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Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfill the requirements of Master of Science degree in Biomedical Engineering

by
André Alves Moreno Sampaio Lobo

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i. Abstract

Periprosthetic joint infections are one of the leading challenges in the orthopedic field today. This specific type of infection surrounds bone's prosthesis and the tissues adjacent to it, occurring mainly when a total joint arthroplasty is performed. When successful, this joint replacement provides pain relief and restores function and independence to the body, improving the patients' quality of life. The full dynamic of these infections is still unclear, but as the number of joint arthroplasties keeps rising due to the aging population, so does the incidence of infections.

The selection of materials to interact with hard tissues, such as bone, must possess two essential characteristics for the proper healing of the tissue surrounding the material, which are osteoconduction and osteoinduction. Osteoconduction is the ability of bone to grow on a surface, in this case, the material's surface. Osteoinduction is the process of inducing new bone formation, also known as osteogenesis, by the mobilization of immature and undifferentiated cells, differentiating them into pre-osteoblasts.

Currently, calcium phosphate ceramics, such as hydroxyapatite (HAp), are promising substitutes for large orthopedic defects' remodeling and regeneration. This biomaterial presents osteoconductive and osteoinductive properties. However, pure HAp is a material that can be limited by its brittleness, low fracture toughness, bad tensile strength and poor wear resistance. A lot of effort has been put into modifying HAp with the use of polymers in order to enhance its clinical applications. The polymer used in this work was chitosan, which possesses an intrinsic antimicrobial nature, it is biodegradable and biocompatible, and it can be molded into all sorts of structures.

This biocomposite was tested in L929 fibroblast cells in other to assess the toxicity of the material. Besides that, the paste was tested in a pre-osteoblastic cell line (MC3T3) by several techniques, to determine the differentiation, proliferation, and calcium production of the cells when in contact with the biocomposite. This work presents a new biocomposite that should have the appropriate characteristics, not only to promote faster regeneration of the bone defect, but also to reduce the incidence rate of periprosthetic joint infections, through the antimicrobial properties of chitosan.

Keywords: Hydroxyapatite; Chitosan; Osteogenesis; Bone Regeneration; Periprosthetic Join Infection.

i.i. Resumo

Atualmente, as infeções articulares periprostéticas são um dos principais desafios no campo ortopédico. Este tipo específico de infeção que envolve a prótese óssea e os tecidos adjacentes ocorre principalmente após uma artroplastia total da articulação. Quando bem-sucedidas, estas substituições articulares proporcionam alívio da dor, restauram a função e a independência do corpo, melhorando assim a qualidade de vida do paciente. Ainda não é claro como estas infeções se estabelecem, no entanto como o número de artroplastias continua a aumentar, devido ao envelhecimento populacional, a incidência destas infeções também continua a aumentar.

Na escolha dos materiais para interagir com tecidos duros como o osso, existem duas características inerentes a esses materiais que devem estar presentes, que são a osteocondução e a osteoindução. A osteocondução é a capacidade de o osso crescer numa superfície, neste caso, na superfície dos materiais, e a osteoindução é o processo que induz a formação de osso novo, também conhecido como osteogénese, mobilizando células imaturas e diferenciando-as em pré-osteoblastos.

Atualmente, os cerâmicos de fosfato de cálcio, como a hidroxiapatite (HAp), são substitutos promissores para a remodelação e regeneração de defeitos ortopédicos. Este biomaterial apresenta osteoconductividade e osteoindutividade. No entanto, a HAp pura é um material que pode ser limitado pela sua fragilidade, baixa resistência a fraturas, à tração e ao desgaste. Tem sido feito um grande esforço para modificar HAp com polímeros, a fim de melhorar as suas aplicações clínicas. O polímero utilizado neste trabalho foi o quitosano, que possui propriedades antimicrobianas intrínsecas, é biodegradável e biocompatível e pode ser moldado em vários tipos de estruturas.

Este biocompósito foi testado com a linha celular de fibroblastos L929, com o objetivo de inferir sobre a toxicidade do material. Para além disso, o biocompósito foi testada em células de uma linha pré-osteoblástica (MC3T3) através de várias técnicas, de forma a determinar a diferenciação, proliferação e produção de cálcio das células em contacto com o composito. Este trabalho apresenta um novo biocompósito que deve ter as características necessárias para, não só promover uma regeneração mais rápida de defeitos ósseos, mas também ser capaz de reduzir a taxa de incidência de infeções articulares periprotéticas, graças às propriedades antimicrobianas do quitosano.

Palavras-chaves: Hidroxiapatite; Quitosano; Osteogénese; Regeneração Óssea; Infeção Periprostética Articular.

i.i.i. Acknowledgements

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Lastly, I need to disclose my deepest gratitude towards Dra Ana Rita Pinto, who gave me unbroken support and guidance over all my performance. Through all my mistakes and doubts, it was her aid and encouragement that allowed me to keep going, and without which this work would have not been possible.

i.v. Contents

1. Lists of Figures and Tables

2. List of abbreviations

PJI	Periprosthetic Joint Infection
TJA	Total Joint Arthroplasty
THA	Total Hip Arthroplasty
TKA	Total Knee Arthroplasty
SPARCS	Statewide Planning and Research Cooperative System
MRSA	Methicillin-resistant *staphylococcus aureus*
BMI	Body Mass Index
HAp	Hydroxyapatite
DLS	Dynamic Light Scattering
PDI	Polydispersion Index
MTT	(3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide)
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal Bovine Serum
Pstrep	Penicillin-Streptomycin
DMSO	Dimethyl sulfoxide
Col I	Collagen type 1
UV	Ultraviolet light
CO_2	Carbon dioxide
dsDNA	Double stranded DNA
ALP	Alkaline phosphatase
MUP	4-Methylumbelliferyl phosphate disodium salt

3. Introduction

3.1. Bone

Bones are a type of connective hard tissue that becomes mineralized and is needed for several functions of the body, such as locomotion, support, and protection of soft tissues. They are also used as calcium and phosphate storage and harbor bone marrow.[1]

At the microscopic level, bone can be seen as being made of cells and a matrix composed of organic and inorganic substances. The organic component of this matrix is mainly collagen type one (Col I). The inorganic substances present in the matrix are calcium and phosphorus in the form of hydroxyapatite crystals.[2] The bone cells are originated in the bone marrow, which is continuously producing new bone and blood cells for the correct development of bone tissue.[2,3]

There are four different cell types: osteoblasts, bone lining cells, osteocytes, and osteoclasts, whose origins are depicted in figure 1. The osteoblasts are the cells responsible for the creation of the organic part of the bone matrix.[3] These cells produce a material called osteoid, which is mainly composed by Col I.[4] The arrangement in which the Col I fibers are deposited in the matrix enables the bone tissue to be shock absorbent. This "architecture" can be seen in lamellar bone, which is the mature bone, and it's what makes the structure of the bone resilient instead of brittle.[3]

Once an osteoblast stops producing osteoid, it has three pathways that can follow. The cell will either die through apoptosis or change into another bone cell type. If the cell continues to live, it will either become an osteocyte embedded in the osteoid matrix or a bone lining cell.[3]

The last bone cell type is the osteoclast. Unlike the osteoblasts, which came from a mesenchymal cell line from the bone marrow, osteoclasts are created from macrophages or monocytes, as figure 1 illustrates.[3] According to recent studies, mesenchymal and hematopoietic stem cells form a unique niche inside the bone marrow.[5] These cells are responsible for the reabsorption of dead bone (i.e. fractured bone).

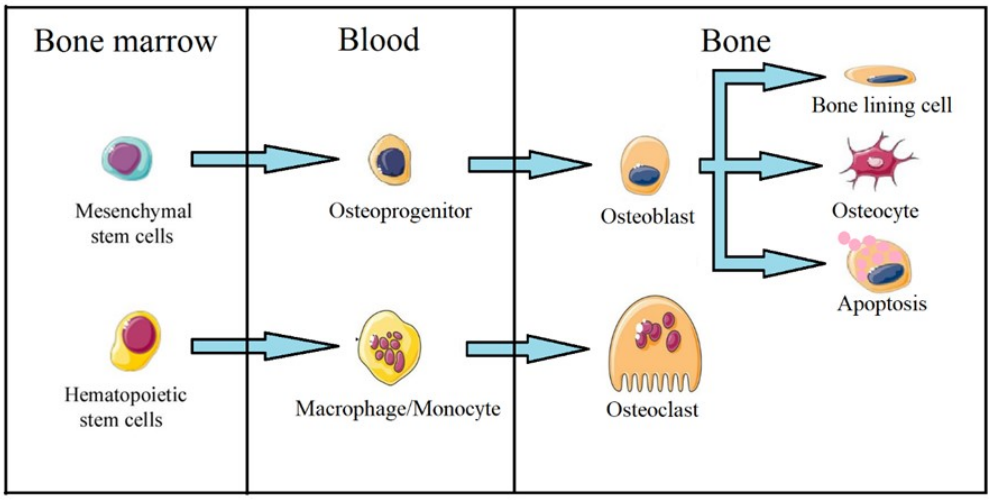

Figure 1. Bone cells differentiation.

Bone tissue starts to be developed in the embryo by the osteoblasts on a pre-existing mesenchymal tissue framework of Col I, creating calcified cartilage or woven bone. Once these bones are formed, they act as a new framework on which the remodeling can occur, creating the mature lamellar bone.[6] Bones are in a continuous remodeling process, being reabsorbed by the osteoclasts, with the help of osteocytes, and reformed by osteoblasts. Additionally, bone lining cells are thought to play a role in the connection between bone remodeling and reabsorption.[7,8]

The differences between immature bone (woven) and mature bone (lamellar) are depicted in figure 2, where histological cuts were made in both lamellar and woven bone. In both types of bone, the pink stained areas are the bone matrix, as they get the color from the presence of Col I. Not only that, but the purple spots present inside of the matrix are osteoblasts that got embedded into the matrix and became osteocytes.[6][9]

In the woven bone from Figure 2, there are purple-stained spaces alongside the matrix, which are cartilage remnants, as the bone is still immature, and has not gone through the complete process of ossification. Unlike with the lamellar bone (mature bone), the bone matrix does not seem to display any kind of pattern, having either random or homogeneous deposition of Col I.

