

Faculdade de Engenharia da Universidade do Porto



**Effects of Static Magnetic Fields on *In Vitro* Bone
Cell Behaviour and *In Vivo* Angiogenesis in
Magnetic Bone Implants**

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Resumo

A regeneração do tecido ósseo é um campo de investigação em evolução que procura desenvolver estratégias inovadoras para a reparação e regeneração de tecido ósseo danificado ou perdido. As abordagens tradicionais, como os autoenxertos e aloenxertos, têm limitações e desafios associados à morbilidade do local do dador e à disponibilidade limitada. Consequentemente, os investigadores voltaram a sua atenção para abordagens baseadas em biomateriais que podem promover a regeneração de tecido ósseo funcional.

Nos últimos anos, a integração de campos magnéticos, nanopartículas magnéticas e scaffolds magnéticos surgiu como uma via promissora para melhorar a regeneração do tecido ósseo. Foi demonstrado que os campos magnéticos exercem vários efeitos biológicos, incluindo a promoção da proliferação celular, a modulação do comportamento celular e a melhoria da regeneração dos tecidos. As nanopartículas magnéticas, frequentemente compostas por ferro ou óxido de ferro, possuem propriedades únicas que as tornam adequadas para aplicações biomédicas. Estas nanopartículas podem ser facilmente funcionalizadas e manipuladas sob a influência de campos magnéticos externos, permitindo a administração orientada de agentes terapêuticos e a libertação controlada de factores de crescimento. Além disso, os scaffolds magnéticos, que incorporam nanopartículas magnéticas numa matriz biocompatível, oferecem a possibilidade de controlar espacialmente e temporalmente o comportamento celular e os processos de regeneração de tecidos.

Neste estudo, explorámos o potencial dos grânulos de hidroxiapatite revestidos com colagénio tipo I e nanopartículas magnéticas, que foram sintetizados utilizando hidroxiapatite dopada com ferro.

Para avaliar os grânulos, foi efectuada uma caracterização exaustiva utilizando várias técnicas. A espectroscopia de infravermelhos com transformada de Fourier (FTIR) foi utilizada para avaliar a composição química. Foram efectuados testes mecânicos, incluindo testes de resistência à compressão e de friabilidade, para determinar as propriedades mecânicas do material. A microscopia electrónica de varrimento (SEM), a tomografia microcomputorizada

(micro-CT) e a análise através do dispositivo de interferência quântica supercondutora (SQUID) foram utilizadas para a caracterização morfológica, estrutural e magnética, respectivamente.

Para além da caracterização do material, foram realizados estudos biológicos *in vitro* e *in vivo*. Os estudos *in vitro* realizados neste estudo envolveram a utilização de células MC3T3-E1 com os grânulos durante 7, 14 e 21 dias, onde o ensaio de resazurina revelou que existe uma maior proliferação celular no 14º dia e que a utilização de MNPs com SMF teve impacto quando utilizado com factores osteogénicos. A microscopia confocal mostrou que existe migração celular e nenhuma toxicidade. A fosfatase alcalina (ALP) revelou que a diferenciação teve lugar ao 21º dia e que o SMF com MNPs teve uma influência positiva. A alizarina revelou não ser o melhor teste para a mineralização, uma vez que forma complexos com o ferro.

Para avaliações *in vivo*, foi realizado o ensaio da membrana corioalantóica de galinha (CAM), que demonstrou que o SMF juntamente com as MNPs promove a angiogénese e confirma a não toxicidade.

Em conclusão, esta investigação apresenta uma alternativa promissora para a regeneração do tecido ósseo, utilizando grânulos com MNPs combinados com estimulação externa através da aplicação de um SMF. A caracterização exaustiva, juntamente com as avaliações biológicas positivas, apoia a potencial aplicação clínica do material compósito desenvolvido como substituto dos enxertos ósseos.

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Abstract

Bone tissue regeneration is an evolving field of research that seeks to develop innovative strategies for the repair and regeneration of damaged or lost bone tissue. Traditional approaches, such as autografts and allografts, have limitations and challenges associated with donor site morbidity and limited availability. As a result, researchers have turned their attention to biomaterial-based approaches that can promote the regeneration of functional bone tissue.

In recent years, the integration of magnetic fields, magnetic nanoparticles, and magnetic scaffolds has emerged as a promising avenue for enhancing bone tissue regeneration. Magnetic fields have been shown to exert various biological effects, including promoting cell proliferation, modulating cellular behaviour, and enhancing tissue regeneration. Magnetic nanoparticles, often composed of iron or iron oxide, possess unique properties that make them suitable for biomedical applications. These nanoparticles can be easily functionalized and manipulated under the influence of external magnetic fields, enabling targeted delivery of therapeutic agents and controlled release of growth factors. Furthermore, magnetic scaffolds, incorporating magnetic nanoparticles within a biocompatible matrix, offer the possibility of spatially and temporally controlling cellular behaviour and tissue regeneration processes.

In this study, we explored the potential of hydroxyapatite granules coated with collagen type I and magnetic nanoparticles, which were synthesized using iron-doped hydroxyapatite.

To evaluate the granules, a comprehensive characterisation was performed using various techniques. Fourier Transform Infrared Spectroscopy (FTIR) was employed to assess the chemical composition. Mechanical tests, including compressive strength and friability tests, were conducted to determine the material's mechanical properties. Scanning Electron Microscopy (SEM), micro-Computed Tomography (micro-CT), and Superconducting Quantum Interference Device (SQUID) analysis were employed for morphological, structural, and magnetic characterisation, respectively.

In addition to the material characterization, biological studies were conducted both *in vitro* and *in vivo*. *In vitro* studies conducted in this study involved the use of MC3T3-E1 cells with the

granules during 7, 14 and 21 days, where resazurin assay revealed that there is greater cell proliferation at day 14 and that the use of MNPs with SMF had an impact when used with osteogenic factors. Confocal microscopy showed cell migration and no toxicity. Alkaline Phosphatase (ALP) revealed that the differentiation took place on day 21 and that the SMF with MNPs had a positive influence. Alizarin red, proved not to be the best test for mineralisation as it forms complexes with iron.

For *in vivo* assessments, the Chick Chorioallantoic Membrane (CAM) assay was performed and showed that SMF together with MNPs promotes angiogenesis and confirms non-toxicity.

In conclusion, this research presents a promising alternative for bone tissue regeneration by using granules with MNPs combined with external stimulation through the application of an SMF. The comprehensive characterization, along with the positive biological evaluations, supports the potential clinical application of the developed composite material as a substitute for gold-standard bone grafts.

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Acronyms, Abbreviations and Symbols

List of abbreviations

ALP	Alkaline Phosphatase
bAp	biological Apatite
BCA	Bicinchoninic Acid Assay
bFGF	basic Fibroblast Growth Factor
BMP	Bone Morphogenetic Proteins
BSA	Bovine Serum Albumin
CAM	Chorioallantoic Membrane
CO ₂	Carbon Dioxide
COL	Collagen
EDC	1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride
EDS	Energy Dispersive Spectroscopy
EED	Embryonic Development Day
eNos	endothelial Nitric oxide synthase
FBS	Fetal Bovine Serum
FCUP	Faculdade de Ciências da Universidade do Porto
FFUP	Faculdade de Farmácia da Universidade do Porto
FTIR	Fourier Transform Infrared Spectroscopy
Hap	Hydroxyapatite
HUVEC	Human Umbilical Vein Endothelial Cell
HCL	Hydrochloric acid
IONP	Iron Oxide Nanoparticle
MicroCT	Micro Computer Tomography
MNP	Magnetic Nanoparticle
MRI	Magnetic Resonance Imaging
NHS	N-Hydroxysuccinimide
NIR	Near-infrared
PBS	Phosphate-buffered Saline

PCL	Polycaprolactone
PCR	Polymerase Chain Reaction
PEMF	Pulsed Eletromagnetic Field
PFA	Paraformaldehyde
PMMA	Polymethylmethacrylate
P/S	Penicillin/Streptomycin
PUR	Polyurethane
RMF	Rotating Magnetic Field
ROS	Reactive Oxygen Species
RT	Room Temperature
SEM	Scanning Electron Microscope
SMF	Static Magnetic Field
SPION	Superparamagnetism Iron Oxide Nanoparticle
SQUID	Superconducting Quantum Interference Device
UK	United Kingdom
USA	United States of America
VEGF	Vascular Endothelial Growth Factor
XRD	X-ray Powder Diffraction
α MEM	alpha Minimum Essential Medium

List of symbols

A	Ampere
Cm	Centimeter
g	Gram
K	Kelvin
Kg	kilogramme
kPa	Kilopascal
M	Molar
m	Meter
mg	Miligram
min	Minute
mL	Mililiter
mm	Milimeters
mT	Militesla
N	Newton
nm	Nanometers
nT	Nanotesla
Oe	Oersted

Pa	Pascal
rpm	Rotation per minute
s	Seconds
T	Tesla
V	Voltage
w/v	Weight per volume
μL	Microliter
μT	Microtesla
°C	Celsius Degrees
%	Percentage

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Chapter 1

Introduction

1.1 - Bone Tissue

The human body is made up of 206 bones, which are constantly remodelled to adapt to the biomechanical stresses to which they are subjected throughout their lives. This remodelling process takes place by replacing old and damaged tissue with new tissue capable of better supporting the loads to which it is subjected[1]. The human skeleton has several functionalities, including structural support for the entire body, allowing locomotion and movement, protecting internal structures such as organs, maintaining mineral homeostasis, providing space and environment for hematopoiesis within the bone marrow, as well as serving as a reservoir for cytokines and growth factors (nutrients and minerals)[2]. Bones can be classified and divided into four categories: long bones (femurs, radii, tibiae, metatarsals, phalanges), short bones (patellae, carpal, and tarsal bones), irregular bones (vertebrae, coccyx, sacrum) and flat bones (sternum, ribs, skull, scapulae, mandible).

For bones to fulfill all these functions, they have a unique architecture in which the trabecular and cortical zones are distinguished[3].

Cortical bone, also known as compact bone, is a type of bone tissue that makes up the outer layer of bones. It is characterized by its dense, hard structure, composed mainly of mineralized collagen fibers. Cortical bone provides structural support and protection for the body and gives bones their shape. It is denser more robust than trabecular bone and has a lower surface-area-to-volume ratio. This type of bone is less metabolically active than trabecular bone and is not as responsive to changes in mechanical stress. Still, it can withstand higher loads and provide better protection[2]-[4].

Trabecular bone, also known as cancellous or spongy bone, is characterized by its highly porous, spongy structure, which is made up of a network of thin, interconnecting trabeculae. It is also highly adaptable and responsive to changes in mechanical stress, making it a key

player in maintaining bones' overall strength and integrity. It plays a vital role in bone remodelling process, which helps maintain the balance between new bone formation and bone resorption. Trabecular bone is more susceptible to osteoporosis and brittle fractures than dense cortical bone. Still, it has a higher area-to-volume ratio that allows better blood flow and remodelling[2]-[4].

1.2 - Bone Grafts

When a bone is subjected to a traumatic event in which there is an external load that exceeds the bone strength, or when it is subjected to cyclic and accumulated damage to a bone structure, the bone characteristic dynamic balance is exceeded, and a fracture initiates. bone can repair itself, as it is nothing more than a recapitulation of the ontological process during the embryonic phase of the development and growth of the skeleton so that a damaged bone can also recover its shape and its mechanical characteristics and functionalities[5].

However, when the size to be repaired is bigger than a critical size, the regenerative process needs external "help" so that this process can be carried out. This situation happens when there is a delayed union, non-union, and malunion or in cases of bone filling caused by tumours[6]. In such cases, the gold standard is to make use of bone grafts.

Bone grafting is nowadays a common surgical procedure in orthopaedics. It is estimated that around two million surgeries are carried out every year around the world using bone grafts, thus making it, after blood transfusion, the most used tissue transplant[5].

Given the various existing injuries and traumas, from the simplest to the most complex, each one is different from one organism to another. Therefore, bone grafts aim to combine mechanical characteristics and functions, as well as osteo-regeneration, which includes three critical biological properties: osteoconduction, osteoinduction, and osteogenesis[5]. Osteoconduction is a material's property that promotes apposition between the surface and bone, acting as a scaffold, hosting the necessary cells to facilitate bone formation. Osteoinduction refers to the ability of a material to stimulate the differentiation of stem cells into bone cells. It's the process of initiating new bone formation by recruiting mesenchymal stem cells and their differentiation into osteoblasts. Osteoblasts are bone cells that produce and deposit the extracellular matrix that forms bone. Osteogenesis is the bone formation process and a combination of osteoconduction and osteoinduction[6]-[8].

There are several bone grafts, including autografts, allografts, and synthetic grafts. An autograft is a bone graft taken from one area of the patient's body and transplanted to the area that needs to be repaired. This type of bone graft is considered the gold standard because it is the patient's bone and has the least risk of rejection. Autografts are usually taken from the patient's hip, shinbone, or ribs. Allografts are bone grafts taken from a donor and transplanted to the patient. This type of bone graft is less invasive than an autograft and can be used when the patient's bone is unavailable or is not healthy enough to be used. Allografts

are usually taken from cadavers but can also be taken from living donors. Synthetic grafts are artificial bone substitutes made from various materials, such as ceramics, polymers, and biologics. These bone grafts are used when patient's bone or a donor's bone is not available or suitable. Synthetic bone grafts are designed to mimic the properties of natural bone and promote bone growth. The success of a bone graft depends on several factors, including the type of bone graft used, the health of the patient, and the location of the injury or disease. Bone grafts can help to repair bone damage and promote bone growth, but they do not guarantee a complete cure. The recovery period after bone grafting may vary depending on the type of bone graft and the location of the injury or disease, but it can take several months for the bone to fully heal[5], [9].

1.3 - Bone Tissue Engineering

Tissue Engineering was defined in 1993 by Joseph P. Vacanti and Robert Langer as "... an interdisciplinary field which applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function." [10]. After 30 years, tissue engineering continues to be one of the most researched fields, with constant discoveries in the most wide areas of science and human health.

Bone tissue engineering uses biological, chemical, and physical methods to repair or regenerate bone tissue. This can include the use of stem cells, growth factors, scaffolds, and other biomaterials to promote the growth of new bone tissue. The goal of bone tissue engineering is to repair or replace damaged or missing bone tissue to improve the activity and strength of the affected area. This field has many potential applications, including treating bone fractures, osteoporosis and bone tumours[11].

