerative capabilities. [41]

These advances open new perspectives in ceramic science and encourage further research, thanks to their excellent biocompatibility, avoiding adverse body reactions, and providing a natural source of calcium ions to promote bone mineralization. Future research should focus on understanding the mechanisms and processes that guide bone formation to better mimic the natural healing process. This includes material strength for supporting hard tissues, degradation rates of implanted biomaterials or devices, drug release profiles, and biological cues necessary for cell differentiation. Achieving adequate mechanical properties is critical, particularly to regenerate load bearing critically sized bone defects. [40]

In conclusion, combining β-TCP with akermanite represents a significant advancement in bone tissue engineering. This combination offers improved mechanical strength, controlled degradation rates, and enhanced bioactivity, making it a promising solution for bone regeneration and repair. Future research should continue to explore the mechanisms guiding bone formation to better mimic the natural healing process, focusing on material strength, degradation rates, drug release profiles, and biological cues necessary for cell differentiation. Achieving adequate mechanical properties is critical, especially for regenerating load-bearing bone defects, thus opening new avenues for effective bone regeneration therapies.

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