In the case of the lamellar bone from figure 2, as it is mature, there is no more cartilage present (no big stains of purple). The bone matrix is not homogeneous, as the Col I was deposited in a tree-like pattern, creating parallel lines that can be seen in the

pink areas of the lamellar bone.[6][9] There are also purple cells that surround the osteoid matrix, which are osteoblasts that differentiated into bone lining cells.[9]

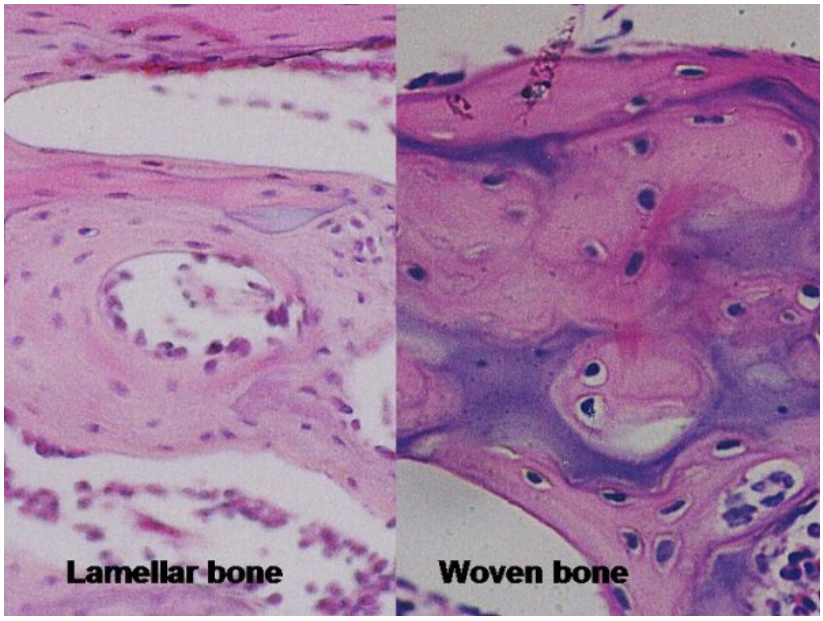

Figure 2. Histological cut showing details of lamellar bone and woven bone.[9]

The mineralization of the osteoid matrix begins two weeks after the matrix is formed. During this time, with the help of cytokines and other cell signaling molecules, the mineral content of the bone tissue (calcium and phosphorus) increases rapidly.[10] It is thought that the osteocytes incorporated in the matrix have a crucial role in the homeostasis of these minerals, ensuring their proper deposition and crystallization.[6] Figure 3 depicts how the deposition of the mineral materials creates new bone on the top of the old bone over time.

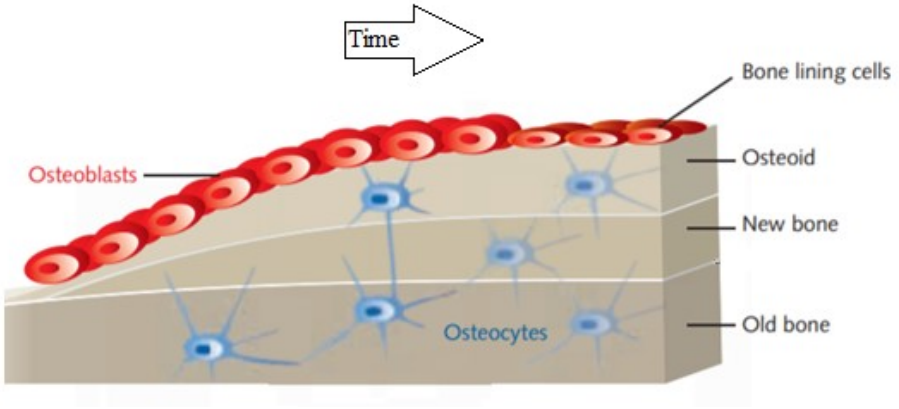

Figure 3. Differentiation and mineralization of bone cells over time.

Once mineralization is complete, the hydroxyapatite crystals, which are the final stage of the bone mineral, will account for 70% of the bone's final weight.[10] The bond formed between ceramic calcium phosphate and collagen gives the bone tissue a composite nature, creating an unique microstructure, which allows for most of the mechanical properties of bone, being rather challenging to replicate.[11]

Despite its inert appearance, bone is a very dynamic organ. The perceived equilibrium between formation and absorption appears to be dependent on the actions of several local and systemic factors such as hormones, growth factors, cytokines, chemokines, and biomechanical stimulation.[7] This remodeling is important for the proper healing of fracture complications of bones, adjustment of the whole skeleton to adapt to its mechanical use and to maintain calcium homeostasis in the body. Osteoporosis is an example of a bone disease related to an imbalanced performance between bone formation cells and bone absorption cells.[3][7]

3.2. Bone tissue transplants

Two important properties of bone tissue that current implants try to mimic are osteoconductivity and osteoinductivity. Osteoconductivity is the ability to provide an appropriate scaffold or structural framework for bone formation. This scaffold is triggered by specific chemical stimulations that allow the bones to grow in specific directions or shapes.[12,13] Osteoinductivity is the process that stimulates osteogenesis, which is the differentiation of pre-osteoblastic cells into mature bone cells. Both of these properties are crucial for the regeneration and healing of bone.[14]

There are three types of bone tissue transplants: autografts (from the patient's own body), allografts (from cadavers) and xenografts (bone from animals). All these types of tissue transplant present disadvantages that limit their application in the clinic area. The current gold standard for bone grafts is the autograft, even though the limited supply and risk of donor site morbidity are huge drawbacks.[11][15] In the case of allografts, the biggest downsides are the possible pathogen transmission and rejection of the tissue by the patient's body.[11] Lastly, in the case of xenografts, there is a higher chance of triggering an immunologic response, as the transplanted tissue is from another species.[11]

The increase in demand for bone transplants encouraged tissue engineering and regenerative medicine to come up with promising strategies for bone reconstruction over the last decades, such as the usage of scaffolds.[16] Scaffolds are defined as artificial structures created to support three dimensional (3D) tissue formation that, when implanted, allow for the host's cells to colonize it and foster tissue regeneration.[16,17] The combination of scaffolds with cells, for example, derived from bone marrow, can promote *in vivo* bone formation, with cells differentiating into the osteogenic lineage and/or by the release of specific molecules that will boost bone regeneration.[18]

Bone diseases issuing from traumas, tumors, bone fractures or defects still affect millions of people every year. [11] It is estimated that around 1.5 million individuals suffer a fracture due to bone disease each year.[19] In the last decade, much attention has been brought towards implants regarding bioactive fixation, which means, the creation of interfacial bonding between the implant and the patient bone tissue.[11]

Tissue engineering is an expanding field, especially bone tissue augmentation and repair by using artificial bone materials, such as scaffolds to replace the damaged or diseased bone.[20] One of the biggest challenges of hard tissue engineering is the creation of scaffolds with enough porosity and mechanical strength, allowing cell adhesion, migration, growth and proliferation.[11]

Bone is a highly vascularized and dynamic tissue with self-regenerative capacity, though it can be limited by the size of the bone defect.[21] Joint arthroplasties, long bones, and vertebral fractures are usually healed with metal implants, like titanium alloy or stainless steel materials used to provide mechanical and structural support to the fragile bone. Unfortunately, these implants are still associated with periprosthetic joint infections (PJIs), which reveals a poorly achieved interface between the implant and the patient's body.[11][22]

3.3. Periprosthetic Joint Infections

Periprosthetic Joint Infections (PJIs) are a specific type of infection that surrounds a bone prosthesis and the adjacent tissue of the patient, mainly occurring after total joint arthroplasties (TJAs).[23] The most commonly performed TJAs are total hip arthroplasties (THAs) and total knee arthroplasties (TKAs), which are elective surgical procedures with a survival rate of 95%.[24] When successfully implanted, this joint replacement provides pain relief, restores function and independence to the patient, significantly improving their quality of life.[24]

In the US alone, one million of THAs and TKAs are performed annually.[25] This number is expected to rise to four million by 2030.[23,24,25] There is evidence of a substantial rise in the revision cases of TJAs. The New York State's Statewide Planning and Research Cooperative System (SPARCS) inpatient database identified that, between 1993 and 2010, the revision procedures for THAs and TKAs increased 24.6% and 44%, respectively. These revisions are mostly related to the growth of PJIs cases. [24][27]

The most common microorganisms responsible for PJIs are methicillin-resistant *staphylococcus aureus* (MRSA). In the US and Europe, this Gram-positive bacteria is responsible for 46.7% and 15% of all cases of PJIs, respectively.[24] MRSA is a subgroup of *Staphylococcus aureus* that has shown resistance to methicillin and all beta-lactamic antibiotics. Some of these strains present frequently reduced susceptibility to vancomycin, which is a broad-spectrum antibiotic used to treat several Gram-positive bacterial infections.[24] This fact created an urgent need to find alternative solutions to fight these infections.[27]

The full dynamics of PJIs is still unclear, but as the number of TJAs keeps rising, so does the incidence of PJIs. These infections' rates range between 0.5 to 1% for THAs and between 0.5 to 2% for TKAs.[23,24][27] In the first two years after surgery, 60 to 70% of all PJIs take place.[23] They are normally caused by the growth of bacteria in the form of biofilms, and its treatment is performed by antibiotic therapy and by the complete removal of the implant.[28] The revision of infected prosthesis can be, by itself, a dangerous procedure for the patient. Sometimes, these infections lead to several revision surgeries for the same implant, enhancing the risk to the patient and the costs associated.[29] The revision process involves additional surgeries to remove all infected

tissue and the implant, severely diminishing the quality of life of the patients, and can be 3.4 to 6 times more expensive than the first implantation.[23][30] A TJA revision after infection leads to mortality rates between 8 to 25% after 2 years, and 45% after 7 years.[24]

3.4. Risk factors of infection

When patients present a combination of features such as the presence of pathogens, the surgical procedure itself and possible comorbid conditions (obesity, arthritic rheumatoid, diabetes, etc.), the percentage of patients that can develop the initial stage of PJIs can increase up to 20%.[25]