One of the key components of bone tissue engineering is the use of stem cells. Stem cells are undifferentiated cells that can differentiate into various cell types, including osteoblasts, the cells responsible for building new bone. Stem cells can be obtained from several sources, including bone marrow, adipose tissue, and umbilical cord blood. Once obtained, the stem cells can be induced to differentiate into osteoblasts and then used to repair or regenerate damaged bone tissue[12], [13].

Another essential component of bone tissue engineering is the use of scaffolds. Scaffolds are three-dimensional structures that provide a framework for the growth and differentiation of cells[11]. They can be made from various materials, including ceramics, polymers, and natural materials such as collagen[4], [14]. Scaffolds can be designed to mimic the structure and composition of natural bone. To achieve this, the three-dimensional structure must fulfill specific characteristics to succeed. It needs to be biocompatible, not containing toxic materials or generating an immune response due to possible inflammation. It is required that it shall be biodegradable, so the material must degrade over time, in a controlled manner, allowing the host cells time enough to produce their extracellular matrix. The scaffold needs to be

1 Introduction

bioactive, with osteoconductive and osteoinductive properties, with the addition of growth factors, thus enabling cell growth and differentiation. Its mechanical properties should also be considered since these are scaffolds for bone tissue regeneration; for that reason, this should correspond to the characteristics of bone, and it is, therefore, essential that the material might have compressive, elastic, and fatigue strengths, to prevent the stress-shielding phenomena. Its architecture is also one of the most critical points, paying special attention to its micro and macro porosity so that there is cell interaction and, migration, interconnectivity to allow cell diffusion and pore size to reach all tissues and cells without compromising the structure. Inbuilt vascular channels activate and help angiogenesis *in vivo*[15].

Growth factors are also an essential component of bone tissue engineering. These are naturally occurring proteins that play a critical role in the regulation of cell growth and differentiation. When applied to damaged or missing bone tissue, growth factors can promote the proliferation and differentiation of stem cells into osteoblasts, which can then be used to repair or regenerate the damaged bone[11], [14].

Finally, bone tissue engineering also involves the use of various biomaterials. Biomaterials can create scaffolds that deliver growth factors and other therapeutic agents to the site of bone injury. These materials, as said before, must be biocompatible, meaning they should not cause any adverse reactions in the body and should be able to promote the growth and repair of bone tissue[16].

Overall, bone tissue engineering is a multi-disciplinary field involving stem cells, scaffolds, growth factors, and biomaterials to repair or regenerate damaged or missing bone tissue. With the continued advancements in regenerative medicine, it is expected to play a more significant role in the field of orthopedic and trauma surgery.

1.3.1 - Internal and External Stimuli for Bone Regeneration

In its conventional form, tissue engineering is the combination of cells, scaffolds, and growth factors, always to promote cell growth and differentiation in the regeneration and/or repair process of damaged bone tissue. However, the most recent studies have focused on the possibility of adding internal and external stimuli to accelerate the biological process of bone regeneration[17]. The internal stimuli consider the specific pathological microenvironments. Therefore, traumas which it possible to develop a biomaterial capable of responding autonomously to changes in its environment. External stimuli, as the term suggests, is the application of an external source stimulating stimulate cells to adhere, proliferate, and differentiate inside the scaffold. This is usually achieved by integrating biomaterials, usually nanoparticles, which can be externally influenced and stimulated. There are several examples of stimuli, internal and external, as shown in figure 1.1.

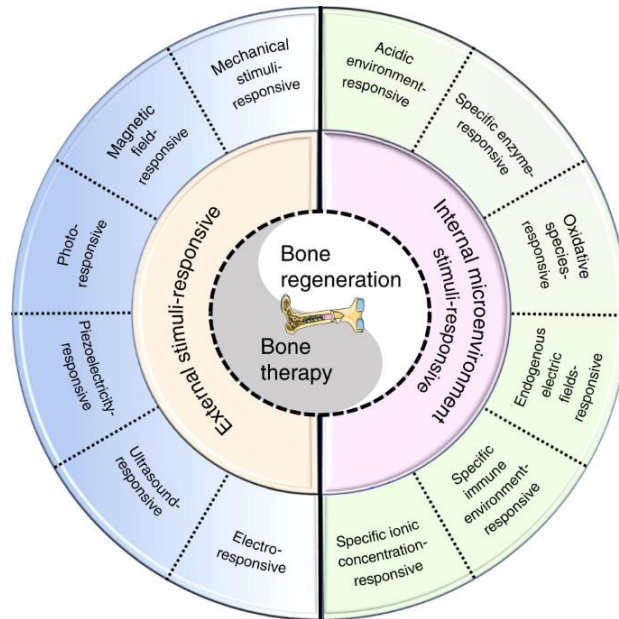


Figure 1.1 - Different types of internal and external stimuli for bone regeneration[18].

However, focusing more on external stimuli, these can be of various types, including:

- **Mechanical** - Mechanical stimuli are used because they influence the osteocytes, which are sensitive to mechanical stress. In addition, it can be stimulated, leading to cell proliferation, migration, and differentiation. However, there are some barriers to implement this type of stimulus, such as the optimization of the mechanical parameters, such as amplitude, frequency, and timing of the loading events [18], [19].
- **Ultrasound** - stimulation through ultrasounds as they can penetrate the tissues well, making it possible to stimulate the proliferation of cartilage cells and accelerate the process of bone formation and mineralization. However, this method has some limitations, similar to mechanical stimulation, mainly in the choice of parameters, so that cells and normal tissue are not damaged by the increase in temperature [19], [20].
- **Electrical** - The use of electrical stimuli is due to their capacity to activate voltage-gated Ca^{2+} channels, which modulate osteogenesis through calmodulin pathways. This, together with the development of materials that conduct electrical signals to the injured area, leads to an acceleration in the process of bone regeneration. However, issues have arisen, such as the difficulty in controlling material degradation [17].
- **Piezoelectrical** - Piezoelectric stimulation is achieved by transforming mechanical energy into electrical energy. As seen in the previous point, electrical power can influence osteogenesis. This stimulus involves a combination of mechanical stresses and their transformation into electrical energy because bone when under

mechanical stress can generate electrical signals. This stimulus, combined with the integration of piezoelectric biomaterials, can influence the proliferation and differentiation of stem cells. However, recent studies show some limitations, such as alkali volatilization, synthesis temperature process, and the lack of knowledge of biosafety and toxicity in the long term[19].

- **Magnetic** - Magnetic stimulation has not yet been much developed in bone regeneration. However, there is some evidence, particularly in cancer, in which this method can eliminate cancer cells without damaging adjacent tissues, leading to a belief that this stimulus can influence proliferation and cell differentiation. This stimulus is applied through exposure to a magnetic field, combined with the inclusion of magnetic nanoparticles in scaffolds so that the magnetic field controls them. This stimulus is promising since it is a non-invasive and highly controllable method[4], [19].
- **Light irradiation** - The use of stimulation through light irradiation is based on the use of a laser, in which the depth to be reached is determined to choose the most suitable wavelength and therefore understand in which category we are working, which can be: ultraviolet (200-400 nm), visible (400-700 nm) and near-infrared (700-1300 nm). Some studies using this stimulus tend to be based on the use of near-infrared (NIR), as it has the greatest capacity to penetrate the tissues. This method is allied to photoresponsive nanoparticles that, when exposed to light, are activated, promoting osteogenesis. This method presents some limitations in which greater control and less invasive means capable of improving tissue penetration are sought[17], [19], [20].

As external stimuli have been raising a lot of interest and curiosity in bone tissue regeneration, this report will address and focus on magnetism as an external stimulus. This method has been studied and investigated; however, there is still the need for consensus regarding its capacity to stimulate osseointegration. This work, therefore, aims to reach some conclusions about its fundamental importance and use in bone tissue regeneration.

Chapter 2

Magnetic Fields Alone Effects on Bone Regeneration

As previously mentioned, stimulation through magnetism has been arousing curiosity, leading to more and more studies being carried out to understand its advantages when applied to bone tissue regeneration. This is based on creating and applying a magnetic field responsible for the stimulus to be applied to the injured area. This magnetic field can be generated in four ways: pulsed magnetic fields (PEMFs), static magnetic fields (SMFs), alternating magnetic fields, and rotating magnetic fields (RMFs)[21]. Although all of them have great capacities to generate a magnetic field, there is a clear choice and preference for the use of SMFs, because their application is more accessible. It does not need to be connected to a power supply since its production derives from a simple magnet [22], [23].

The static magnetic field is categorized according to its strength, as ultra-high (above 5T), high SMF (from 1T to 5T), moderate SMF (between 1 mT and 1T), low SMF (from 5 μ T to 1mT) and hypo SMF (below five μ T), in most cases, applied magnetic fields are low and moderate as they do not cause any adverse effect in humans or animals[4], [21].

Some studies carried out in this area with the use of SMFs in bone regeneration have shown to be very promising, showing that when bone traumas are exposed to this stimulus, an acceleration in the healing and mineralization process during bone repair is observed, and also in cases of osteoarthritis and spinal fusion [24]. Studies carried out by Aydin et al. on rabbits showed that the use of SMF helped the healing process in the first two weeks and influenced the bone mineral density values, something that had also been observed by Q.C Yan et al., who found that the use of the stimulus could prevent the decrease in bone density values[22], [25].

2.1 - Effects on Osteoblast Cells

Osteoblasts are cells that are responsible for bone formation. They are a type of bone cell that produces and deposits the matrix (the non-mineralized, organic part and the mineralized one) of bone. Osteoblasts are derived from mesenchymal stem cells, that can differentiate into various types of connective tissue cells, including osteoblasts, chondrocytes, and adipocytes. Once they differentiate into osteoblasts, they begin to produce and deposit the bone matrix, composed of collagen and other proteins from the BMP family (bone morphogenetic proteins). They also produce growth factors and other signaling molecules that regulate bone formation. As osteoblasts deposit the matrix of bone, they become trapped within it and are transformed into osteocytes, which are mature bone cells responsible for maintaining the bone matrix[4], [26].

Since magnetic stimulation is shown to impact bone tissue regeneration, it also influences osteoblastic cells, which are extremely important in bone tissue formation, as said before.

In the literature, it is well evidenced that the influence of a magnetic field enhances osteoblast differentiation. This happens due to the distortion and reorientation of the cell membrane and proteins of the extracellular matrix, which alters the isotropic diamagnetic properties[22], [27].

In the study by Bekhite et al., it was found that exposing Flk-1⁺ cardiac progenitor cells to magnetic fields of 1mT (SMF) can induce cardiomyogenesis. This process is activated by the influx of calcium and the production of reactive oxygen species (ROS). The magnetic fields activate stretch-activated cation channels, which in turn leads to an increase in calcium levels. The resulting ROS plays a crucial role in the differentiation of the cells. Similarly, the magnetic field strength can affect bone tissue's differentiation by modulating intracellular mineral ions levels [28].

Zhang et al. conducted a study in which they exposed MC3T3-E1 cells to different strengths of static magnetic field (SMF) (500 nT, 0.2 T, and 16 T). They found that as the field strength increased, so did the concentration of minerals such as calcium, magnesium, and iron inside the cells[29]. This led to a higher degree of differentiation. Iron and magnetic fields have a natural relationship, as described by Yang et al., who found that under high SMF (16 T), iron accumulation inside MC3T3-E1 cells was due to the increased expression of transferrin receptor one and ferroportin 1. This effect was also associated with increased alkaline phosphatase (ALP) activity and mineralization[30].

In an in vivo study, Zhang et al. showed that moderate SMF (4mT) could prevent bone deterioration in diabetic mice. The study found that the SMF treatment led to higher levels of osteocalcin, an increase in the number of osteoblasts, and an upregulation of BMP2 and Runx2 genes, while not affecting osteoclast-related biochemical factors[31].

2.2 - Effects on Endothelial Cells

Endothelial cells play a vital role in the process of bone regeneration. They are involved in forming new blood vessels, a process known as angiogenesis, which is essential for the growth and repair of bone tissue. During bone healing, endothelial cells migrate to the site of injury, forming new blood vessels to provide the necessary oxygen and nutrients for the proliferation and differentiation of bone-forming cells. This cell type also produces various growth factors and cytokines that promote bone formation and repair, such as Vascular Endothelial Growth Factor (VEGF) and the basic Fibroblast Growth Factor (bFGF). Moreover, they are responsible for modulating the activity of bone-resorbing cells, such as osteoclasts, for maintaining a proper balance between bone formation and bone resorption[32], [33].

Martino et al. study shows that weak magnetic fields positively impact the growth and expression of eNOS (endothelial nitric oxide synthase) in HUVECs. The authors found that endothelial cells are sensitive to both the strength of the magnetic field and the length of time they are exposed to it. After 2 days at 120 μ T, the proliferation of HUVECs increased by 40%. In contrast, at 60 μ T, with only 24 h of exposure, a 40% increase in the number of cells was observed. Such an increase was justified by the augmented endothelial nitric oxide synthase (eNOS) expression. When compared with the control, the percentage of expressed eNOS was 54% against 78% with magnetic stimulation [34].

In another study, Wang et al. found that PEMF with specific settings prevented bone loss in a mouse model of postmenopausal osteoporosis. This effect was accompanied by an increase in a specific type of endothelial cells in the metaphysis of long bones. The study suggests that this coupling of PEMF-induced osteogenesis and expansion of types H vessels may be mediated by HIF-1 α signaling in these endothelial cells. These findings suggest new possibilities for using PEMF to target the endothelium in the treatment of osteoporosis and the need for further research to understand the underlying mechanisms of HIF-1 α signaling for optimal results[35].

The application of magnetic fields has increased in importance and curiosity in the most varied scientific areas. Through the literature, we can see that this importance is since the application of external stimulus promotes good indicators, especially in the area of bone regeneration, with the promotion of cell proliferation and differentiation.

A summary of these studies can be seen in Table 2.1.

Table 2.1 - Summary of studies reporting the influence of magnetic fields in bone regeneration and angiogenesis.