The microorganisms responsible for PJIs formation can come from the patients´ skin, surgical team, surrounding environment or even the implant itself.[31] Loosening of the bone cement interface, periprosthetic fracture and implant malposition or dislocation are often reasons associated with aseptic failures of prosthesis.[23]

Obesity is expected to increase the risk of infection.[23] A patient with a higher body mass index (BMI) will likely need a prolonged operation, which by itself boosts the risk of infection. On the other hand, someone with a low BMI would probably have a poor nutritional reserve, which also places the patient at a higher risk of infection.[23][27]

Patients with rheumatoid arthritis, diabetes or immune suppression were reported to have an increased risk of infection that ranges from 2 to 8 times higher than healthy patients, depending on the study.[23][27][31] Hyperglycemia, anemia and wound drainage have also shown to be risk factors.[23][27] Ischemic necrosis, hematomas, abscess cellulite or local granulocytes in the wound are able to surround the implant, slow down the healing process and to stop deeper tissues from forming barriers against infections.[31] Lastly, aging is also a risk factor. The most common patients for these surgical procedures have already advanced age and a suboptimal level of health, which further enhances the chances of developing PJIs.[27][31]

3.5. Hydroxyapatite

Calcium phosphate ceramics are promising substitutes for large orthopedic defects' remodeling and regeneration. These biomaterials show osteoinductive and osteoconductive properties, and besides being used *per se* in many applications, they can also be used to coat metallic implants, giving it a better long-term performance, and minimizing the micro-motions between bone and the implant.[1]

Hydroxyapatite (HAp) is a ceramic material, widely used in the area of hard tissue repair and regeneration, due to its similarities to human apatite. It possesses chemical and crystallographic similarities to the inorganic components of bone.[1] HAp porous structure allows a fast proliferation of the cells.[32] It presents good compatibility with natural and synthetic polymers as well as proteins, which makes it easier to attach to several materials.[11] This ceramic material has been used in the coating of dental and orthopedic implants for years now, allowing for the adhesion and proliferation of osteoblasts at the surface of the prosthesis.[1]

Naturally occurring HAp has a hexagonal shape, having each unit the following chemical formula (Ca10(PO4)6(OH)2) (Figure 4). The hydroxyl (OH) group can be replaced by fluor, calcium or carbon trioxide.[32]

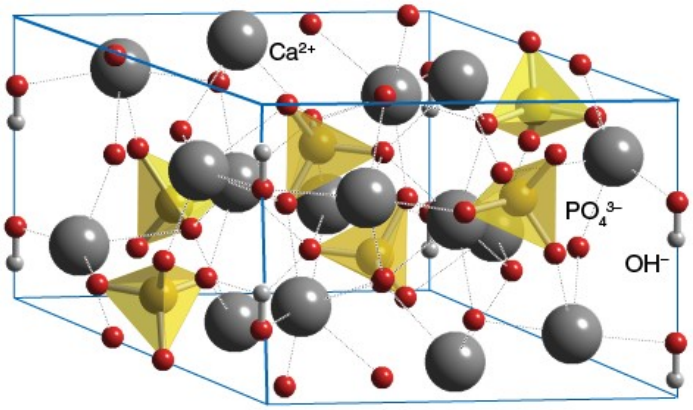

Figure 4. Representation of the hydroxyapatite chemical formula. (https://socratic.org/questions/human-bone-is-made-up-of-a-compound-known-as-calcium-hydroxyapaptite-ca-10-po-4-).

New methods for manufacturing synthetic HAp are being investigated, due to the importance to develop economic and versatile methods that can have a wide application in biomedical treatments. Synthetic HAp has a similar chemical and crystal structure to the bone HAp and it is thermodynamically stable at a pH similar to the human body.[32]

Besides synthetic HAp, these materials (HAp and other calcium phosphates) can also be produced from natural sources.[33] Piccirillo *et al* was able to produce calcium phosphate-based materials from the byproducts of Atlantic codfish (*Gadus Morhua*), which is the most consumed fish in Portugal.[33] HAp is the main component of fish bones, accounting for 60 to 70% of their weight, making them a high-value product that is currently seen as byproducts from the food industry, being directed to animal feed without any differentiation.[33] In other words, these bones could be used as a raw material for the production of commercial HAp.[33]

Piccirillo *et al* washed the bones supplied by Pascoal & Filhos S.A. (Aveiro, Portugal) and crushed them manually into a fine powder. Then the powder passed through an annealing process, becoming a calcium phosphate biphasic material consisting of hydroxyapatite and β-tricalcium phosphate (molar proportion of 75:25), which is a composition analogous to biomaterials used in current bone implants.[33] This powder was the source of HAp used in this work.

Nonetheless, HAp alone presents low fracture toughness, bad tensile strength, and poor wear resistance, which may compromise its use in clinical applications.[1] β-tricalcium phosphate is often added to HAp, improving the material's resorbability.[33] it is also known that elements like sodium, chloride, and fluor, which are all present in the human skeleton, can influence the biocompatibility of the implanted material.[33]

The application of pure HAp can also be limited by its brittleness.[34] A lot of effort has been put into modifying HAp with the use of polymers in order to enhance its tensile strength. The polymers most commonly used are polylactic acid, collagen, polyethylene and chitosan.[34]

3.6. Chitosan

Chitosan possesses an intrinsic antimicrobial nature, it is biodegradable, biocompatible and it can be molded into all sorts of structures.[35,36] This biomaterial is a polysaccharide originated from chitin, which is the second most common polysaccharide in the world, just after cellulose. It is found in fungi, yeast and it is the main constituent of the exoskeleton of crustaceans.[35,36]

Chitosan is the deacetylated form of chitin, which means that it loses at least 50% of its amino groups. Thus, the amount of deacetylation is going to affect the number of free amino groups present in the polymer chain (Figure 5). The free amino groups provide the chitosan a positive charge, which allows it to interact with negatively charged molecules such as cholesterol, lipids, proteins, metal ions and cells. These amino groups, along with the hydroxyl groups confer to chitosan a highly reactive polymer chain.[35,36]

The chitosan used in industrial applications is produced by chemical or enzymatic treatment of chitin present in the shells of shrimps and crabs. The waste from crab and shrimp hunting has progressively become a serious issue in coastal areas, which makes the exploitation of these sources for chitin not only economically viable but also environmentally friendly.[36]

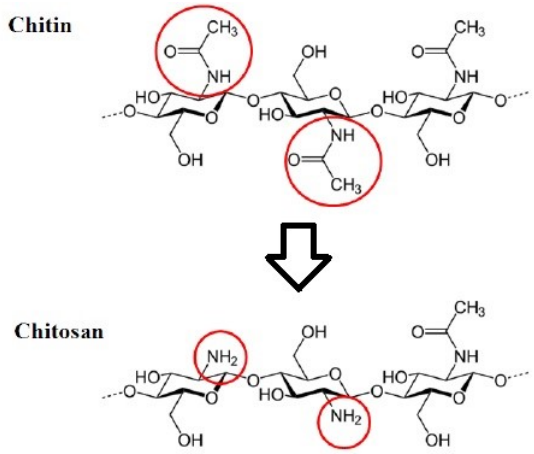

Figure 5. Chitin deacetylation process.
(https://www.researchgate.net/figure/Molecular-structures-of-chitin-and-chitosan-13-Permission-to-reproduce-the-figure_fig1_236911235)

One of the leading issues in the production of functional derivatives of chitosan is to obtain a product that can be dissolved in acidic aqueous solvents. As the number of amino groups affects the interaction of chitosan with other molecules, it also influences its crystalline structure, which is associated to the ability of chitosan to be soluble in acidic aqueous solutions, such as acetic or lactic acid.[35]

The precise mechanism of the antimicrobial properties of chitosan is not fully understood. Regardless, Gram-negative and positive bacteria and fungi are known to be highly susceptible to this polymer.[37,38] The most accepted method for the antibacterial and antifungal actions of chitosan are thought to arise from the interaction of the cationic polysaccharide and the anionic molecules on the surface of bacteria and fungi.[28] In other words, the cationic nature of chitosan allows it to bind to growth factors and to release them at a controlled rate.[21] This interaction is mediated by electrostatic forces between the protonated NH^{+}_{3} groups of chitosan and the negative residues, such as lipopolysaccharides, teichoic and teichuronic acids, and capsular polysaccharides of the microorganisms.[37] This process destroys the microorganisms by changing the permeability of the cell wall, creating osmotic imbalance and leakage of intracellular electrolytes such as potassium ions, consequently inhibiting cell growth.[38] Alternative mechanisms by which chitosan is thought to be antimicrobial is by binding to the microorganisms´ DNA, which leads to the inhibition of the mRNA and protein synthesis.[38]

3.7. Hydroxyapatite and Chitosan Composite

In the orthopedics field, the functional groups of chitosan allows it to interact with many materials, such as HAp or other calcium based minerals, forming composites for several applications.[20] These composite materials have been widely used as substitutes for bone grafting.[20]

In recent years, there has been an increased number of studies on chitosan and HAp composite materials. This composite is biodegradable and considered to be biocompatible.[36]

Natural polymers are one of the best options to produce biomaterials for bone tissue engineering, due to their similarities with the bone extracellular matrix, chemical versatility, biological performance and cellular interactions.[39] The development of biodegradable implants is severally affected by the inadequate availability of bioactive materials and the growing need to find alternatives to auto and allografts for bone regeneration.[40] In this work, chitosan was selected to produce the paste with the inclusion of calcium phosphate, in the form of HAp. This type of composite can be used as bioresorbable bone substitutes. The physical structure of the paste makes it highly favorable for the particles' immobilization upon its implantation.[41,42]

This composite presents interesting mechanical properties due to the attachment of amino and hydroxyl groups of the chitosan to the calcium ions present at the surface of the HAp crystals.[36] This composite can be used as a bioactive interface between the implant and the patient bone tissue, and to prevent the development of PJIs after a TJA is performed. Furthermore, by enhancing the osteoconduction and osteoinduction of the implant, which is inert, this composite boosts the formation of new bone.

4. Objectives

The main objective of this work is to formulate a composite in the form of a paste of chitosan and HAp, that can be used as a coating for metal joint prosthesis used in TJAs to prevent PJIs and, at the same time, promote bone regeneration. The combination of a naturally derived polymer - chitosan and a ceramic - HAp should exhibit the strengths of both materials.

This work will show the development of the paste and the cytotoxic evaluation using fibroblastic cells. Moreover, the osteogenic potential of the paste is also assessed using human pre-osteoblastic cells.

5. Materials and methods

5.1. Materials

5.1.1. Methodology

The hydroxyapatite was extracted from codfish bones kindly provided by Pascoal & Filhos, S.A. Portugal, according to Piccirillo *et al* [33]. The type of chitosan used was medical-grade and was bought from Heppe Medical (Heppe Medical Chitosan GmbH).

The HAp powder was dissolved in MQwater and the solution homogenized using 300 watts ultrasonic processor (Thomas Scientific) for 45 minutes. The concentration of the HAp that was dissolved was 20% (v/v). This concentration was optimized so that when the chitosan solution is added, the HAp concentration becomes 1%.

The chitosan powder was dissolved in a solution of 1% acetic acid (Fisher Scientific) for 24 hours in a magnetic hot plate stirrer at 50 ºC, in order to obtain a 3% chitosan solution (v/v).

5.1.2. Characterization of the developed paste by dynamic light scattering

Dynamic light scattering (DLS) works by inserting the sample in a cuvette that is then targeted with light beams. Once these beams reach the sample, they can be absorbed or scattered by it. The machine detects the scattered light, distinguishing the size of the particles, electrokinetic potential and sample polydispersion index (PDI).

As the paste is extremely opaque (because of the white color of HAp), it was diluted in MQwater at 0.5%, 0.2%, and 0.1% concentrations before being added to the cuvettes and scanned through the machine.

5.2. Cells

5.2.1. L929 fibroblast like cells

The L929 cell line derives from mouse adipose tissue (Sigma-Aldrich). These cells are adherent and present a fibroblastic morphology. This type of cell is routinely used and recommended by international standards (ISO 10993-5) for cytotoxic evaluations in the screening procedures.[43] In this work, these cells were used to study the eventual cytotoxicity of the paste. The cytotoxicity was evaluated by MTT viability assay.

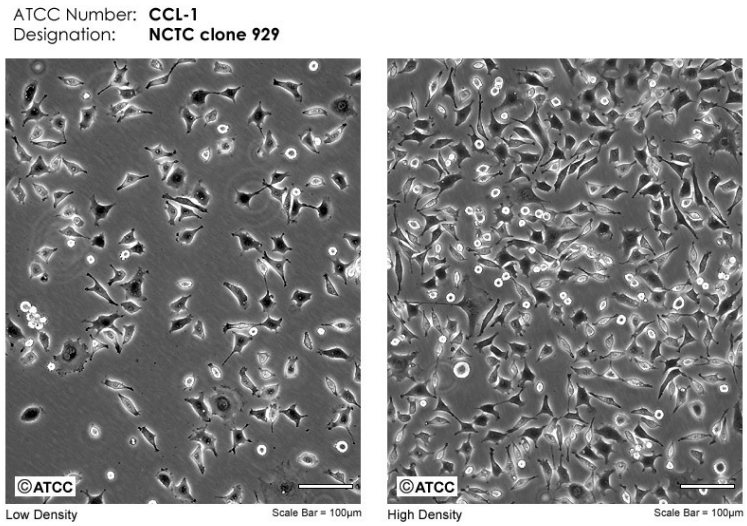

Figure 6. L929 cells micrographs. Images obtained from American Type Culture Collection (ATCC) website. (https://www.lgcstandards-atcc.org/~/media/Attachments/Micrographs/Cell/CCL-1.ashx)

5.2.2. MC3T3 pre-osteoblastic like cells

The MC3T3 cell line is originated from newborn mice calvaria bone.[44] These cells express osteoblastic markers and produce a mineralized extracellular matrix,[45] exhibiting osteoblastic characteristics that are adequate for the study of osteoblastic differentiation.[46,47] They also present the characteristics of bone progenitor cells, being able to differentiate into mature osteoblasts. This cell line is a good model for studying *in vitro* osteoblast differentiation, since they behave similarly to primary calvaria osteoblasts.

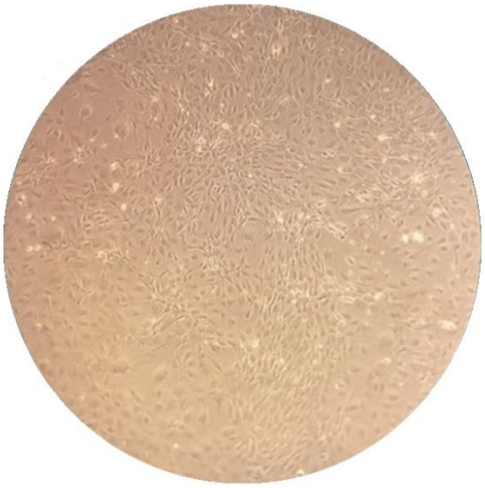

Figure 7. Image of MC3T3 cells observed under an optical microscope, with a 10x objective.

5.3. Cell seeding

5.3.1. L929 cells

The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific), 2mM Glutamine (Thermo Fisher Scientific), 10% Fetal Bovine Serum (FBS) (Thermo Fisher Scientific) and 1% Penicillin-Streptomycin (PStrep) (Thermo Fisher Scientific). Cells between passages 6 and 8 were used in this work.

The L929 cell line was used to determine the viability of the paste diluted in culture medium. The cells were seeded at $5x10^4$ cells/well in 96 well adherent plates (Thermo Fisher Scientific). Previous optimizations revealed that a concentration of paste higher than 50% was not adequate for cell survival. Thus, it was decided to expose the cells to lower concentrations of the paste, ranging from 3 to 50% diluted in DMEM.

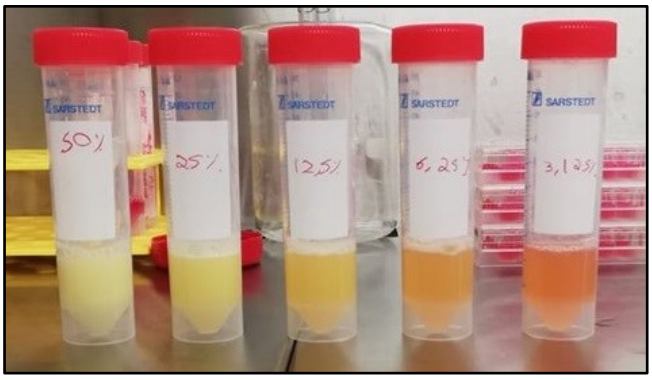

Figure 8. Representative image of the different concentrations of the paste used in this work.

The cells were incubated with the paste for 24, 36 and 48hours in an incubator at 37 ºC and 5% CO2. The following Table sums up all the conditions tested.

Table 1. Conditions used to assess L929 cells' viability

Conditions	Description
1	50% Paste + 50% DMEM + cells
2	25% Paste + 75% DMEM + cells
3	12.5% Paste + 87.5% DMEM + cells
4	6% Paste + 94% DMEM + cells
5	3% Paste + 97% DMEM + cells
6	100% DMEM + cells
7	DMSO 20% + 80% DMEM + cells
8	100% DMEM
9	50% Paste + 50% DMEM
10	25% Paste + 75% DMEM
11	12,5% Paste + 87.5% DMEM
12	6% Paste + 94% DMEM
13	3% Paste + 97% DMEM

Conditions 1 to 5 correspond to the tested concentrations of the paste. Condition 6 is the negative control for cell death, where the cells were incubated with DMEM, without any paste, the ideal condition for cell growth. Condition 7 is the positive control

for cell death, where the cells were exposed to a 20% (v/v) of dimethyl sulfoxide (DMSO), leading to their death. Lastly, conditions 8 to 13 describe the blanks for the experiment itself, in which everything from conditions 1 through 6 was added, except the cells.

5.3.2. MC3T3 cells

These cells were also cultured in DMEM with 2 mM Glutamine, 10% FBS and 1% PStrep. The cells used in this work were between passages 9 to 12.

This cell line was used to assess cell proliferation, osteoblastic differentiation, and calcium production when in contact with the paste. Although the tests mentioned before are the core reasons for the use of the MC3T3 cell line, a cytotoxicity test was also performed to study the cytotoxicity of the paste in direct contact with the cells (paste was deposited into coverslips).

5.3.2.1. Preparation of Chitosan-HAp paste coatings on TCPS coverslips

The coverslips with the paste were freshly prepared prior to cell seeding. An optimization process was required in order to achieve the ideal volume of paste to cover the coverslip surface and the time needed to achieve the desired consistency. In the end, 30 µl of paste was deposited per coverslip and let it dry for 30 minutes. After that, the coated coverslips were sterilized for 45 minutes under UV radiation in the laminar flow chamber.

5.3.2.2. Osteogenic medium preparation

The osteogenic medium is based on DMEM basal medium supplemented with β-glycerophosphate, ascorbic acid and dexamethasone. β-glycerophosphate serves as a source of inorganic phosphate to promote mineralization; ascorbic acid increases the secretion of Col I, leading to Col I/α2β1 integrin-mediated intracellular signaling; and

dexamethasone induces Runx2 expression, which is a key transcription factor associated with osteoblast differentiation.[40]

Stock solutions (100x concentrated) of each constituent were filtered through a 0.22 µm filter and stored at -20 ºC. The β-glycerophosphate and the ascorbic acid were diluted in ultrapure water to 0.216 g/ml and 0.005 g/ml, respectively, and dexamethasone diluted in pure ethanol to 2.5 mg/ml. Whenever needed, the osteogenic supplements were taken from the stock solutions and added to DMEM medium, resulting in the osteogenic medium. The amount of the stock solutions added to the basal medium were 0.01 µl of β-glycerophosphate and the ascorbic acid per µl of DMEM for the and 0.1 x 10^3 µl of dexamethasone per µl of DMEM.

5.3.2.3. MC3T3 cell seeding

1,5x10^5 cells were seeded on top of coverslips (Sarstedt) covered with 30 µl of paste and placed in 24 well non-adherent plates (Greiner Bio-One). Then they were incubated with DMEM or osteogenic medium. The cells were incubated with the coverslips for 1, 7 and 14 days in a 37 ºC incubator with 5% CO2. The following Table (Table 2) presents the conditions tested.