			Type of Cell	Results	Results <i>in vivo</i>	Mechanism	Reference
Static	Pulsed	Intensity					
X	—	500 nT 0.2 T 16 T	MC3T3-E1	Concentration of minerals increase alongside the strength of SMF; Higher degree of differentiation	—	Osteoblasts in differentiation accumulated more mineral elements than non-differentiated cell cultures	[17]
X	—	16T	MC3T3-E1	Iron accumulation inside the cells	—	Expression of transferrin receptor one and ferroportin 1; Associated with increased ALP activity and mineralization	[13]
X	—	4 mT	—	—	Prevent bone deterioration in diabetic mice	Treatment led to high levels of osteocalcin; increased number of osteoblasts; upregulation of BMP2 and Runx2 genes	[20]
X	—	60uT 120 uT	HUVECs	40% increase in proliferation	—	Endothelial cells' functionality increased with the application of SMF, which upregulated eNOS expression	[22]
—	X	—	—	Increase of endothelial cells in the metaphysis of long bones	Prevent bone loss in a mouse model of postmenopausal osteoporosis	PEMF-induced osteogenesis and expansion of types H vessels may be mediated by HIF-1 α signalling in these endothelial cells	[23]

Chapter 3

Magnetic Nanoparticles on Bone Regeneration

Magnetic nanoparticles (MNPs) have been studied to understand their role and influence on bone tissue regeneration. MNPs are a type of nanomaterial that is composed of metals that are influenced in the presence of a magnetic field. This type of particle can be made of metals such as iron and cobalt, onto which a biocompatible coating is then applied to provide good acceptance by the organism/tissue in which it is placed. In addition, this coating can also serve to enhance some extra functionality depending on its application[36].

Iron-oxide-based MNPs (magnetite (Fe_3O_4), maghemite ($\gamma\text{-Fe}_2\text{O}_3$), and hematite ($\alpha\text{-Fe}_2\text{O}_3$)) are the most used and studied in bone regeneration in the literature, as they have superparamagnetic properties. Superparamagnetism happens when you have ferromagnetic material of small dimensions, which in the presence of a weak magnetic field, can generate strong magnetism, disappearing when it is no longer under the influence of external stimulus. Superparamagnetic Iron Oxide Nanoparticles (SPIONs) present, therefore, some important properties such as biocompatibility; they are controllable, biodegradable, and chemically stable[4], [37].

Regarding toxicity, some studies show some discrepancies; on the one hand a 35 mg/kg dose of SPIONs showed significant levels of toxicity in some organs, such as the kidney and liver[38]. On the other hand, Pham et al. stated that it had no considerable toxicity results when using 90 mg/kg SPIONs conjugated with diblock copolymers[38].

A study in rats developed by Verma et al. shows that some rats presented some weight loss and significant liver damage when using IONPs over a long period of time [39]. However, when comparing toxicity between mouse and human stem cells, human-derived stem cells show a more remarkable ability to metabolize iron ions before they become toxic[36].

Regarding the toxicity of MNPs, some criteria should be considered before their possible use/testing. One of the aspects to be taken into account is the type of cell to be used (cell-

specific), the dosage (dose-dependent), the particle size, the aggregation time at the same site, and the choice of surface coating[40].

Additionally, SPIONs presented significant potential in supporting bone tissue regeneration. They have been shown to promote cell migration, the osteogenesis process and exhibit angiogenesis properties that encourage the formation of blood vessels in damaged bone tissue, both with and without the use of a magnetic field. [40].

3.1 - Effects on Osteoblast Cells

Iron oxide nanoparticles are taken up by stem cells and directed using a strong magnetic field (SMF) in stem-cell magnetic targeting. A study by Silva et al. found that while stem cells remained functional after being targeted with a magnetic field of 0.3-0.45 T, there was a temporary decrease in cell proliferation and viability. This research indicates that using SMF and nanoparticles can impact the behaviour of cells[41].

Also, Jiang et al. found that when Fe_3O_4 /BSA nanoparticles of 200 nm and SMF of 1T were used separately, they did not affect stem cell differentiation. However, when they were used together, it resulted in a higher level of nanoparticle internalization, which led to a significant increase in the differentiation of stem cells into osteogenic cells. Even though cell proliferation decreased when the nanoparticles and SMF were used together, it was expected as the cells were in the process of differentiating[42].

Magnetic nanoparticles can also impact pre-existing bone cells, not just stem cells. Marycz et al. discovered a new magnetic core-shell nanocomposite, $\text{Co}_{0.5}\text{Mn}_{0.5}\text{Fe}_2\text{O}_4\text{@PMMA}$, made of Cobalt-manganese ferrite nanoparticles and polymer polymethylmethacrylate (PMMA), combined with SMF (0.2 T), could restore the balance of osteoblasts and osteoclasts activity in the condition of osteoporosis. The research showed that the combination of the nanocomposite and SMF stimulated integrins, improving preosteoblast activity and inhibiting osteoclasts[43].

3.2 - Effects on Endothelial Cells

Regarding the use of MNPS and endothelial cells, the existing literature indicates that much remains to be explored. However, an in vivo study by Hu et al. implanted gelatinous sponges with SPIONs in the incisor sockets of rats. The results indicated that SPIONs-containing gelatine sponges showed an increase in bone density and trabecular volume/tissue volume, as well as increased new bone formation according to histological analysis, which also indicates an increase in blood vessel formation in conjunction with bone development. The SPIONs were observed to have been taken up by osteoblasts and vascular endothelial cells, leading to improved bone formation and blood vessel formation[44].

Mulens-Arias et al., through an in-vitro study, found that using polyethyleneimine-coated (PEI) SPIONs negatively affected the functioning of primary HUVECs, impairing endothelial cell

migration and tube formation, reducing blood vessel numbers on tumor sites. The SPIONs caused the cells to produce more reactive oxygen species, disrupting the cells' actin cytoskeleton activity[45].

Despite the differing results presented by the previous two works, it is possible to conclude that MNPs can be directed toward multiple targets, such as bone regeneration and tumors. Hence, MNPs play a crucial role in tissue engineering, displaying remarkable versatility.

A summary of these studies can be seen in Table 3.1.

Table 3.1 - Summary of studies reporting the influence of magnetic nanoparticles in bone regeneration and angiogenesis.

Material	Methods	Type of magnetic field			Type of Cell	Results	Results <i>in vivo</i>	Mechanism	Reference
		Static	Other	Intensity					
Iron Oxide NPs	Coprecipitation of Fe(II) and Fe(III) in aqueous solution by alkaline hydrolysis	X	—	0.3 - 0.45 T	Stem Cells	Temporary decrease in cell proliferation and viability	—	Iron in magnetized MSCs aggravated the loss of viability	[41]
Fe ₃ O ₄ /BSA NPs (200 nm)	Desolvation technique	X	—	1 T	Stem Cells	Increase in the differentiation of stem cells into osteogenic cells	—	Higher level of nanoparticle internalization; Proliferation decreased probably due to cell differentiation	[42]
Co _{0.5} Mn _{0.5} Fe ₂ O ₄ @PMMA	Microwave-driven non-hydrolytic approach	X	—	0.2 T	Pre-existing bone cells	Restore the balance of osteoblasts and osteoclasts activity in the	—	Stimulated integrins, improving preosteoblast activity and	[43]

3.2 Effects on Endothelial Cells

						condition of osteoporosis		inhibiting osteoclasts	
Gelatinous sponges with SPIONs	Coprecipitation and following synthetization	—	X	1.5 T 7 T	—	—	Increase bone density, trabecular volume, new bone formation and blood vessel formation in the sockets of rats	SPIONs have been taken up by osteoblasts and vascular endothelial cells, leading to improved bone formation and blood vessel formation	[44]
PEI-coated SPIONs	Coprecipitation and following synthetization	—	—	—	HUVECs	Negatively affected the functionality of primary HUVECs; Decreased blood vessel number on tumor sites	—	SPIONs lead the cells to produce more reactive oxygen species, disrupting the cells' actin cytoskeleton activity	[45]

Chapter 4

Magnetic Scaffolds on Bone Regeneration

One of the strategies that have been studied is the combination of MNPs with scaffolds, given the already demonstrated capacity of MNPs in bone regeneration.

The production of these scaffolds can be separated into two categories, conventional and advanced. The big difference between these two types of production is in the resulting architecture, with the first having a randomized architecture, which cannot be controlled. In contrast, with the second method, it is possible to have very high control over the architecture desired in the final product, such as pore size and geometry, which ends up being very positive in terms of improving the mechanical properties that can be achieved. Another significant difference is undoubtedly the cost, since, through advanced methods, the equipment needed to produce scaffolds is very expensive. Examples of conventional methods are direct foaming, replica technique, freeze drying, gas foaming, sacrificial model, phase separation, casting, and particle leaching, and melt-quenching. Advanced methods can be electrospinning, stereolithography, and 3D printing[46], [47].

Recent studies have been proving the actual capacity of magnetic scaffolds. Shuai et al., through *in vitro* tests using Fe_3O_4 MNPs in scaffolds, observed that there was stimulation in cell adhesion, and proliferation, and proved through *in vivo* tests that these scaffolds accelerated the cell regeneration process, promoting increased cell growth inside the scaffold[48].

Another study by Safari et al., using MNPs introduced into a phosphorylated polymer (PCL-P) scaffold, showed cell development, attachment, and proliferation. Furthermore, increased alkaline phosphatase (ALP) activity, a good indicator of bone formation, was denoted in scaffolds with MNPs inclusion. In addition, no cytotoxicity effects were detected[49].

Singh et al. detected, using *in vivo* rat models, a substantial increase in the formation of new blood vessels inside magnetic scaffolds, which had already been achieved also by *in vitro* tests[50].

4.1 - Effects on Osteoblast Cells

In a study by Xia et al., it was reported that a scaffold made of calcium phosphate and containing iron oxide nanoparticles could support stem cell growth in a lab setting. Even more significantly, when a specific type of magnetic field was applied to the scaffold in living organisms, the formation of bone was enhanced by 22.2%. This effect was believed to be due to the physical forces generated by the magnetic field and the presence of magnetic nanoparticles within the scaffold[51].

Alternatives to calcium phosphate scaffolds are those made of electrospun polymer fibers. Li et al. found that incorporating Fe_3O_4 nanoparticles into poly(caprolactone) fiber scaffolds resulted in improved growth of MC3T3-E1 cells when exposed to a specific type of magnetic field (15 mT)[52].

A separate study involving polymeric scaffolds found that incorporating Fe_3O_4 nanoparticles not only added magnetic properties but also improved the mechanical strength of the scaffold - a crucial aspect for bone regeneration. Additionally, increasing the concentration of nanoparticles led to greater cell attachment as it increased surface roughness providing more points for attachment. Most significantly, the study discovered a synergistic effect when the magnetic scaffold was used in combination with a specific type of magnetic field (1.3 T); the groups exposed to the magnetic field exhibited increased mineralization in a lab setting (up to 50%) and, in initial animal testing, faster bone regeneration[53].

Yun et al., in their research, showed that using PCL-MNP scaffolds, along with the application of an SMF (15 mT), could enhance the differentiation of cells that create bone (osteoblasts) and increase bone formation. The positive effects of the combined magnetism were likely due to integrin- and BMP-mediated signaling pathways[54].

4.2 - Effects on Endothelial Cells

As seen previously, the literature shows that the conjugation of magnetic fields with nanoparticles enables good results and indicates that it could be a viable pathway in bone tissue engineering. However, it is increasingly being realised through the implantation of these magnetic fields and magnetic scaffolds that it may be a good solution, especially for bone regeneration.

As above said, co-culture studies between osteoblast and endothelial cells have revealed the interplay between molecules that they secrete, such as BMP2 and VEGF [55]. In an *in vitro* study by Yun et al., in which a culture of osteoblasts on PCL magnetic scaffolds was externally stimulated by SMF for 3 days, and subsequently treated for HUVECs, tubular cell formation was detected. The combined magnetic stimuli significantly increased the expression of key angiogenic genes, suggesting that the osteoblasts' increased functional activity due to magnetism may positively affect endothelial functions [54].

A summary of these studies can be found in Table 4.1.

Based on the current literature, it can be seen that the introduction of MNPs in scaffolds, externally stimulated by magnetic fields can promote bone regeneration. Besides, it also shows promising results when osteoblastic cells are used, giving good cell proliferation and differentiation indicators. This bone tissue engineering approach also promotes angiogenesis, which is crucial for vascularization and the development of new bone, cell proliferation and differentiation indicators.

In conclusion, several research works suggest that magnetic fields may play a role in regulating the growth of blood vessels, which are critical for tissue healing and regeneration. While more research is needed to fully understand the relationship between magnetism, bone cells, and angiogenesis, these findings promise to develop new therapies and treatments for various conditions, from bone fractures to osteoporosis.

With that in mind, this work aims to explore the application of magnetic scaffolds in osteoblastic cells and *in vivo* and thus provide more information about an area that still raises some doubts. Since the application of an SMF as an external stimulus may become a solution and an ally in bone regeneration, thus replacing the use of chemical solutions, in a safer, more stable way, and readily available aid in bone healing. The same was observed in coatings for metallic implants, where the synergistic strategy improved implant osteointegration.

In bone tissue engineering, innovation plays a crucial role in developing new solutions for the future advancement of medicine. One innovative solution involves the use of hydroxyapatite granules with a collagen coating and MNPs (magnetic nanoparticles) made of hydroxyapatite doped with Fe^{2+} and Fe^{3+} . Hydroxyapatite, naturally present in the body, particularly in bone tissues, is chosen for its biocompatibility and similarity to bone mineral components[55]. By incorporating MNPs with low iron content, these nanoparticles can still be stimulated by magnetic fields, providing an alternative to magnetite, which has higher iron content and is primarily used for contrast MRI purposes[56].

This innovative approach holds expectations for bone tissue engineering due to several factors. Firstly, hydroxyapatite, a critical mineral in the human body, offers unique characteristics that make it suitable for bone tissue engineering and drug delivery systems[56]. Although hydroxyapatite has good biocompatibility and osteoconductive properties, its poor mechanical strength limits its use in non-load-bearing applications[55]. The addition of collagen coating and MNPs provides a means to enhance mechanical properties and stimulate bone regeneration.

The use of MNPs made of hydroxyapatite doped with Fe^{2+} and Fe^{3+} is particularly noteworthy. These nanoparticles contain small amounts of iron but can still respond to magnetic fields, allowing for potential magnetic stimulation in bone tissue engineering applications[56]. This innovation overcomes the need for magnetite, which has higher iron content and is typically used only in small quantities for contrast MRI purposes.

Table 4.1 - Summary of studies reporting the influence of magnetic scaffolds in bone regeneration and angiogenesis.