Table 2. Conditions used to assess MC3T3 cells viability, proliferation, osteogenic differentiation, and calcium production

Conditions	Description
1	coverslip + DMEM + cells
2	30 µl paste + coverslip + DMEM + cells
3	30 µl paste + coverslip + DMEM
4	coverslip + osteogenic medium + cells
5	30 µl paste + coverslip + osteogenic medium + cells
6	30 µl paste + coverslip + osteogenic medium

5.4. Cytotoxicity tests

5.4.1. Cell viability assay

MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) (Thermo Fisher Scientific) is a reagent commonly used in viability assays. This compound has a yellow color that when it comes into contact with live cells is metabolized into formazan, which has a purple color.[49] The amount of color is dependent on the quantity of formazan produced, which means that it is directly proportional to the number of live cells. This color can be quantified by analyzing its absorbance in a plate reading spectrophotometer at 570 nm. This methodology is one of the most used in cytotoxicity tests due to its sensitivity and reproducibility, being a reliable way to quantify cell viability.[50,51]

The MTT stock solutions were made by dissolving 0.5 mg/ml of the MTT powder in MQwater and storing them at -20 ºC in 1 ml Eppendorfs. The stock solutions were then diluted 1:10 in DMEM and added to the wells. The plates were then incubated for 4 hours at 37 ºC and 5% CO_2. After this time, cells were lysed with 350 µl of DMSO per well in order to release the produced formazan. After that, the plates were agitated for 15 minutes to assure that all content of the cells was released. Then, the samples were pipetted to a 96 well clear plate and the absorbance read in a plate reader (BioTek Instruments) at 570 nm.

5.5. Functionality tests

For these tests, MC3T3 cells were used. Being a pre-osteoblastic cell type, it was used to determine if the paste would induce the osteogenic differentiation of the cells. For that, it was evaluated cell proliferation rate, alkaline phosphatase activity (early osteogenic marker), and calcium content (late osteogenic marker). For all the assays, the samples were collected at each time point and were stored at -80 ºC into 1 ml Eppendorfs, until further use.

5.5.1. Cell proliferation

Cell proliferation was assessed using a DNA quantitation kit (Sigma-Aldrich). The assay uses a fluorescent dye - bisBenzimide H 33258 - that binds to AT sequences at the minor groove of double stranded DNA (dsDNA). According to the kit, this dye when excited at 360 nm gives a detectable fluorescence emission at 460 nm. The proliferation rate of the cells is directly correlated to the amount of dye that binds to AT sequences.

The dye was prepared by diluting the bisBenzimide H 33258 solution (1 mg/ml) in 10X fluorescent assay buffer and MQwater, to a concentration of 0.1 µg/ml. The samples were collected from the wells to 1ml Eppendorfs. For the assay, the samples were taken from the Eppendorfs, placed in a multiwell plate (50 µl per well) and exposed 150 µl of the dye for 30 minutes inside the incubator and read in a multiplate reader (BioTek Instruments) at 460 nm, once excited at 360 nm.

In other to determine the DNA content of the samples, a standard calibration curve was determined with calf thymus DNA. For that, a stock solution was prepared, in which the DNA standard (1 mg/ml) was diluted from the kit in 100 µl of 10X fluorescent assay buffer and 890 µl of MQwater, to a concentration of 10 µg/ml. These DNA stock solutions were kept in the dark until they were needed for the calibration curve. For that, several concentrations of the DNA standard were added to the multiwell plate, from 2 µl to 100 µl, leading to a calibration curve ranging from 20 to 1000 ng of calf thymus DNA.

5.5.2. Alkaline phosphatase (ALP) activity - Early osteogenic marker

Alkaline phosphatase (ALP) is an enzyme-linked to cellular metabolism and formation of bone matrix. It enhances the hydrolysis of phosphate esters, producing inorganic phosphates, which are essential components to promote bone mineralization. It is highly produced during osteogenesis, which means that it can be used as an early marker for osteogenic differentiation.[52,53] In the case of pre-osteoblastic cells like MC3T3, an increase of ALP is related to the differentiation into an osteoblastic phenotype.[54]

In this assay, 4-methylumbelliferyl phosphate disodium salt (MUP) (Abcam) was added to the samples. The ALP produced by the cells stimulates the hydrolysis of the phosphate group of the MUP substrate, which results in a fluorescent signal.

The MUP reaction mix was produced by adding 2 µl of the MUP standard to 8 µl of the assay buffer. The samples were resuspended in the assay buffer and then treated with the MUP reaction mix before being incubated for half an hour, at room temperature and away from light. After that, 20 µl of stop solution was added to each well and read in a microplate reader (BioTek Instruments USA) at 440 nm, once the samples were excited at 360 nm.

In order to make a standard calibration curve, different concentrations of the ALP enzyme solution had to be prepared. First, 5 µl of the MUP standard (5 mM) was diluted in 495 µl of assay buffer to 50 µM. Different standard concentrations (from 0 to 30 µl) were produced by adding different volumes of the ALP enzyme solution to the MUP standard.

5.5.3. Calcium quantification

In this assay, the calcium production is detected by o-cresolphthalein, which is a chromogenic reagent that binds to calcium molecules. Along with phosphorus, calcium plays a crucial role in bone modeling and growth. It is an essential element for many intracellular regulations, and it can be found in the form of free ions or in the form of complexes like calcium phosphate or calcium carbonate. The structural development of the skeleton is regulated by calcium signaling, and its malfunction can be associated with many human diseases. When osteoblastic cells develop, they produce calcium in order to create bone mineral, which is fundamental for the bone structure and strength.[55,56]

This assay was performed with a calcium colorimetric assay kit (Sigma-Aldrich). 50 µl of every sample was added to a 96 well plate. To these samples, 90 µl of chromogenic reagent and 60 µl of assay buffer were added before incubating them for 5 to 10 minutes at room temperature.

In order to quantify the calcium content of the samples, a standard calibration curve with calcium standards was performed from the assay kit. The calcium standard

solution (500 mM) was diluted in MQwater in order to get a 5 mM standard solution. Then, different volumes of the standard solution were added, from 0 µl to 10 µl, to the 96 well plates and MQwater was added to all standard solutions to reach 50 µl. The same procedure was applied to the samples. All absorbances were measured by a plate reading spectrophotometer at 575 nm (BioTek Instruments).

5.6. Statistical analysis

The statistical analysis was performed with the Graphpad Prism 8 software. The variables were analyzed independently through ANOVA two-way statistical method and all the data was expressed in the form of means and standard deviations.

The absorbance data of the samples from the viability tests with the L929 cell line were calculated in function of the negative control for cell death. This control did not receive any type of treatment and can be used to establish unknown variables. Therefore, the data from all the samples was calculated through the following equation:

$$Sample\ value = \frac{Sample\ absorbance\ \times 100}{Average\ of\ all\ control\ absorbances\ for\ that\ time\ point}$$

Only after the values from all samples were calculated (for all time points) were they analyzed through the ANOVA two-way method, in which the time points and the conditions of the samples were the independent variables and viability levels were the dependent variables.

The data obtained from the assays with the MC3T3 cells were analyzed using ANOVA two-way statistical method, being the time points and the conditions of the samples the independent variables in all the assays. For MTT, DNA quantification, ALP and Calcium colorimetric assays, the dependent variables were, respectively the cell viability levels; the amount of AT sequences; the ALP expression and the calcium concentration.

6. Results and Discussion

6.1. Characterization of the paste

The DLS was used to determine the size of HAp particles, their electrokinetic properties, and PDI. The samples were diluted in MQwater to different concentrations (0.5, 0.2 and 0.1%) and analyzed by the DLS 3 times for each concentration (Table 3).

Table 3. Characterization of the paste through dynamic light scattering.

Dilution	Size (nm)	PDI	Zp (mV)
0.5% (1:200)	$7.05x10^7 \pm 0.012x10^7$	1	69.3 ± 2.84
0.2% (1:500)	$2.6x10^7 \pm 0.245x10^7$	0.753 ± 0.133	61.4 ± 1.45
0.1% (1:1000)	$1.7x10^7 \pm 0.449x10^7$	0.355 ± 0.156	59.6 ± 1.28

Three different dilutions were prepared in order the equipment be able to measure the size, PDI and zeta potential. The HAp has a white strong opaque color that does not allow light beams of DLS to be scattered. Light-scattering techniques have the limitation that only solutions that are transparent to light or highly diluted, can be properly analyzed. Therefore, it was necessary to prepare dilutions of the paste.

The average size of the particles obtained for all tested dilutions were in the same size range – 10^7 nm, which is in the range of 10^3 μm. This result is not accurate, because the addition of water contributed to the formation of clusters, which led to the presentation of high values. The chitosan-HAp paste mixed with water results in a non-homogeneous solution, which was the reason for these numbers.

The polydispersion index, which recognizes how homogeneous the sample is at the nanoscale, helps to determine if the materials are well mixed inside the paste. At 0.1% dilution the index level was 0.355 ± 0.156, which suggests that the solution is homogeneous. At 0.2% dilution, the index level is 0.753 ± 0.133, meaning that the paste is more heterogeneous than homogeneous. At 0.5% dilution, the index is 1, meaning that the heterogeneity of the sample is too high to be quantified by the machine, displaying the maximum value. This can be explained, not only by the formation of HAp clusters in the solution, as previously mentioned but also by the opacity of the HAp particles. These particles were harder for the light beams from the DLS to pass through the cuvette, and

subsequently, to give a reading of the particles inside the sample. This means that the higher the amount of HAp particles, the harder it is to analyze the sample.

The zeta potential, which is the electrokinetic potential of the material, of the three dilutions of the Chitosan-HAp paste was positive. This result was in accordance with what was expected, as chitosan is a cationic polymer. The absolute values of the three dilutions were similar, and not affected by the HAp clusters present in the solutions.

6.2. Viability of L929 cells

The cells were incubated for 1, 2 and 3 days with the 5 tested concentrations of the paste (3, 6, 12, 25 and 50%). As previously mentioned, a positive control (DMSO) and negative control (DMEM) for cell death were also added to the experiments. The results were obtained in the function of negative control for cell death, the ideal environment for cell growth (Figure 9).