Material	Methods	Type of magnetic field			Type of Cell	Results	Results <i>in vivo</i>	Mechanism	Reference
		Static	Other	Intensity					
Calcium phosphate Scaffold with IONPs	Compression	X	—	35 mT	hDPSCs	Formation of bone enhanced by 22,2%	—	Probably due to physical forces generated by the magnetic field and the presence of magnetic nanoparticles within the scaffold	[51]
PCL fiber scaffold with IONPs	Depressurization of subcritical CO ₂ fluid	X	—	15 mT	MC3T3-E1	Cell growth	—	Great cell penetration, collagen deposition and angiogenesis	[52]
Polymeric scaffold with IONPs	Laser direct writing via two-photon polymerization (LDW via TPP)	X	—	1.3 T	MG-63	Increased mineralization up to 50%	Faster bone regeneration in initial animal tests	SMF promote cell attachment and the early stage mineralization of nanoparticle-free osteoblast-like cells	[53]
PCL-MNP scaffold	Space holder	X	—	15 mT	HUVECs	Tubular cell formation	—	Increase expression of key angiogenic genes	[54]

Chapter 5

Materials and Methods

This chapter describes the materials and preparation methods for the experimental tests.

5.1 - Materials and Reagents

For the scaffold production polyurethane sponge SupraCell AR 4234 BR (Euroespuma, Portugal), hydroxyapatite micro aggregates nanoXIM-Hap202 (Fluidinova SA, Portugal), Dolapix CE64 (Zschimmer & Schwarz, Germany), ultrapure type I Milli-Q water with a resistivity of 18,2M Ω , were used. To obtain the superparamagnetic nanoparticles calcium hydroxide (Ca(OH)₂, 98%), phosphoric acid (HPO₄, 85% in water), iron II chloride tetrahydrate (99+%), iron III chloride hexahydrate (99+%), were used, all provided by Acros Organics, USA.

For the coating and crosslinking were used type I collagen, from bovine Achilles tendon, MES buffer (0,05 M, pH=5.4), N-hydroxysuccinimide (NHS), purchased from Sigma-Aldrich, USA, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) from Fluka, USA.

To support the cell cultures: α -MEM Alpha medium powder, Penicillin/Streptomycin 100x, Fetal Bovine Serum (FBS), fungizone and 0,25% trypsin purchased from Gibco (Thermo Fisher Scientific, USA), were chosen. For supplement complete medium with osteogenic factors: B-glycerophosphate, dexamethasone and ascorbic acid (2-Phospho-L-ascorbic acid) were provided by Sigma-Aldrich, USA. Also, for cell culture purposes, PBS (phosphate buffered saline) and trypan blue solution were acquired from Sigma-Aldrich, USA.

For cell metabolic activity, resazurin was acquired from Sigma-Aldrich, USA.

To observe the scaffolds at confocal microscopy, Alexa Fluor 488 and DAPI were acquired from Invitrogen.

For ALP staining, Rippa Buffer was used to lysate the cells and to quantify total protein BCA both from Thermo Fisher, USA. The Alkaline Phosphatase yellow substrate was from Sigma-Aldrich.

To perform mineralization test, alizarin red staining was used from Sigma-Aldrich, USA.

5.1.1 - Hydroxyapatite Scaffold Production

The production of scaffolds starts with a polyurethane sponge (with density of 42 Kg/m³ and hardness of 3.4 kPa), cut with the dimensions 5x10x10 mm, as shown in figure 5.1(A). A solution containing hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$), Dolapix CE64 dispersant, and ultrapure type I water is prepared. After optimization, the ideal proportions of the ceramic slurry to produce around 35 scaffolds were 27.75 g, 888 μL and 25 mL, respectively. The sponge was subsequently immersed in the solution and squeezed a few times so that the solution became well impregnated. Then the sponge was removed and pressed against an aluminum foil to remove the excess solution, as seen in figure 5.1(B).

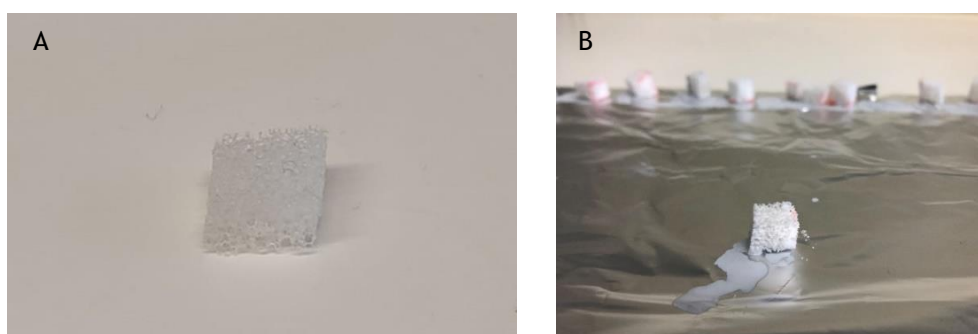


Figure 5.1 - Preparation scaffolds. (A) Cube of polyurethane sponge. (B) Sponge immersed in the slurry.

After this procedure, the cubes were kept for about 30 minutes at room temperature. Then, they were heat treated, in a sintering furnace (Termolab, Portugal), where the temperature raised 600 °C at a rate of 1 °C/min and was left at this temperature for 60 minutes plateau. Afterwards that the rate changes for 5 °C/min until it reaches the maximum temperature of 1300°C for 180 minutes. To finalize, the scaffolds were left inside the furnace to cool down.

However, there were issues with the cubic-shaped scaffolds as some of them were damaged, leading to wastage. Additionally, when handling these scaffolds, some of them fell apart, which raised concerns regarding their use in surgical procedures for implantation in patients. These difficulties prompted the question of how surgeons would cope with such challenges. Consequently, it was decided to resize the scaffolds since it appeared that smaller ones offered increased rigidity and improved handling characteristics. Therefore, and since the smaller the scaffolds became, they apparently presented greater stiffness and better behaviour when handled, they were resized.

For this, a sieve system was used, where scaffolds were transferred and then manually broken until they reached granule dimensions between 1.88 mm and 2.0 mm.

5.1.2 - Magnetic Nanoparticles

Hydroxyapatite nanoparticles doped with Fe^{2+} and Fe^{3+} ions were produced by chemical precipitation. A calcium hydroxide solution $\text{Ca}(\text{OH})_2$ (1.4818 g in 60 mL of dH_2O), which was subsequently prepared overnight under magnetic stirring, is heated to 40 °C in an oil bath. To remove the oxygen from the solution and create an inert system, an inflow of nitrogen was added. Five minutes later, two sources of Fe^{2+} and Fe^{3+} ions ($\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ and $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) are added 0.376 g and 0.510 g, respectively. Then, through a peristaltic pump at a rate of 0.500 mL/min, a phosphoric acid (H_3PO_4) solution (0.892 mL in 20 mL of dH_2O) was added.

After the final solution was complete, it was kept in suspension, constant stirring, and heating for one hour. At the end of this hour, the heating was turned off and only the stirring remained until the temperature of the solution reaches room temperature (RT). The solution was left to rest for 24 hours. Then the nanoparticles are washed 3 times with ultrapure type I water in centrifugation for 10 minutes and left to dry for 48 hours.

After drying, the fine powder was grounded in an agate mortar pestle, placed in a sintering furnace (Termolab, Portugal) at 600 °C for 1 hour with a heating rate of 5 °C/min, and then stored in a silica gel dissector.

5.1.3 - Coating and Crosslinking

To perform the coating of the MNPs it was necessary to produce the collagen solution. In this, type I collagen from bovine Achilles tendon was used, which was added to a hydrochloric acid (HCL) solution and kept at low temperatures (4 °C) for 48 hours under magnetic stirring until it reached the %0.5 (w/v) portions. Afterwards, that the solution was homogenized for 1 hour at 10.000 rpm with Ultra Turrax (T25 D, IKA).

This coating was developed by a concentrated suspension of type I collagen with the $\text{FeHAp}^{2+/3+}$ nanoparticles. Three solutions, each containing 2mL of the collagen solution, were prepared, and 0, 15, and 30 mg of nanoparticles were added, which, to achieve homogeneous suspension, were then sonicated with VibracellTM (Sonics&Materials Inc., USA) until full dispersion of the MNPs.

After the suspension were finalized, 18 mL of 0.01M HCl were added for each MNPs concentrated solution, 6,5 µL of the final suspension was placed in each scaffold of each concentration. To ensure the full integration of the solution in the scaffold, they were kept in a vacuum oven (Raypa, Spain) for 48 hours at room temperature.

Later crosslinking was performed, by putting for 2 hours at 4°C the scaffolds in a solution of MES buffer (0.05 M, pH=5.4), which contained N-Hydroxysuccinimide (NHS) and also 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC), that were used to bind an amine to a carboxyl group. Collagen has both of these groups, so it binds to each other and creates a lattice that does not degrade so easily, avoiding denaturation and making it more similar to what is present in the human body. MES Buffer is required to promote the reaction

keeping the pH at a stable value. Then, the scaffolds were washed three times each with dH₂O and left for drying at room temperature.

The final result was small scaffolds (Hap-COL) with 3 different concentrations of MNPs that from this point onwards are referred to as 0MNP, 1.5MNP, and 3MNP.

5.2 - Hap-Col Granule Characterization

The characterization process was carried out to gain a comprehensive understanding of the material and its properties. The primary objective was to study the physical and chemical properties of hydroxyapatite, collagen, and MNPs collectively. By analyzing the data obtained through characterization, it becomes feasible to determine whether the biomaterial possesses the necessary characteristics to serve as a viable medical solution.

5.2.1 - Fourier Transform Infrared Spectroscopy

Fourier Transform Infrared Spectroscopy (FTIR) is a technique used to analyse and identify the chemical composition of materials. It measures the interaction of infrared light with a sample, providing information about how different bonds, molecular structure and functional groups absorb different frequencies leading to a spectrum revealing the chemical characteristics of the scaffold. This technique was performed on 4 samples, the first being the hydroxyapatite granule alone, followed by granules with the various concentrations of MNPs (0MNP, 1.5 MNP and 3MNP).

To perform this technique the samples must be converted into pellets. For this, each pellet was reduced to powder using a mortar, where 200 mg of potassium bromide (KBr) was subsequently added. Then the powder was placed and compressed (8 tones) in a hydraulic press (Grasedy Specac, UK). Finally, each pellet was inserted into the Perkin-Elmer 2000 spectrometer (Perkin-Elmer, USA), where a wavelength between 400 cm⁻¹ and 4000 cm⁻¹ with a spatial resolution of 4 cm⁻¹ was used, resulting in an average of 64 scans.

5.2.2 - Mechanical Tests

After reducing the size of the scaffolds, it was important to understand what physical characteristics they presented. For this purpose, mechanical tests were performed at Faculdade de Farmácia da Universidade do Porto (FFUP) where it was possible to analyse the 4 types of granules, without collagen impregnation and with different concentrations of collagen and MNPs (0 MNP, 1.5 MNP and 3 MNP).

Two types of mechanical tests were carried out, friability tests using the Friabilator (USP) EF-1W (Electrolab, India) and compression tests using the TA-XT2i Texture Analyser (Stable Micro Systems, UK), as shown in figure 5.2.

For the friability tests, approximately 2 g of granules were weighted and inserted into a graduated glass beaker of 15 cm in length. The beaker was placed inside the equipment and

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attached. The test was carried out for 5 minutes at a speed of 25 rpm. In the end, the dust that results from the shock of the granules was removed, and at the end the granules were weighed again. The tests were repeated 3 times for each condition.

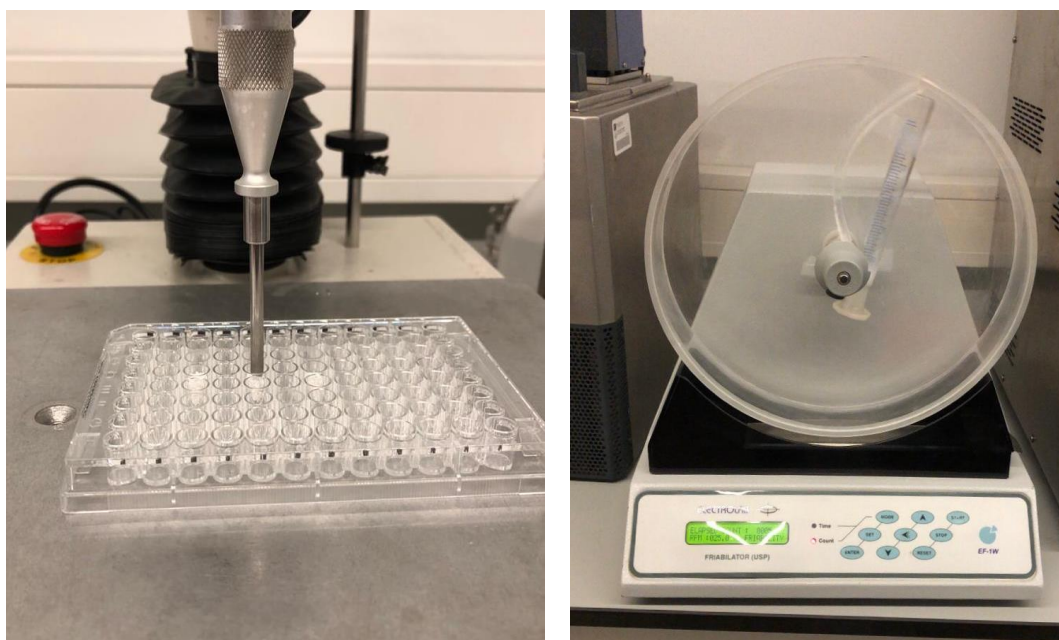


Figure 5.2 - Mechanical Tests. (A) Compressive tests using a texturometer. (B) Friability tests.

Compression tests were performed on a 96-well plate where granules were placed until they reached about half of the well. A 30 kg load cell was used, and a 4 mm stainless-steel cylinder probe was applied vertically at a displacement rate of 0.1 mm/sec over 2 mm of sample. The tests were repeated 3 times for each condition.

5.2.3 - Scanning Electron Microscopy

Scanning Electron Microscopy (SEM), it's a very common technique used to visualize the surface of samples at high resolution, providing information regarding the morphology, topography of the materials. X-ray Energy Dispersive Spectroscopy (EDS) it's a complementary technique used for elemental analysis, to better understand the composition of the materials.

These two techniques were carried out using Phenom XL G62 (ThermoFisher Scientific, USA) with an acceleration voltage of 15 kV. Thus, the samples were fixed with epoxy resin (Araldite™) and sputter-coated with palladium-gold alloy film for about 100 s (SPI Module Sputter Coated).

5.2.4 - Micro-Computed Tomography

Micro-computed tomography (micro-CT) is based on the same principles as conventional computed tomography but on a smaller scale (mm to μm). It is used to visualise internal structures with a greater resolution and detail, through three-dimensional images.

This technique was performed on all types of granules, without hydroxyapatite and on samples with various concentrations of MNPs (0MNP, 1.5MNP and 3MNP) using the Skyscan 1276 scanner (Skyscan, Belgium).

The granules were introduced and immobilised in 0.2 mL micro-centrifuge tubes and scanned for 18 minutes. An X-rays source voltage of 70 kV and a current of 200 μ A were used for the acquisitions. Images were acquired using a high-resolution mode of 0.99 μ m and a pixel size of 4.04 μ m with an exposure of 700 ms.

Three different software from SkyScan were used for reconstruction, processing and analysis. For image reconstruction, NRecon was chosen. For the processing of the cross sections, DataViewer was used. For the analysis of the images, where the segmentation of the images was made with the application of thresholding between 115 and 255 and some filters to help in the identification of pores through the highlighting of the ceramic material, the CTAn was used.

5.2.5 - Superconducting Quantum Interference Device

Understanding and characterizing how different materials respond to external magnetic fields is consistently valuable. By studying their behavior in such conditions, we can gain insights into their magnetic properties and potential applications.

Since not all materials have the same response to these stimuli, sometimes not all equipment/techniques can provide the best information. Superconducting Quantum Interference Device (SQUID) is an equipment that can provide a reliable response and characterization of the magnetic behaviour of the materials, with the highest sensitivity of all techniques.

The SQUID analysis was performed at Faculdade de Ciências da Universidade do Porto (FCUP), Physics Department, using the MPMS3 SQUID Magnetometer (Quantum Design, UK), where FeHap^{2+/3+} NPs were tested. Before starting the analysis of the sample, it was weighted to know the mass value, 0.0202 g of FeHap^{2+/3+}.

To make correct use of SQUID, some steps must be followed so that the analysis is done correctly. So, the sample must be placed in a kind of plastic straw/flask because it is a material that has a low magnetic susceptibility so that it does not interfere with the sample results. In addition, the sample must be placed in the middle of that same straw for this, an accessory was used as shown in figure 5.3, and fixed using a special adhesive tape called Kapton, which has thermal and magnetic insulator properties withstanding temperatures between 5 and 300K. Each sample takes about 1 hour to be analysed, in which a magnetic field from -5 T to 5 T is applied.



Figure 5.3 - SQUID accessory to place the sample in the right spot.

5.3 - *In vitro* Cell Culture

For cell culture, an osteoblast precursor cell line, MC3T3-E1, was used, which previously had to be thawed and expanded in a T-75 flask with complete alpha medium, kept in a 5% CO₂-humified atmosphere incubator at 37 °C, which was changed every 2 days until it reached about 80% confluence. Then the cells were trypsinised with 0.25 % trypsin and expanded to new T-75 flasks.

Before starting the cell culture, it was necessary to prepare the scaffolds. With this aim, two days before seeding the cells, the scaffolds were sterilized with 70% ethanol and left overnight. The day before the start of cell culture, the scaffolds were washed 3 times with PBS to remove excess ethanol and complete medium, alpha Minimum Essential Medium (α MEM) with 10% Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin (P/S), was placed overnight to leach hydroxyapatite and allow protein absorption.

The granules were placed in 96-well flat bottom non-treated plates (Avantor-VWR, USA), and the cells were seeded according to the concentration of 9.3×10^4 cells/mL together with complete alpha medium or complete alpha medium with osteogenic factors (1% β -glycerophosphate, 1% dexamethasone and 1% ascorbic acid) according to the distribution that can be seen in figure 5.4, being B0, B1.5, and B3, scaffolds with the respective concentrations of MNPs without cells, and 0, 1.5, and 3 with cells. These MNPs concentrations were also cultured with complete alpha medium but without containing osteogenic factors and with or without an external magnetic stimulus 1 T (OZ Biosciences, France). The medium was changed every two days. The aim is to understand how the presence of the magnetic field and osteogenic factors can influence the cells.

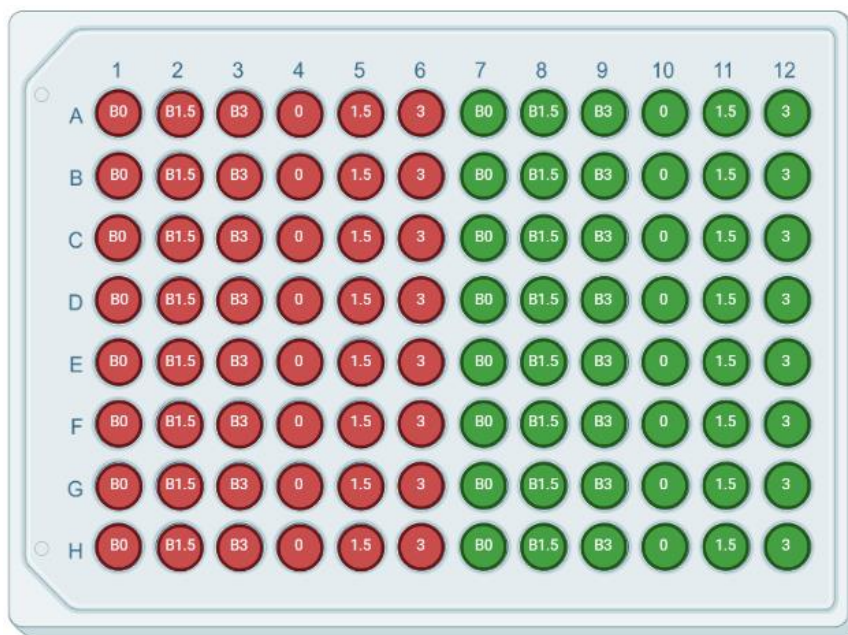


Figure 5.4 - Distribution of cells for cell culture. Created with BioRender.com.

5.3.1 - Resazurin Assay

To determine the cell viability and metabolic activity of MC3T3-E1, resazurin tests were performed after 7, 14, and 21 days. Resazurin (blue dye) is added and metabolised by the cells. If this happens, the blue dye turns pink.

At the end of each timepoint (7, 14 and 21 days), the media were removed, new media with 10% resazurin added and placed in the 5% CO₂ incubator at 37°C for 4 hours, as resazurin is light-sensitive, all these steps had to be carried out with the light off. After this time, 100 µL was transferred to a 96-well black reading plate, and fluorescence was measured at 550-605 nm with a sensitivity of 60 in the SynergyMx Microplate Reader (BioTek, VT). The results were obtained using Gen5 1.11 Data Analysis Software.

5.3.2 - Immunostaining for Confocal Microscopy

Immunostaining was performed to be able to observe the samples in confocal microscopy. This technique allows the visualization of three-dimensional samples with high-resolution capacity.

After cell culture, explained in section 5.3, samples were washed with PBS and 3.7% Paraformaldehyde solution was added to fix the cells for 15 min at room temperature. The samples were then washed 3 times with PBS and 0.2% Triton X-100 was added in PBS for 10 minutes under stirring (50 rpm) in an orbital shaker. The triton was removed and 3 washes with PBS were performed again. The samples were incubated with 1% BSA in PBS for 30 minutes at room temperature, again on stirring (50 rpm). Then, diluted Alexa Fluor 488 phalloidin (1:100) was added to the BSA/PBS solution for 30 minutes in the dark over the same agitation (50 rpm).

Samples were washed 3 times with PBS and subsequently incubated in the dark with diluted DAPI (1:10 000) in PBS for 10 minutes at room temperature under shaking (50 rpm). Finally, the samples were again washed with PBS and were observed under a confocal microscope.

5.3.3 - Alkaline Phosphatase Activity

Alkaline Phosphatase (ALP) is an enzyme that is particularly present in high concentrations in bone. The expression of this enzyme is associated with the appearance of bone cells phenotype and the maturation of the extracellular matrix before mineral deposition.

The ALP assay was performed after the cell culture explained in section 5.3. After each timepoint (7, 14 and 21 days) samples were lysed with 100 μ L of Rippa Buffer and were placed in the sonicator on ice for 5 minutes and then transferred to 1.5 mL micro-centrifuge tubes. Afterwards, 20 μ L of lysate were transferred to each well of a 96-well plate and 80 μ L of ALP substrate were added to each well. To determine total protein, BCA was used, where 25 μ L of lysate were taken from each well to another 96-well plate and 200 μ L of BCA were added.

Then, the 96-well plates were incubated for 24h at 37 °C. Finally, the absorbance was measured at 405 nm for ALP and at 562 nm for BCA in the SynergyMx Microplate Reader (BioTek, VT). The results were obtained using Gen5 1.11 Data Analysis Software.

5.3.4 - Alizarin Red Assay

Alizarin Red is a technique used to quantify calcium deposition, very common to observe osteogenic differentiation and mineralization. After the cell culture, in section 5.3, the samples were fixed with 4% Paraformaldehyde in PBS and incubated for 15 minutes at 37 °C. Next, 3 washes were made with dH₂O and 200 μ L of 40 mM Alizarin red solution were added for 20 minutes under stirring (50 rpm). Finally, 4 washes were performed with dH₂O and the samples were visualized and photographed with the SZX10 stereoscopic microscope (Olympus, Japan).

5.4 - *In Vivo* CAM Assay

After the *in vitro* tests, we proceeded to the *in vivo* tests, where Chorioallantoic Membrane (CAM) Assay was performed.

The CAM assay is an experimental technique based on the principle of using fertilised chicken eggs that are incubated for a few days until they have time to form a vascular network in the chorioallantoic membrane, which surrounds the embryo.

In this experiment the eggs were prepared (window opening) at embryonic development day (EED) 3, to allow the growth of the CAM detached from the shell. At EDD10, CAMs were inoculated with the granules with different concentrations of MNPs (0 MNP, 1.5 MNP and 3 MNP) and the application of a magnetic plate 1 T (OZ Biosciences, France) was also tested. The experiment ended at EDD13. At the endpoint, CAMs were fixed (10% neutral-buffered formalin),

excised from the embryo and photographed *ex ovo*. Further, granules were removed from the excised CAMs and processed for histological analysis, to understand the structure, composition and organization of the blood vessels, and possible inflammations at a microscopic level.

Note that, according to the European Directive 2010 63 / ethical approval is not required for experiments using embryonic chicken. Correspondingly, the Portuguese law on animal welfare does not restrict the use of chicken eggs for experimental purposes.

5.5 - Statistical Analysis

Statistical analysis and graphs were done using GraphPad Prism 8 software (GraphPad, USA). Regarding the data obtained, resazurin, ALP and CAM assay were tested with a variance two-way ANOVA, and statistical significance between groups was analysed by post hoc Tukey's test with a statistically significant level of $p < 0.05$. Mechanical test results were analysed with a variance one-way ANOVA, and statistical significance between groups was also analysed by post hoc Tukey's test with a statistically significant level of $p < 0.05$. The presented graphs show the mean $\pm$ standard deviation (SD).

Chapter 6

Results and Discussion

This chapter will present the results of the several experimental tests carried out, their resulting outcomes, and a corresponding analysis/discussion.

6.1 - Characterization of Hap-Col-MNPs granules

6.1.1 - Fourier Transform Infrared Spectroscopy

The chemical characterization of the Hap-Col-MNPs granules was performed through FTIR analysis, as shown in figure 6.1.

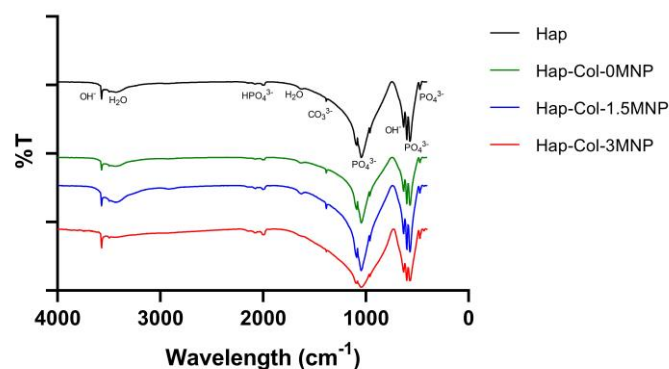


Figure 6.1 - FTIR spectra of the granules with peak identification.

The FTIR spectra show peaks at $\sim 3575\text{ cm}^{-1}$ and $\sim 635\text{ cm}^{-1}$ that characterise and correspond to the lattice and vibrations of hydroxyl group (OH^-). Between the bands $3380\text{--}3480\text{ cm}^{-1}$ and the peak $\sim 1630\text{ cm}^{-1}$ is the presence of water. The spectrum shows sharp peaks for the phosphate groups (PO_4^{3-}) ($\sim 1092\text{ cm}^{-1}$, $\sim 1045\text{ cm}^{-1}$, $\sim 960\text{ cm}^{-1}$, $\sim 604\text{ cm}^{-1}$, $\sim 572\text{ cm}^{-1}$ and $\sim 475\text{ cm}^{-1}$). A small peak is observed at $\sim 1385\text{ cm}^{-1}$ which indicates the presence of the carbonyl group (C-O) that results from the atmospheric adsorption of CO_2 by the apatite. There are also

two peaks ($\sim 1995\text{ cm}^{-1}$ and $\sim 2080\text{ cm}^{-1}$) in all spectra that indicate the presence of the HPO_4^{3-} groups[57]-[59].

Despite some minor differences in terms of wavelength and transmittance values, the spectra evaluated with the different concentrations of MNPs compared to the spectra of the granules with only Hap revealed that they do not show significant differences in terms of their chemical composition.

6.1.2 - Mechanical Tests

The mechanical properties of a material are always important, especially in those that are used as biomaterials that are intended to be implanted in humans. Not only must it match the mechanical characteristics of the part that it is going to replace, but also the whole process up to the moment of replacement must be considered. Therefore, the handling of the material, in this case, the ceramic granules, by the surgeon during the surgical procedure, as well as the motion done until it may reach the medical institutions should also be considered.