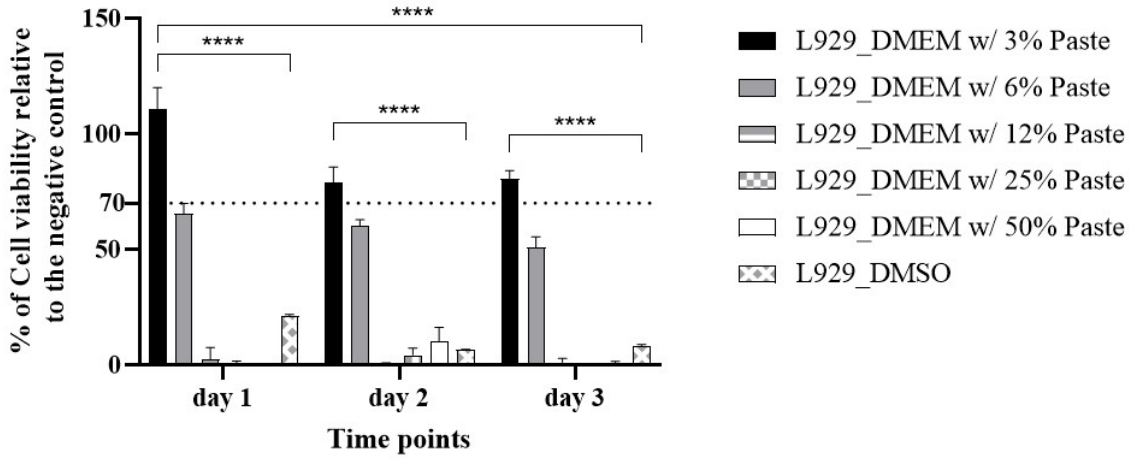

Figure 9. Viability of the L929 cell line in function of the negative control for cell death, when exposed to DMEM with paste diluted in it, by MTT assay.**** ($P \leq 0.0001$)

L929 cells exposed to the lowest concentration of the paste (3%) present the highest viability level for all time points tested. When exposed to the 6% concentration of the paste, cells showed intermediate values of viability, which slightly decreased with time. For higher concentrations of the paste (12, 25 and 50%) cell viability was significantly lower, similar to the positive control of cell death, which indicates that these concentrations were not adequate for cell culture. According to ISO 10993-5, which determines the standards for cytotoxicity tests, the percentage of cell viability needs to be above 70% to be considered as non-cytotoxic. Furthermore, samples that induce cell viability levels below 40% are considered to possess strong cytotoxicity.[57] The cells that were exposed to concentrations of the paste equal or higher than 12% expressed viability

levels lower than 40%, which means that these samples presented a high level of cytotoxicity through all time points, similar to DMSO condition.

Cells exposed to 6% of the paste in DMEM, presented an absorbance level that starts above 60% in the first time point, decreases to 60% in the second time point, and after 3 days decreases below 60%. Finally, cells exposed to 3% of the paste, displayed viability levels above 70% throughout all time points, which represents that this condition was suitable for cell growth. Therefore, this condition was selected to continue the experiments conducted in this work.

The p-value of all sources of variation of the data (interaction, row factor and column factor) are <0.0001, meaning that the probability that the data was generated by random chance can be disregarded. Thus, the values have statistical significance.

6.3. Viability of MC3T3 cells

MC3T3 cells were seeded on top of coverslips covered with the paste, to be in direct contact with the coating, contrasting with the previous assay, where cells have adhered to the culture plates and exposed to diluted paste concentrations (indirect contact). The time points assessed were also different, longer than the ones used for L929 cells, since the objective was to assess the osteogenic differentiation of MC3T3 cells in direct contact with the paste, and for that, longer time points were required. The cells were incubated with basal medium (only DMEM, without osteogenic supplementation) or osteogenic medium (Table 2) for 1, 7 and 14 days in a 37 ºC incubator with 5% CO_2. The results were not calculated in function of the negative control for cell death (DMEM), but all plotted. The reason for this is that as we wanted to check the effect of direct contact of the paste on the cells, the negative control was TCPS coverslips and contrarily to an indirect test, like the previous one with L929 cells where the DMEM is considered the 100% of cell viability, in the case where we want to study a biomaterial, it might not be.

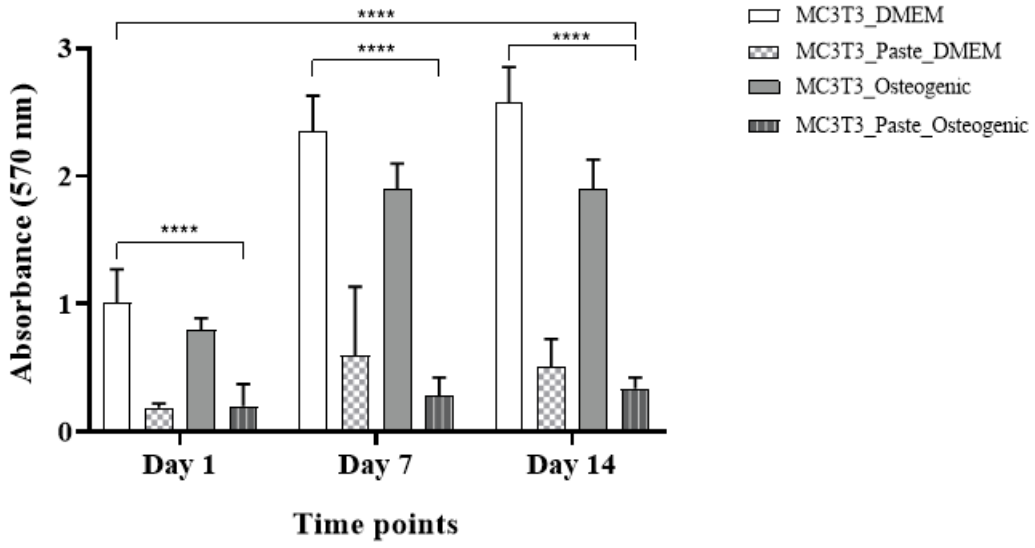

Figure 10. Viability of the MC3T3 cell line when seeded onto the coverslips, by MTT assay. **** ($P \leq 0.0001$)

Figure 10 shows that cells cultured only in basal medium presented the higher values of absorbance, which was expected since this is the ideal condition for cell growth. The condition that followed this trend was the one with cells cultured only with osteogenic medium. For both conditions with paste, either with or without osteogenic supplements, the absorbance values were lower than the controls. In these conditions, cells were seeded on top of coverslips coated with 3% of paste, which is not the ideal condition for cell growth. Cell viability is not a direct method to evaluate cell growth, it only provided a readout of cell health through measurement of metabolic activity.

The p-value of all sources of variation of the data (interaction, row factor and column factor) are <0.0001, meaning that the probability that the data was generated by random chance can be disregarded. Thus, the values have statistical significance.

6.4. Cell proliferation

Cell proliferation was quantified by the amount of AT sequences at which the dye bisBenzimide H 33258 was bound. These sequences have a positive correlation with the proliferation of the cells. Figure 11 shows the results obtained for all conditions tested with MC3T3 cells after 1, 7, and 14 days of culture.

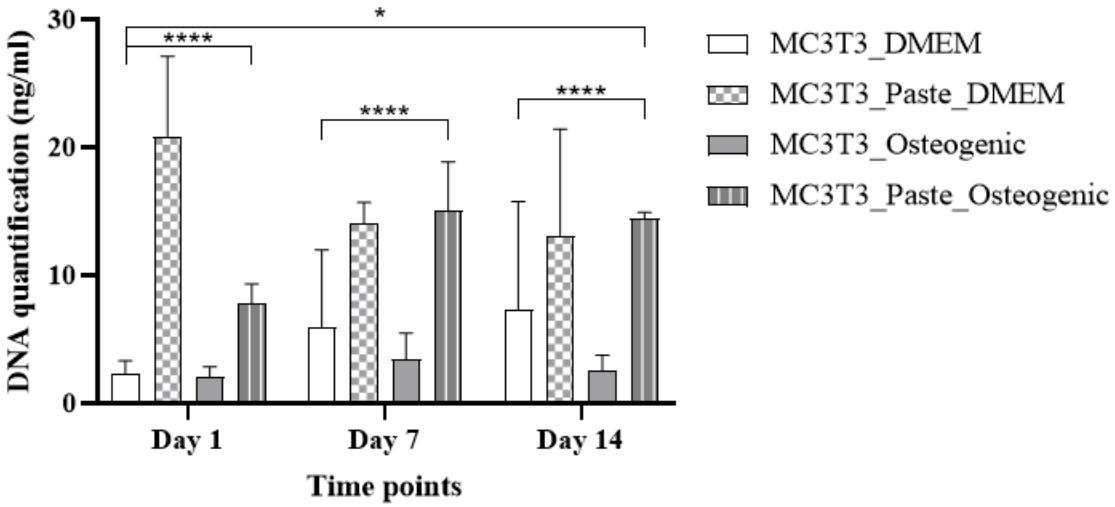

Figure 11. Proliferation rate of the MC3T3 cell line when exposed to the coverslips, by DNA quantification assay. * ($P \leq 0.05$), **** ($P \leq 0.0001$)

Cell proliferation was shown to be higher for cells in contact with the paste at all time points, regardless of the medium used. The cells cultured with paste and DMEM presented DNA values higher at the beginning than any other condition but decreased with the time. The cells cultured with paste and osteogenic medium showed an increase in cell proliferation between the first and second time points, stagnating at 14 days. This trend is visible to all conditions containing the paste, which was expected. When cells are differentiating, their proliferation rate decreases mostly due to the production of a mineralized extracellular matrix.[38]

The values obtained have statistical significance, as the p-value of the column factor is < 0.0001 and the interaction factor is < 0.05, meaning that the probability that the data was generated by random chance can be disregarded.

6.5. Early osteogenic marker differentiation – ALP quantification

This assay allows us to verify if MC3T3 cells, which are pre-osteoblastic cells, differentiate into osteoblasts at a different rate when exposed to the paste. They were incubated with basal medium and osteogenic medium for 1, 7 and 14 days in a 37ºC incubator with 5% CO_2.