The choice of using granules instead of scaffolds is beneficial as it allows for less invasive surgical techniques. Furthermore, as the granules end up having different sizes, always between 1.88 mm and 2 mm, different shapes, and pore locations, they eliminate the need for bone grafts with pre-defined shapes and sizes[60], [61].

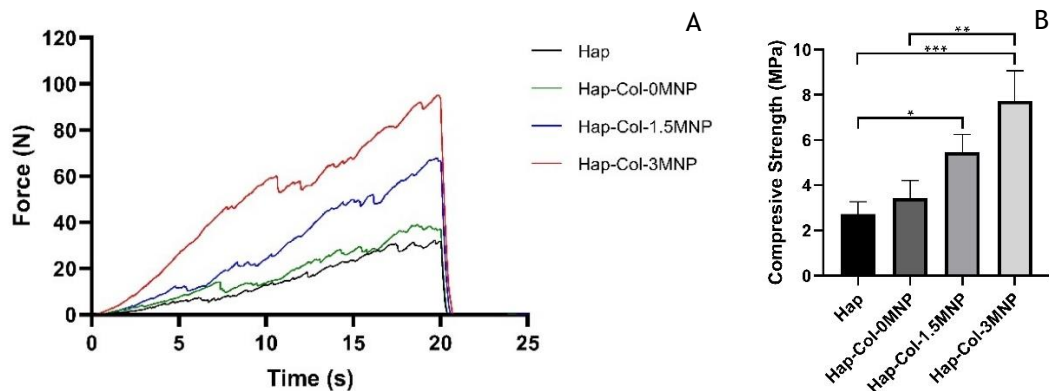


Figure 6.2 - (A) Representative Force (N) vs Time (s) curves of the granules. (B) Results obtained of the Compressive Strength (MPa) for the different type of granules. Statistically significant differences are marked with * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) or **** ($p<0.0001$).

Through the graph of figure 6.2(A), it is observed that the curves of Force (N) vs Time (s) in granules only with hydroxyapatite show the lowest values, reaching its maximum average value of 32.5 N. In the case of the curves with collagen impregnation, the maximum average values were 39.2 N, 68 N and 95.3 N, for 0MNP, 1.5MNP and 3MNP, respectively.

Using the data collected by the texture analyser, the compressive strength for the different types of granules was calculated, as shown in the graph in figure 6.2(B). The average values were 2.72 MPa, 3.42 MPa, 5.45 MPa and 7.73 MPa, for Hap, 0MNP, 1.5MNP and 3MNP, respectively. Through a one-way ANOVA statistical analysis, it was possible to find significant

differences between the hydroxyapatite granule and the concentrations 1.5 and 3 MNPs ($p < 0.05$ and $p < 0.001$, respectively), and also between the granule only with collagen only (0MNP) and with the maximum of MNPs (3MNP) ($p < 0.01$).

Table 6.1 - Results obtained from friability tests, with the percentage of weight lost.

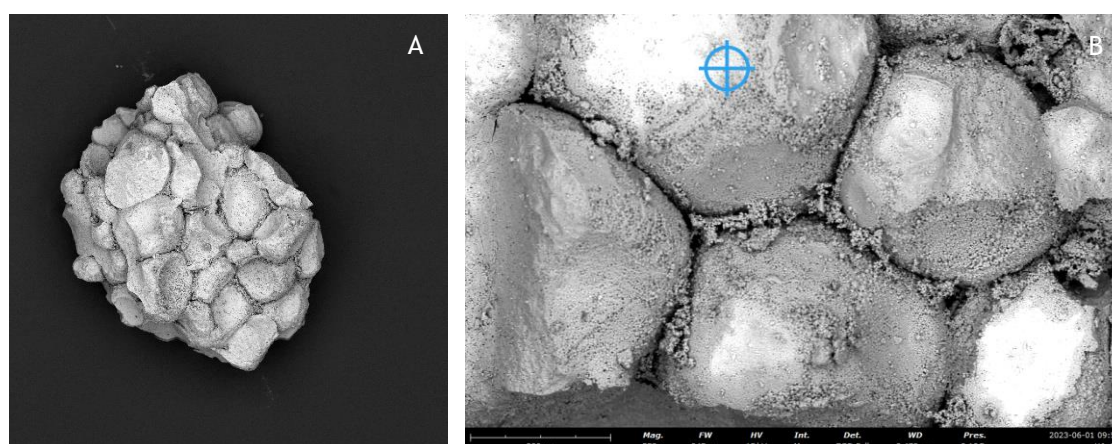
	Friability (Weight Loss)
Hap	0,49%
Hap-Col-0MNP	0,46%
Hap-Col-1.5MNP	0,32%
Hap-Col-3MNP	0,50%

Regarding the friability tests, the values between the different types of granules turned out to be quite similar, never exceeding the limit stipulated by the pharmaceutical industry, that the lost mass should not be greater than 1% of the initial mass, although there is no regulation for this type of material[62].

Since the compressive strengths of trabecular bone range between 1 and 10 MPa, granules turn out to be a good bone substitute since it presents values within the range [63]. Moreover, it is verified that with the increase of the MNPs concentration the mechanical properties of the hydroxyapatite scaffolds improve significantly.

6.1.3 - Scanning Electron Microscopy

SEM was used in order to better understand in approximate detail the morphology and topography of the granules and how the collagen coating with MNPs was distributed. All samples, including the granule with only hydroxyapatite and with the different concentrations of MNPs (0MNP, 1.5MNP, and 3MNP) were analyzed. In addition, EDS was also performed complementing the information obtained by SEM images, helping to understand the composition of the different granules. Figure 6.3, 6.4, 6.5 and 6.6 shows the images obtained by SEM and the information obtained by EDS show in Tables 6.2, 6.3, 6.4 and 6.5.



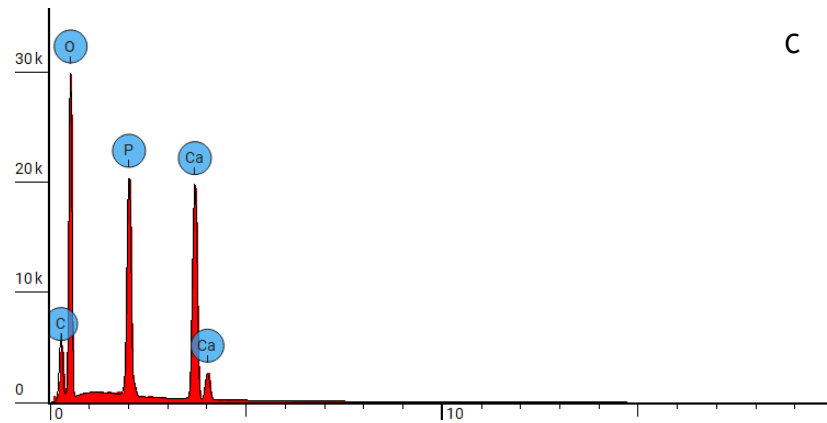
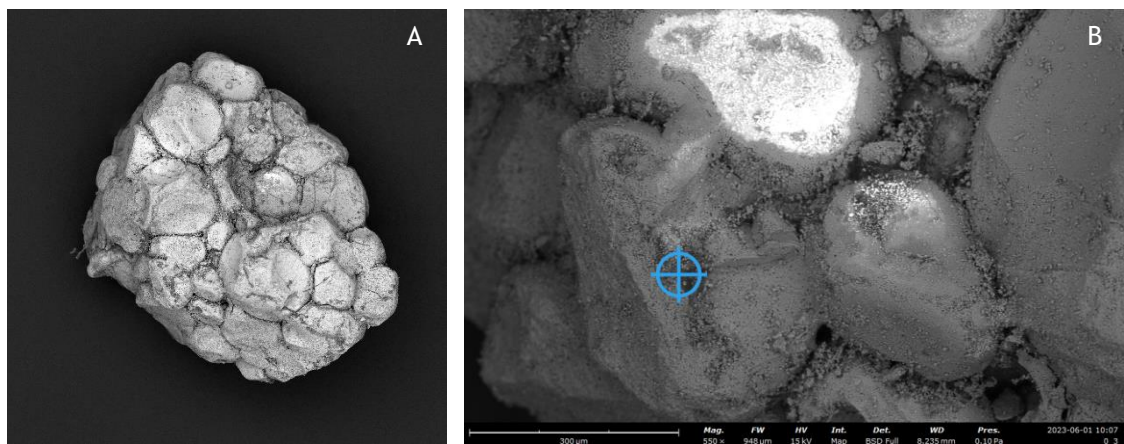


Figure 6.3 - SEM images of Hap granule. (A) Magnification of 100x. (B) Magnification of 550x. (C) EDS spectra of Hap granule with peak identification.

Table 6.2 - EDS analysis with the elements found in Hap granule.

Element Number	Element Symbol	Element Name	Atomic Conc.	Weight Conc.
6	C	Carbon	15.06	9.091
8	O	Oxygen	63.36	50.95
15	P	Phosphorus	7.699	11.99
20	Ca	Calcium	13.89	27.97



6 Results and Discussion

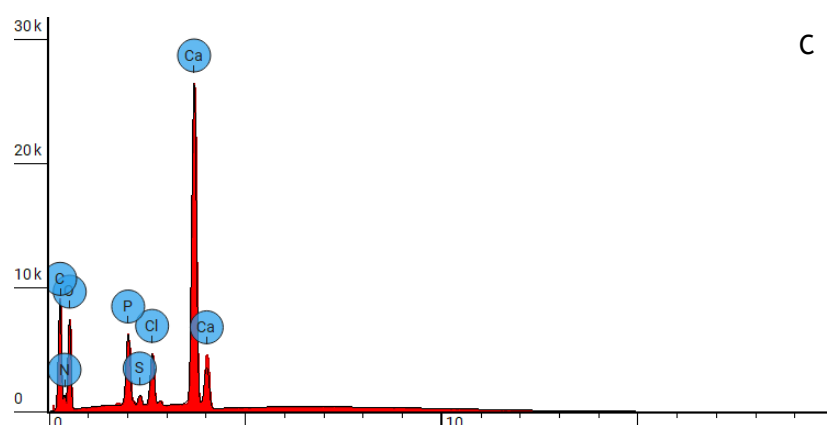
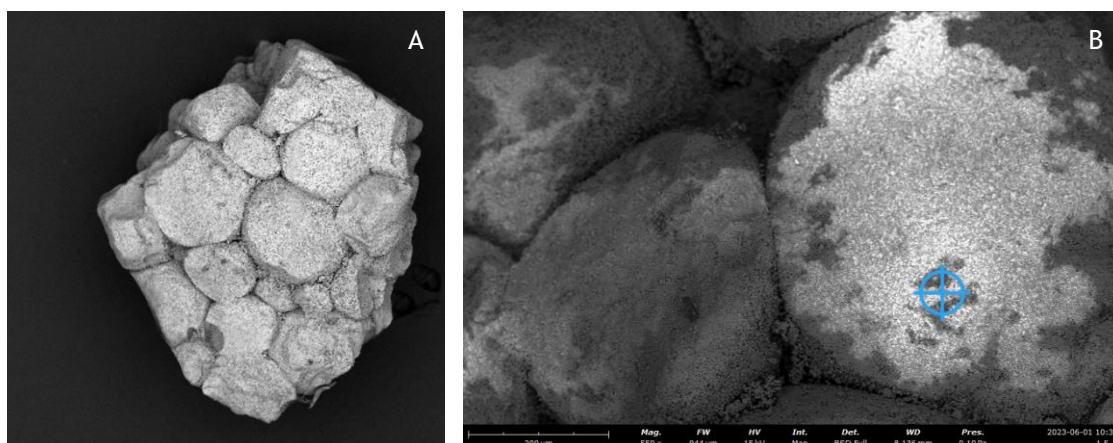


Figure 6.4 - SEM images of OMNP granule. (A) Magnification of 100x. (B) Magnification of 550x. (C) EDS spectra of OMNP granule with peak identification.

Table 6.3 - EDS analysis with the elements found in OMNP granule.

Element Number	Element Symbol	Element Name	Atomic Conc.	Weight Conc.
6	C	Carbon	25.47	15.19
7	N	Nitrogen	20.97	14.59
8	O	Oxygen	28.92	22.98
15	P	Phosphorus	2.338	3.596
16	S	Sulfur	0.4390	0.6990
17	Cl	Chlorine	2.440	4.296
20	Ca	Calcium	19.43	38.66



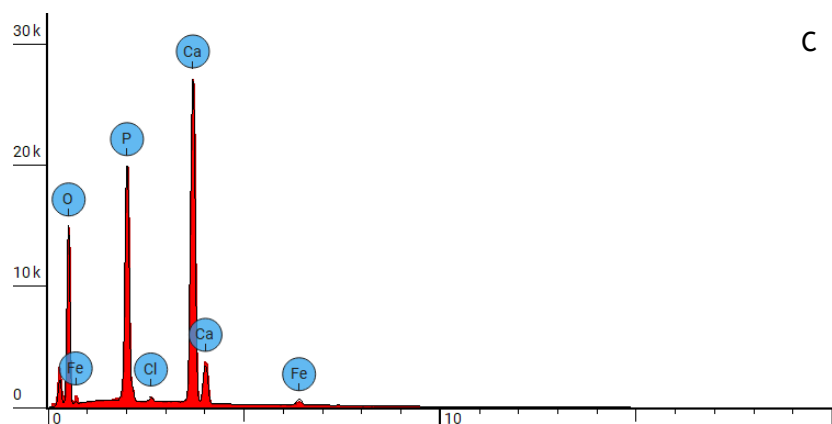
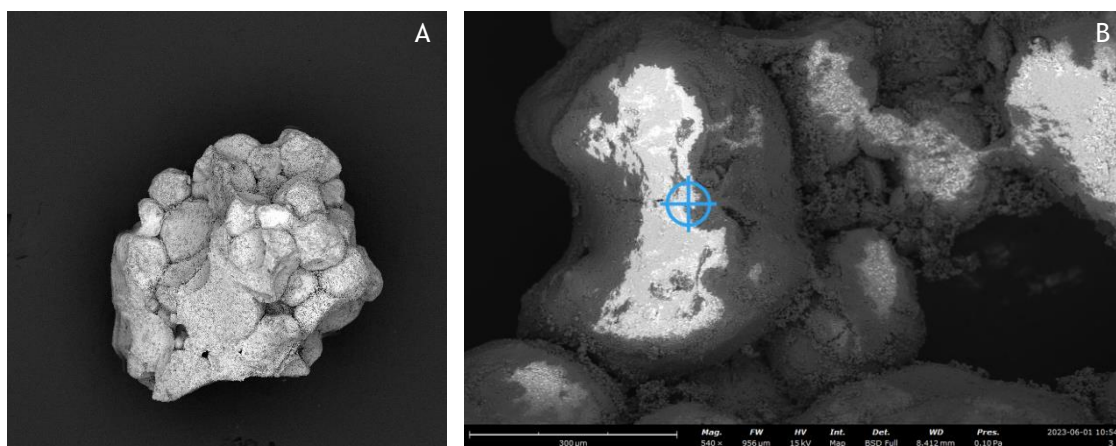


Figure 6.5 - SEM images of 1.5MNP granule. (A) Magnification of 100x. (B) Magnification of 550x. (C) EDS spectra of 1.5MNP granule with peak identification.

Table 6.4 - EDS analysis with the elements found in 1.5MNP granule.

Element Number	Element Symbol	Element Name	Atomic Conc.	Weight Conc.
8	O	Oxygen	60.99	39.76
15	P	Phosphorus	10.84	13.69
17	Cl	Chlorine	0.2770	0.4000
20	Ca	Calcium	26.98	44.06
26	Fe	Iron	0.9220	2.098



6 Results and Discussion

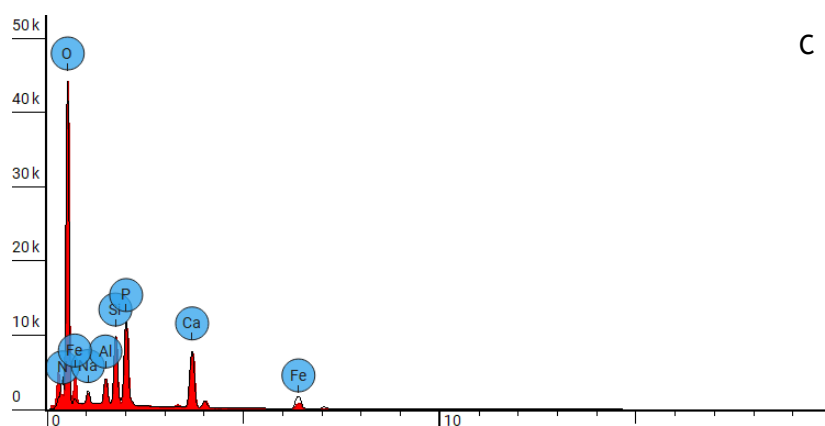


Figure 6.6 - SEM images of 3MNP granule. (A) Magnification of 100x. (B) Magnification of 540x. (C) EDS spectra of 3MNP granule with peak identification.

Table 6.5 - EDS analysis with the elements found in 3MNP granule.

Element Number	Element Symbol	Element Name	Atomic Conc.	Weight Conc.
7	N	Nitrogen	17.36	12.71
8	O	Oxygen	64.51	53.95
11	Na	Sodium	1.249	1.502
13	Al	Aluminum	1.775	2.503
14	Si	Silicon	3.204	4.705
15	P	Phosphorus	4.512	7.307
20	Ca	Calcium	5.160	10.81
26	Fe	Iron	2.229	6.507

As we would expect in all EDS spectra elements such as oxygen (O), nitrogen (N) appears, which is normal since these are among the main constituents of atmospheric air, and also carbon (C) since it is basically everywhere. Since one of the main materials is hydroxyapatite, used not only in the structure of the scaffold but also of the MNPs themselves, elements such as calcium (Ca) and phosphorus (P) appear in all spectra.

In the 0MNP granule, elements such as sulfur (S) and chlorine (Cl) appear, since the former is one of the constituents of collagen and the latter is present in hydrochloric acid (HCl), both used for the coating applied.

In the samples with MNPs (1.5MNP and 3MNP), iron is one of the constituents. Note that in the spots analysed appears a higher amount of iron in the sample 3MNP than in the 1.5MNP, which is to be expected since it contains twice as much MNPs.

In the last sample (3MNP), there are some contaminations of elements such as sodium (Na), due to washing with PBS, aluminium (Al) given that the manipulation of the granules is

done with tweezers, and silicon (Si) present for example in the Petri dishes where the granules were preserved.

6.1.4 - Micro-Computed Tomography

The purpose of micro-CT is to provide information about the interior of the material in greater detail, especially if it has porosity. The four granule types were analysed, hydroxyapatite only and with the different concentrations of MNPs (0MNP, 1.5MNP and 3MNP). Figure 6.7 shows the reconstructed micro-CT images.

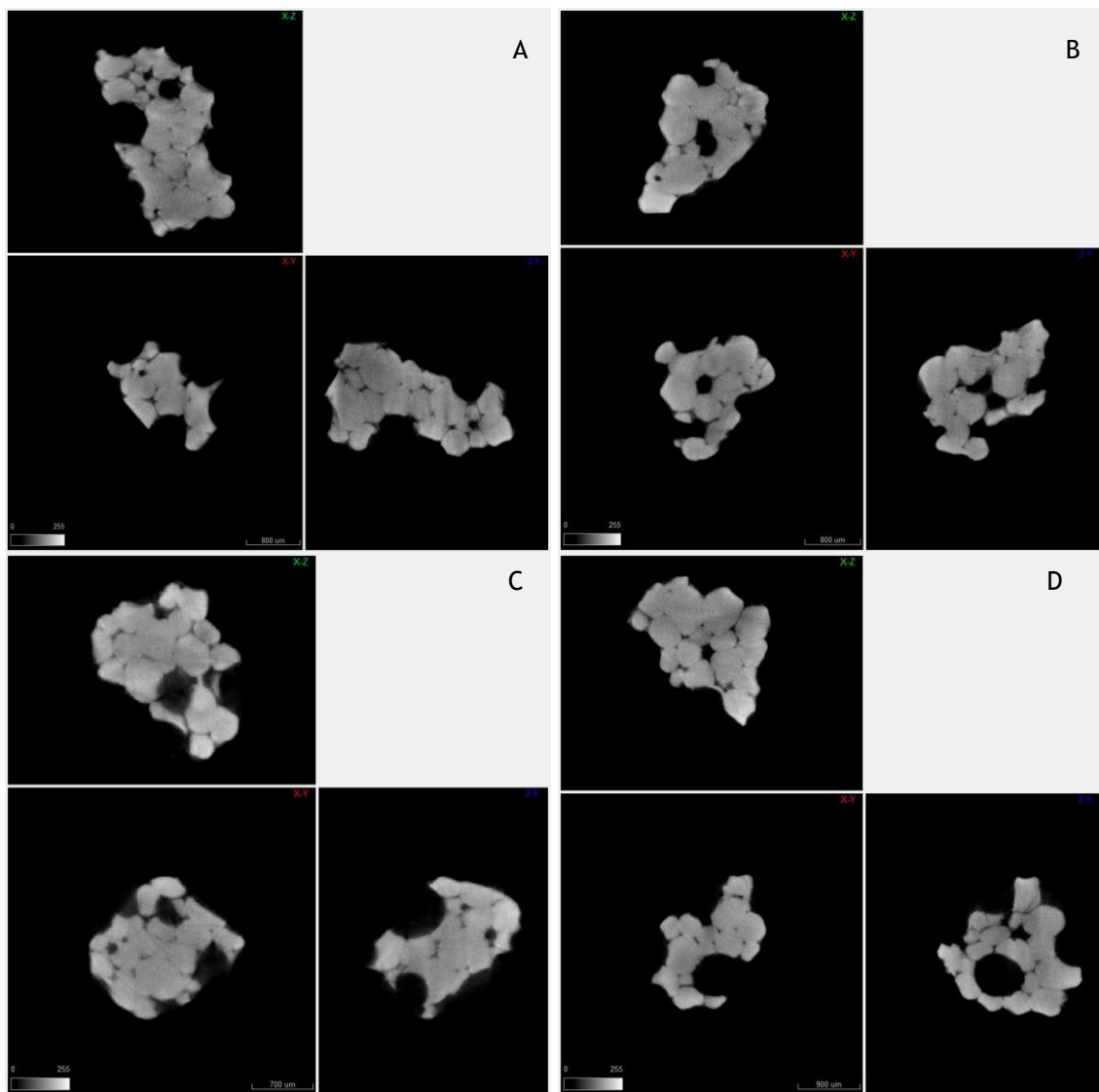


Figure 6.7 - Reconstructive images from micro-CT. (A) Hap granule. (B) 0MNP granule. (C) 1.5MNP granule. (D) 3MNP granule.

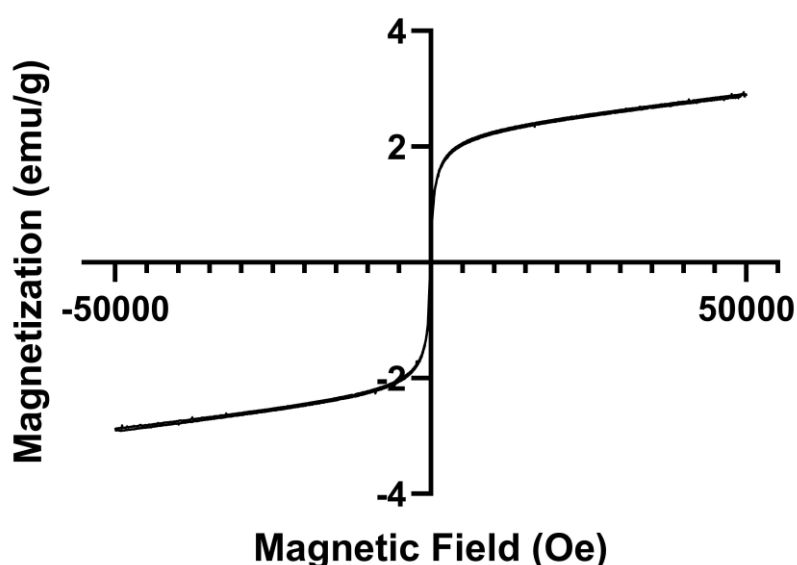
Table 6.6 - Porosity results from the different types of granules.

	Porosity (%)
Hap	7.23%
Hap-Col-0MNP	5.71%
Hap-Col-1.5MNP	3.65%
Hap-Col-3MNP	4.29%

Porosity is an important aspect in the production of biomaterials, especially in bone regeneration, improving the capacity of cell migration, ossification, and angiogenesis[64]. Through the images obtained, we can state that the granules appear to present some pores although through the analysis made in CTAn, presented in Table 6.6, all the granules had less than 10% porosity. Since the main goal of these granules is to reconstruct a space in a bone structure, granule by granule, the porosity will be achieved when the granules are placed side by side, leading to a macroporosity given the irregular structure of the granules allowing vascularisation through these pores.

6.1.5 - Superconducting Quantum Interference Device

Through the analysis made in SQUID it was possible to characterize the magnetic behaviour of MNPs. The graph in Figure 6.8 shows the magnetic response of FeHap^{2+/3+} nanoparticles when exposed to a magnetic field (± 50000 Oe) at a temperature of 300K.

Figure 6.8 - Representative results of magnetic response of FeHap^{2+/3+} MNPs.

When a magnetic field is applied to a ferromagnetic material, it describes a hysteresis curve that is characterised by remanence and coercivity. Remanence is the remaining magnetization in a material after the magnetic field is removed. Coercivity is the measure of the magnetic field strength needed to demagnetize the material completely. Since the coercivity is dependent on the size of the particle analyzed and since below the critical diameter the coercivity goes to zero, these particles are called superparamagnetic [65], [66].

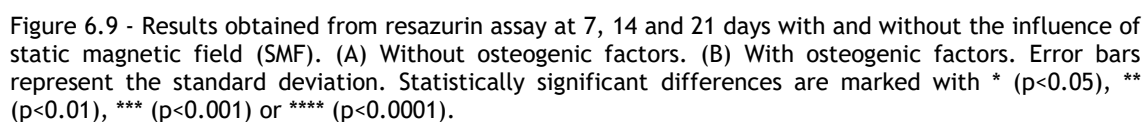
Observing the graph of figure 6.8, it can be seen that there is no hysteresis loop, since when the value of the applied magnetic field drops to values close to zero, even, also the magnetization of the material drops to zero. Since we are in the presence of nanometric particles and there is no hysteresis loop, it is possible to say that the FeHap^{2+/3+} nanoparticles are superparamagnetic. This was also the conclusion of a study by Morsi et al, who tested the same type of nanoparticles (FeHA NPs) and found that they exhibited superparamagnetic behaviour at room temperature[67].

6.2 - *In Vitro* Studies

6.2.1 - Resazurin Assay

The resazurin assay aimed to understand the metabolic activity and proliferation of pre-osteoblastic cells when seeded on the granules with different concentrations of MNPs (0MNP, 1.5MNP and 3MNP), inserted in different types of complete alpha medium (without and with osteogenic factors), with and without the external stimulus generating a 1T SMF. This assay is performed through a fluorescent product, which measures the reduction of blue dye and its intensity, in pink dye. It relates directly with the number of metabolically active cells[68]. Results were collected for 7, 14 and 21 days to assess metabolic viability and cell proliferation evolution.

To better analyse and understand the results obtained, it was important to separate the data with complete alpha medium, figure 6.10(A), and complete alpha medium supplemented with osteogenic factors, presented in figure 6.10(B).



Statistically significant differences were only found at day 14 with osteogenic factors between OMNP and 1.5MNP+Magnet ($p<0.0001$), OMNP and 3MNP+Magnet ($p<0.01$), OMNP+Magnet and 1.5MNP+Magnet ($p<0.01$), 1.5MNP and 1.5MNP+Magnet ($p<0.0001$), 3MNP and 1.5MNP+Magnet ($p<0.0001$).

From these results, the presence of SMF and MNPs in the collagen coating plays an important role in the viability and proliferation of osteoblastic cells, and also, it's noteworthy that the results obtained do not demonstrate any signs of cytotoxicity from either the external influence of the magnetic field or the addition of MNPs in varying concentrations. This is in line with the study by Ehnert et al, which found that applying a magnetic field to human osteoblastic cells improved cell viability and proliferation[69].