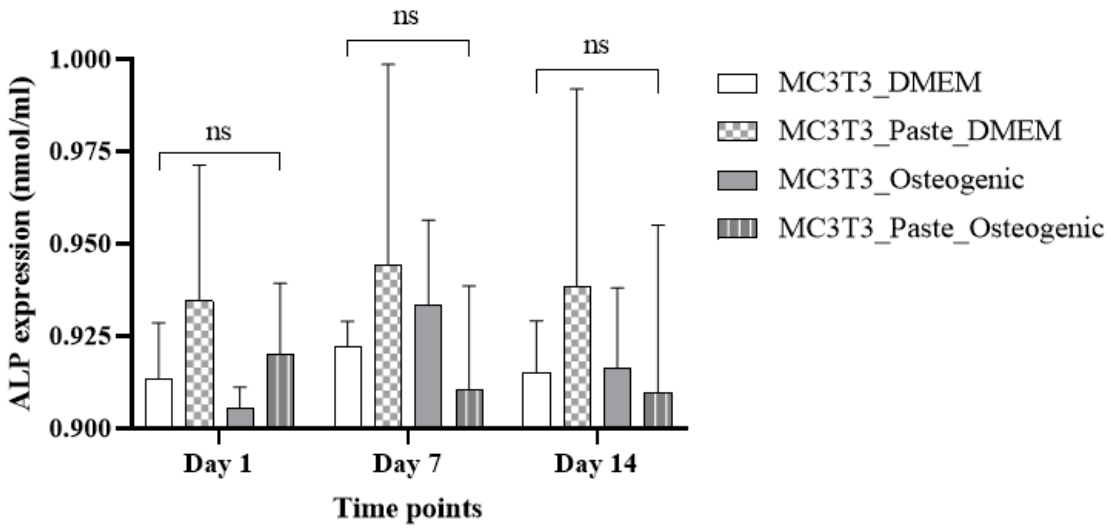

Figure 12. Differentiation of the MC3T3 cell line when exposed to the coverslips, by alkaline phosphatase assay. ns (non-significant, P > 0.05)

In the literature, it is described that MC3T3 cells differentiate into osteoblasts after being treated with ascorbic acid and β-glycerophosphate for 14 days.[22] Cell differentiation is dependent on osteoinduction. For this process to occur, it is needed a minimal level of alkaline phosphatase activity. This makes ALP a suitable maker of early osteogenic differentiation.[54] It was expected that cells grown in osteogenic medium would have a higher differentiation rate since this medium contains materials previously mentioned, which is known to accelerate the differentiation of cells.[48][58]

The results obtained from the ALP assay display high standard deviations and this heterogeneity reflected non-statistical significative results (Figure 12). Regardless of this fact, some observations can be performed. The condition that suggests the higher ALP

values is the cells cultured into coverslips coated with the paste in basal medium, which is a very good result. Even the trend of ALP during time is common for osteogenic differentiation, with an increase to 7 days and a decrease after that. The cells cultured without paste but in osteogenic medium, also presented the same osteogenic differentiating trend, which was the expected, however, the values were lower than the previously mentioned. The results for cells cultured with paste and osteogenic medium were of utmost importance, since this condition was never tested before. We expected that this condition would present the most preeminent results, because the medium was supplemented and the paste had also differentiating agents, but surprisingly the values obtained were lower than for the cell cultured in the paste coated coverslips in basal medium. This fact might be due to some interference of the components of the paste itself with the osteogenic supplements present in the osteogenic medium or the fact that multiple thaw cycles where performed. Cells cultured only with basal medium presented values of ALP, and this fact might be due to these cells being a pre-osteoblastic cell line, have some intrinsic osteogenic differentiation.

6.6. Calcium quantification

Calcium is an important component for the correct development and regeneration of bone tissue. The MC3T3 cells were incubated with basal medium and osteogenic medium for 1, 7 and 14 days in a 37 ºC incubator with 5% CO_2.

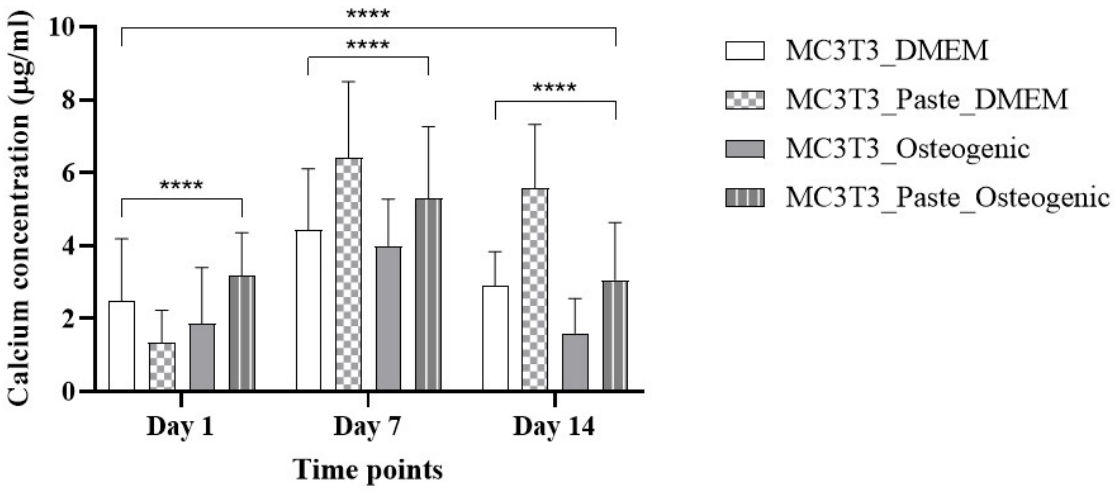

Figure 13. Calcium production of the MC3T3 cell line when exposed to the coverslips, by calcium colorimetric assay. **** ($P \leq 0.0001$)

It is known that calcium and calcium receptors in osteogenic cell lines are important for the correct regulation of cell differentiation and other functions.[59] Cellular interactions with calcium phosphate ceramics, such as HAp induces a biological response analogous to the one generated during bone remodeling, and during osteoclastic resorption. Due to this interaction, substantial amounts of Ca^{+2} should be released from the samples [55][59]

In Figure 13, it is possible to observe that there is a higher concentration of calcium in the cells that were exposed to the paste, which was expected as the paste has calcium phosphate in its composition. For all conditions, increasing production of calcium between the first and second time points is observed. However, between the second and third timepoints there was a decrease in calcium production. This can be

explained by the fact that as the amount of calcium rises, it moves towards resorption sites, in which the Ca^{2+} is used to increase the size of the bone structure.[56][59]

The p-value of all sources of variation of the data (interaction, row factor and column factor) are <0.0001, meaning that the probability that the data was generated by random chance can be disregarded. Thus, the values have statistical significance.

7. Conclusions

Today, periprosthetic joint infections are one of the main challenges in the orthopedic field. The full dynamics of these infections is not yet clear, but as the number of joint arthroplasties continues increasing due to the aging of the population, the same is true with the incidence of infections. The cells and components of biomaterials need to be optimized to produce functional bone tissue engineering therapies. Currently, calcium phosphate ceramics, such as HAp, are a promising substitute for major orthopedic defects regeneration.

A new generation of biodegradable natural biomaterials is emerging, with chitosan being one of the most interesting, thanks to its intrinsic antimicrobial properties. This thesis demonstrates how a composite of these two materials (chitosan and hydroxyapatite) could be used as a bioactive interface between the implanted prosthesis and the adjacent tissues of the patient's body. For that, we developed a biocomposite in the form of a coating paste to cover the metallic prosthetics currently used in the clinical field to prevent the formation of PJI and at the same time promote bone regeneration.

Regarding the formulation of the paste, it can be concluded that the clusters formed were in the range of 10^3 μm. The opacity of the HAp particles interferes with the analysis by DLS methodology, which resulted in an inconclusive PDI value. On the other hand, the zeta potential value of the paste was positive, as expected by the presence of chitosan, which is a cationic polymer.

The cytotoxicity screening of the paste conducted through the MTT viability assay with the L929 cell line demonstrated that concentrations of the paste higher than 3% were toxic for the cells. In accordance, this concentration was the one selected to conduct more specific studies with pre-osteoblastic cells - MC3T3.

Further on, it was carried out relevant assays to study the effects of the paste in direct contact with the MC3T3 cells. In the case of the viability assay, the cells were seeded on top of coverslips coated with 3% of paste, cultured with basal or osteogenic medium. The results obtained under the conditions where the cells were in contact with the paste were not the best in this particular assay, comparing to cells cultured in TCPS, both in basal and osteogenic medium.

The DNA quantification assay allowed to inform about the cell proliferation rate in the different conditions tested. The cells that were exposed to the paste presented higher values of DNA, which reflects a superior cell proliferation. The followed trend was expected, by an increase from first to second time point, followed by a stabilization of the proliferation rate from the second to the third time point.

The ALP assay suggests that when the cells were exposed to the paste they were, in fact, differentiating at a higher rate than the cells not exposed to the paste. The values for the cells exposed to the paste and cultured in basal medium suggested the highest levels of differentiation. The paste seemed to enhance osteogenic differentiation when compared to cells that were not exposed to the paste.

In respect of calcium concentration, the values obtained for the cells exposed to the paste were the highest, as expected. Increasing production of calcium between the first and second time points was observed, but a slight decrease was observed in the last time point.

The paste developed in this work presented the essential characteristics for a proper bone regeneration. The anti-microbial potential of the paste was addressed in another MSc thesis, and the results were positive against the most common microorganisms present in PJI. The further step is to evaluate the paste with primary cells, namely human osteoblasts and human bone marrow stem cells to study its effect over the differentiation of these cells. It should be also studied the coating in small titanium discs similar to the ones used in the clinical practice. Although the study reported in this thesis is preliminary, it presented valuable data for the evaluation of this coating method for future clinical application in metallic prosthetics to promote bone regeneration and prevent PJI.

8. Bibliographic references

(1) Li, Ming et al. (2018). An overview of graphene-based hydroxyapatite composites for orthopedic applications. Bioactive Materials. Volume 3. Pages 1-18

(2) Bjorn R. Olsen (2000). Annual Review of Cell and Developmental Biology. Volume 16, Pages 191-220

(3) Sims, A. et al. (2008). Bone remodeling: Multiple cellular interactions required for coupling of bone formation and resorption. Seminars in Cell & Developmental Biology. Volume 19. Pages 444-451

(4) Osteoid anatomy. https://www.britannica.com/science/osteoid.

(5) Méndez-Ferrer, S. et al (2010). Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. National Library of Medicine. Volume 466. Pages 829-824

(6) Canadian orthopedic association basic science course (2020).

(7) Hedges, R. (2002). Bone diagenesis: An overview of processes. Archaeometry. Volume 44. Pages 319–328.

(8) Bone function, development and growth. https://www.coursehero.com/sg/anatomy-and-physiology/microscopic-anatomy-of-bone/.

(9) Almeida Matos, M. (2008). Histomorphometric evaluation of bone healing in rabbit fibular osteotomy model without fixation. Journal of Orthopaedic Surgery and Research. Volume 3. Pages 4

(10) Elliot, R. et al (2009). Bone Graft and Fusion Enhancement. Section IV: Perioperative Consideration. In Surgical Management of Spinal Deformities. Chapter 27. Pages 433-448.

(11) Pighinelli, L. et al (2012). Chitosan–hydroxyapatite composites. Volume 93. Pages 256-262.

(12) LeGeros, R. (2002). Properties of Osteoconductive Biomaterials: Calcium Phosphates. Current Orthopaedic Practice. Volume 395. Pages 81-98

(13) Bucholz, R. (2012) Nonallograft Osteoconductive Bone Graft Substitutes. Clinical Orthopaedics and Related Research. Volume 395. Pages 44-52

(14) Albrektsson, T. et al. (2001). Osteoinduction, osteoconduction and osseointegration. Department of Biomaterials/Handicap Research, Gothenburg, Sweden. Volume 10. Pages 96-101

(15) Hutmacher, D. (2000). Scaffolds in tissue engineering bone and cartilage. Biomaterials. Volume 1980. Pages 175-189

(16) Black, C. et al. (2015). Bone Tissue Engineering. Current Molecular Biology Reports. Volume 1. Pages 132–140
(17) Burg, K. et al (2000) Biomaterial developments for bone tissue engineering. Biomaterials. Volume 21. Pages 2347-2359
(18) Jiao, L. et al. (2014). Scaffold-based regeneration of skeletal tissues to meet clinical challenges. Journal of Materials Chemistry B. Volume 2. Pages 7263-7466
(19) U.S. Department of Health and Human Services. (2004). The Frequency of Bone Disease. Bone Health and Osteoporosis: A Report of the Surgeon General. Rockville MD. Pages 68-84
(20) Pallela, R. et al. (2011). Biophysicochemical evaluation of chitosan-hydroxyapatite-marine sponge collagen composite for bone tissue engineering. J Biomed Mater Res A. Volume 100. Pages 486-495
(21) Martins, A. et al. (2009). Responsive and in situ-forming chitosan scaffolds for bone tissue engineering applications: an overview of the last decade. Journal of Materials Chemistry. Volume 20. Pages 1609-1820
(22) Kulig, D. et al. (2017). Effect of Film-Forming Alginate/Chitosan Polyelectrolyte Complex on the Storage Quality of Pork. Molecules. Volume 22. Page 98
(23) Tande, A. et al. (2014). Prosthetic Joint Infection. Clinical Microbiology Reviews Volume 27. Pages 302–345
(24) Kapadia, B. et al. (2015). Periprosthetic joint infection. The Lancet Journal. Volume 387. Pages 386-394
(25) Berbari, E. (2019). Prosthetic joint infection: Epidemiology, clinical manifestations, and diagnosis. https://www.uptodate.com/contents/prosthetic-joint-infection-epidemiology-clinical-manifestations-and-diagnosis.
(26) Bansal, A. et al. (2014). Revision total joint arthroplasty: the epidemiology of 63,140 cases in New York State. Journal of Arthroplasty. 2014 Volume 29. Pages 23–27
(27) Parvizi, J. et al. (2013). Anais do Segundo Consenso Internacional em Infecções Musculoesqueléticas.
(28) Tan, H. et al. (2013). Quaternized Chitosan as an Antimicrobial Agent: Antimicrobial Activity, Mechanism of Action and Biomedical Applications in Orthopedics. International Journal of Molecular Sciences. Volume 14. Pages 1854-1869
(29) Ryan, S. et al. (2018) Two-Stage Revision Arthroplasty for the Treatment of Prosthetic Joint Infection. Volume 11. Pages 332–340

(30) D'Elia, C. et al. (2007). Tratamento das infecções pós artroplastia total de joelho: resultados com 2 anos de seguimento. Acta Ortop. Bras. Volume 15.
(31) Kumar Nayak, A. (2010) Hydroxyapatite Synthesis Methodologies: An Overview. International Journal of ChemTech Research. Volume 2. Pages 903-907
(32) Weon Lee, D. et al. (2009). Advances in Chitosan Material and its Hybrid Derivatives: A Review. The Open Biomaterials Journal. Volume 1. Pages 10-20
(33) Piccirillo, C. et al (2013). Extraction and characterisation of apatite- and tricalcium phosphate-based materials from cod fish bones. Materials Science and Engineering. Volume 33. Pages 103-110
(34) Chen, F. et al (2002). Preparation and characterization of nano-size hydroxyapatite particles and hydroxyapatite/chitosan nano-composite for use in biomedical materials. Material Letters. Volume 57. Pages 858-861
(35) Venkatesan, J. et al (2010). Chitosan Composites for Bone Tissue Engineering—An Overview. Mar Drugs. 2010 Aug 2;8(8):2252-66
(36) Muzzareli, R. et al. (2019) Antimicrobial properties of N-carboxybutyl chitosan. American Society for Microbiology. Volume 34. Pages 2019–2023.
(37) Rejane C. et al. (2009). A review of the antimicrobial activity of chitosan. Polímeros. Volume 19. Pages 1678-5169.
(38) Costa-Pinto, A. et al. (2011). Scaffolds Based Bone Tissue Engineering: The Role of Chitosan. Tissue Engineering. Volume 17. Pages 1-18.
(39) Fu, C. et al. (2017), Enhancing Cell Proliferation and Osteogenic Differentiation of MC3T3-E1 Pre-osteoblasts by BMP-2 Delivery in Graphene Oxide-Incorporated PLGA/HA Biodegradable Microcarriers. Sci Rep. Volume 7. Article 12549.
(40) Chesnutt, B. et al. (2009). Composite Chitosan/Nano-Hydroxyapatite Scaffolds Induce Osteocalcin Production by Osteoblasts In Vitro and Support Bone Formation In Vivo. Tissue Engineering. Volume 15. Pages 2571-2590
(41) Murugan, R. et al (2003). Bioresorbable composite bone paste using polysaccharide based nano hydroxyapatite. Biomaterials. Volume 25. Pages 3829-3835
(42) Maruyama, M. et al (1996). In vitro properties of a chitosan-bonded self-hardening paste with hydroxyapatite granules. Journal of Biomedical Materials Research. Volume 10. Pages 1097-4636
(43) ISO 10993-5 (2009) Biological evaluation of medical devices — Part 5: Tests for in vitro cytotoxicity. Third edition

(44) Roth, J. et al. (1999). Melatonin Promotes Osteoblast Differentiation and Bone Formation. The Journal of Biological Chemistry. Volume 274. Pages 22041–22047
(45) Franceschi, R. et al (1992). Relationship between collagen synthesis and expression of the osteoblast phenotype in MC3T3-E1 cells. Journal of bone and mineral research. Volume 7. Pages 235-246
(46) Beck, G. et al. (1998). Relationship between alkaline phosphatase levels, osteopontin expression, and mineralization in differentiating MC3T3-E1 osteoblasts. Journal of Cellular Biochemistry. Volume 68. Pages 269-280
(47) Quarles, L. et al. (1992). Distinct proliferative and differentiated stages of murine MC3T3-E1 cells in culture: an in vitro model of osteoblast development. Journal of bone and mineral research. Volume 6. Pages 683-692.
(48) Yazid, M. et al. (2010) Determination of the differentiation capacities of murines' primary mononucleated cells and MC3T3-E1 cells. Cancer cell international. Volume 10. Pages 1475-2867
(49) Ozdemir, K. et al. (2008). In vitro evaluation of cytotoxicity of soft lining materials on L929 cells by MTT assay. Journal of Biomedical Materials Research. Volume 90B. Pages 82-86
(50) Ferrari, M. et al. (1990). MTT colorimetric assay for testing macrophage cytotoxic activity in vitro. Journal of Immunological Methods. Volume 131. Pages 165-172
(51) Yilmaz, Z. et al. (2012). Evaluation of the cytotoxicity of different root canal sealers on L929 cell line by MTT assay. Dental Materials Journal. Volume 31. Pages 1028–1032
(52) Siffert, R. (1991). The role of Alkaline Phosphatase in Osteogenesis. Journal of Experimental Medicine. Volume 93. Page 415
(53) Golub, E. et al. (2007). The role of alkaline phosphatase in mineralization. Current Orthopaedic Practice. Volume 18. Pages 444–448
(54) Beck, George R. et al (1998). Relationship between alkaline phosphatase levels, osteopontin expression, and mineralization in differentiating MC3T3-E1 osteoblasts. Journal of Cellular Biochemistry. Volume 68. Pages 269-280
(55) Barrère, F. et al (2006) Bone regeneration: molecular and cellular interactions with calcium phosphate ceramics. International Journal of Nanomedicine. Volume 1. Pages 317-332
(56) Anderson, J. (1996). Calcium, Phosphorus and Human Bone Development. The Journal of Nutrition. Volume 126. Pages 1153–1158

(57) Lopez-Garcia, J. et al (2014) HaCaT Keratinocytes Response on Antimicrobial Atelocollagen Substrates: Extent of Cytotoxicity, Cell Viability and Proliferation. Journal of Functional Biomaterials. Volume 5. Pages 43-57

(58) Langenbach, F. et al. (2013). Effects of dexamethasone, ascorbic acid and β-glycerophosphate on the osteogenic differentiation of stem cells in vitro. Stem Cell Research & Therapy. Volume 4. Page 117

(59) Yamauchi, Maki et al. (2005). Involvement of calcium-sensing receptor in osteoblastic differentiation of mouse MC3T3-E1 cells. American Journal of Physiology. Volume 288. Pages 608-616