6.2.2 - Confocal Microscopy

Confocal microscopy is crucial in comprehending the morphology and interactions between cells and biomaterials. In this particular case, it played a key role in understanding the interaction between pre-osteoblastic cells and magnetic scaffolds, in the presence of varying concentrations of MNPs, culture medium, and the application of external stimulation using SMF (1T) [59]. Using staining before microscopic analysis, actin cytoskeleton (green) and nucleus (blue) could be observed and highlighted. Figure 6.12 displays the confocal microscopy images taken after day 7, 14 and 21 of cell culture. Although all granules with the various concentrations of MNPs were tested and microscopic images taken, it was chosen only to present the results of the granules with the highest concentration of MNPs (3MNP) with and without SMF (M and XM), with and without osteogenic factors (F and XF).

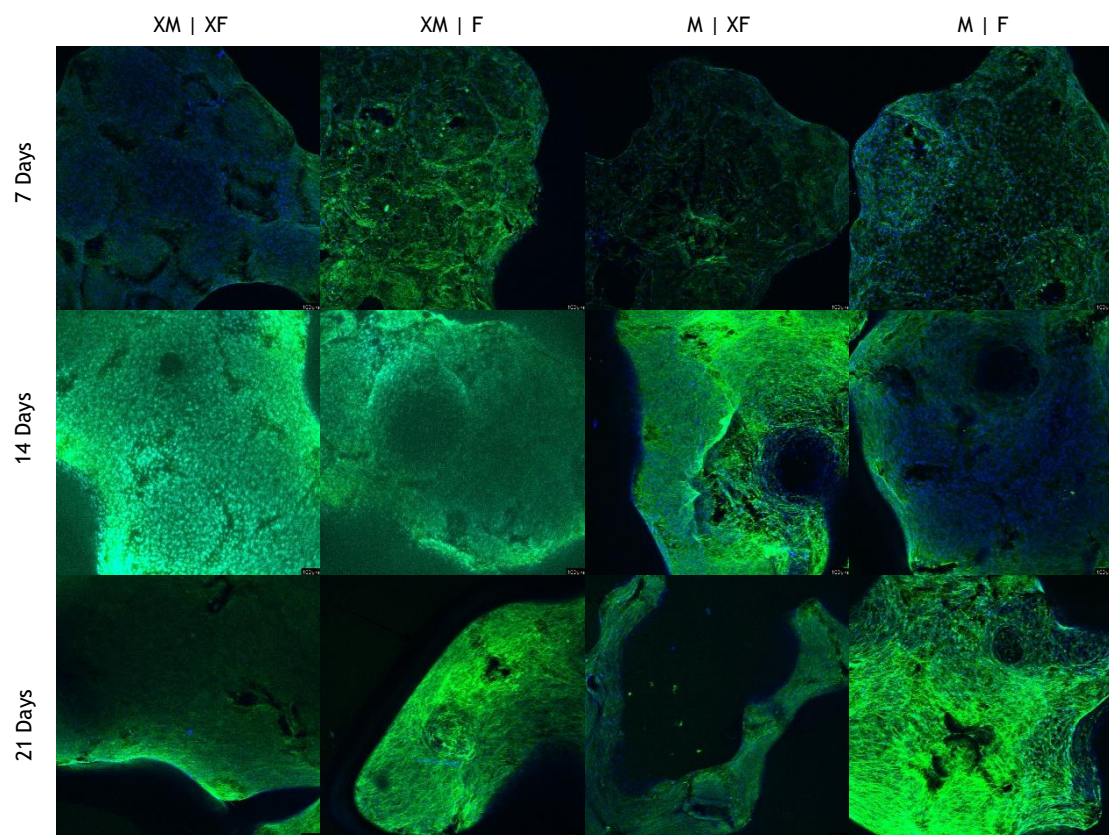


Figure 6.10 - Representative images from confocal microscopy after 7 days and 21 days with and without the influence of static magnetic field (SMF), with and without osteogenic factors.

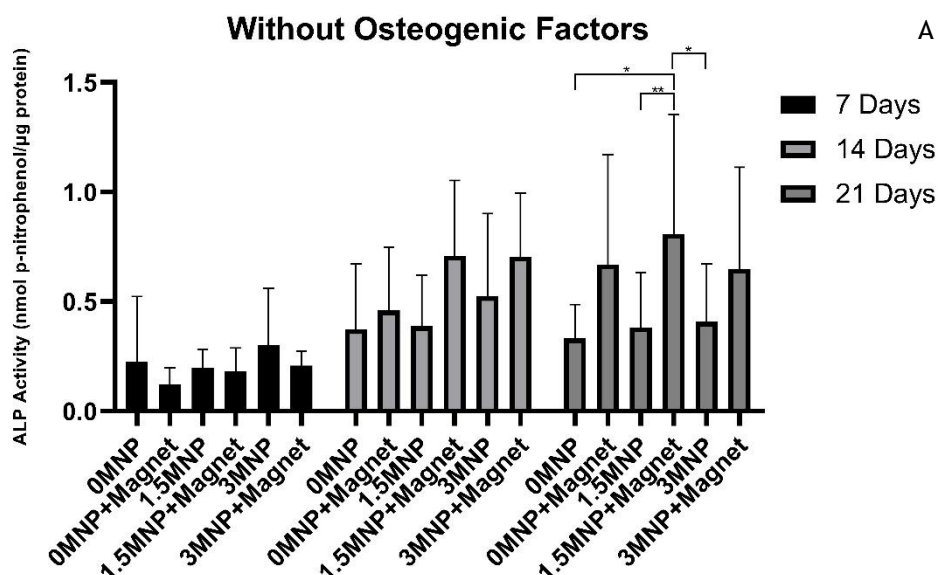
By examining the images captured, it can be observed that after 7, 14 and 21 days the cell layer growth according to granules' own morphology, where the material's boundaries can easily be recognized. Furthermore, cell migration and dispersion are positive, showing an excellent interaction between the magnetic granule and the seed cells.

Although confocal microscopy is a qualitative analysis, the images suggest cell viability as well as proliferation over days in all granule types. From these images apparently there are no major differences in the application of SMF. The same happened in a study by Cunha et al, in which he applied an SMF for 7 days to human osteoblastic cells, but through the images there were no differences in the use of the magnetic field[70].

6.2.3 - Alkaline Phosphatase Assay

Alkaline Phosphatase enzyme is secreted in the early stages of bone regeneration, specifically in the up-regulation of osteoblast differentiation. It serves as a biomarker for osteogenesis, revealing the activity of osteoblasts and is involved in the mineralisation phase[71].

The assay of ALP enzyme activity was done by a quantitative colorimetric test using to detect p-nitrophenol, through absorbance reading. The results are divided into two parts. Figure 6.13 shows the graph without osteogenic factors and Figure 6.13 shows the graph with osteogenic factors.



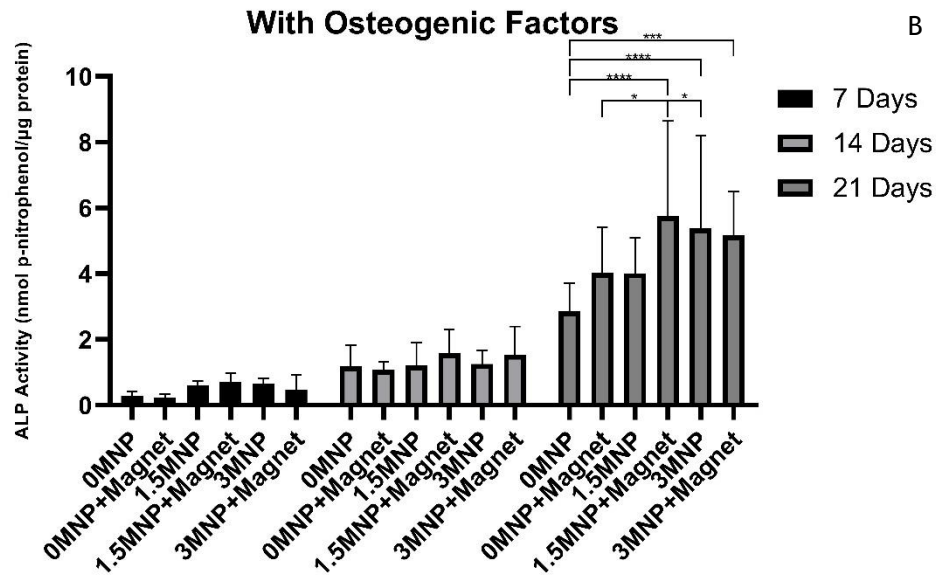


Figure 6.11 - Results obtained from resazurin assay at 7, 14 and 21 days with and without the influence of static magnetic field (SMF). (A) Without osteogenic factors. (B) With osteogenic factors. Error bars represent the standard deviation. Statistically significant differences are marked with * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) or **** ($p < 0.0001$).

Analysing the results present in the graph of figure 6.13, there is a slight increase in the ALP enzyme activity values at day 14 and day 21. While there is a slight upward trend in the values on day 14 when subjected to the external influence of the Static Magnetic Field (SMF), it is on day 21 that this effect becomes more pronounced and clearly visible. Statistically significant differences were only found at day 21 between 0MNP and 1.5MNP+Magnet ($p < 0.01$), 1.5MNP and 1.5MNP+Magnet ($p < 0.05$), 3MNP and 1.5MNP+Magnet ($p < 0.05$).

Observing the graph of figure 6.13, in which osteogenic factors were used, on day 14 there is a small increase in the levels of ALP enzyme activity. However, on day 21 there is a clear increase in the activity of this enzyme, with the values rising considerably. Statistically significant differences were again only found at day 21 between 0MNP and 1.5MNP+Magnet ($p < 0.0001$), 0MNP and 3MNP ($p < 0.0001$), 0MNP and 3MNP+Magnet ($p < 0.001$), 0MNP+Magnet and 1.5MNP+Magnet ($p < 0.05$), 1.5MNP and 1.5MNP+Magnet ($p < 0.05$).

In both cases there is an increase in the activity of the ALP enzyme, but this increase is more visible when osteogenic factors are present, which is normal since these are present in an *in vivo* system. This increase becomes visible from day 14, reaching even higher values on day 21. Yazid et al., also saw the best results with osteogenic factors but in their case after 14 days the osteoblasts were able to differentiate[72].

The presence of MNPs stimulated by an SMF also played an important role, since in the results obtained without osteogenic factors their use allowed higher values to be achieved, showing that the SMF helped the differentiation of osteoblasts. Similarly, a study carried out

by Yamamoto et al., found that after 21 days, stimulation by a magnetic field was effective in osteoblast differentiation[73].

6.2.4 - Alizarin Red Assay

The red resazurin assay is commonly used in in vitro assays as an indicator of bone regeneration by identifying the mineralization phase of osteoblasts. This is a method that is based on the principle of detecting the formation of calcium deposits, since Alizarin Red S, a red dye, binds to calcium ions, forming a calcium complex, which results in the formation of a red precipitate.

Although all granules from all timepoints (7, 14 and 21 days) were tested (0MNP, 1.5MNP and 3MNP), only granules from day 21 are shown in figure 6.12.

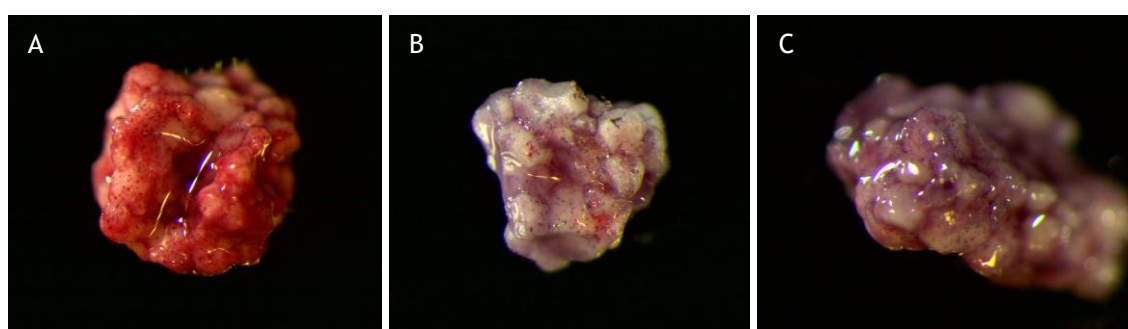


Figure 6.12 - Representative Alizarin red images after 21 days. (A) 0MNP granule. (B) 1.5MNP granule. (C) 3MNP granule.

As previously stated, alizarin dyes with red the nodules with calcium. This is visible in the granule with the lowest concentration of MNPs (0MNP). However, as the concentration of MNPs increases, the colour of the nodules becomes more purple. The reason for this is that the iron that is present in MNPs binds to alizarin by absorption, even when it is in aqueous solutions. This reaction was proven in two studies, carried out by Moriguchi et al. and Fu et al. in which they used iron doped hydroxyapatite and activated clay modified by iron oxide (Fe-clay) in a batch reactor, respectively[74], [75].

Although this was not the most suitable test for our granules, this is an assay that has proven to be able to identify mineralisation, as shown by Kaliannagounder et al, who found that after 21 days, there was increased calcification in pre-osteoblastic cells (MC3T3-E1) when they were seeded with hydroxyapatite MNPs[76]. Another study, by Imaizumi et al., also shown that after 31 days, there was an increase in the number of mineralized nodules in pre-osteoblastic cells (MC3T3-E1) when they were exposed to a 250 mT SMF [77]. Proven once again the power of this assay.

Therefore, in order to perform a quantitative and more accurate analysis, Osteocalcin and Osteopontin ELISA tests should be performed.

6.3 - *In Vivo* Studies

CAM assays are quite common to help understand how an organism might react to a particular material. This ultimately provides important information, especially regarding angiogenesis, new blood vessel formation and toxicity.

For the *in vivo* assays, CAM assays were performed using fertilised chicken embryos and in which granules with the various concentrations of MNPs (0MNP, 1.5MNP and 3MNP) were placed with and without the external stimulation of an SMF. After excision at day 13, the inoculations were photographed as shown in Figure 6.13. Table 6.7 shows the embryos that were used, how many died and how many were finally analysed.

Table 6.7 - Numbers of chick embryos inoculated, dead and used for analysis.

	Inoculated	Dead	Used for Analysis
0MNP	20	3	14
0MNP+Magnet	23	0	18
1.5MNP	38	1	27
1.5MNP+Magnet	24	0	18
3MNP	20	0	19
3MNP+Magnet	23	0	20

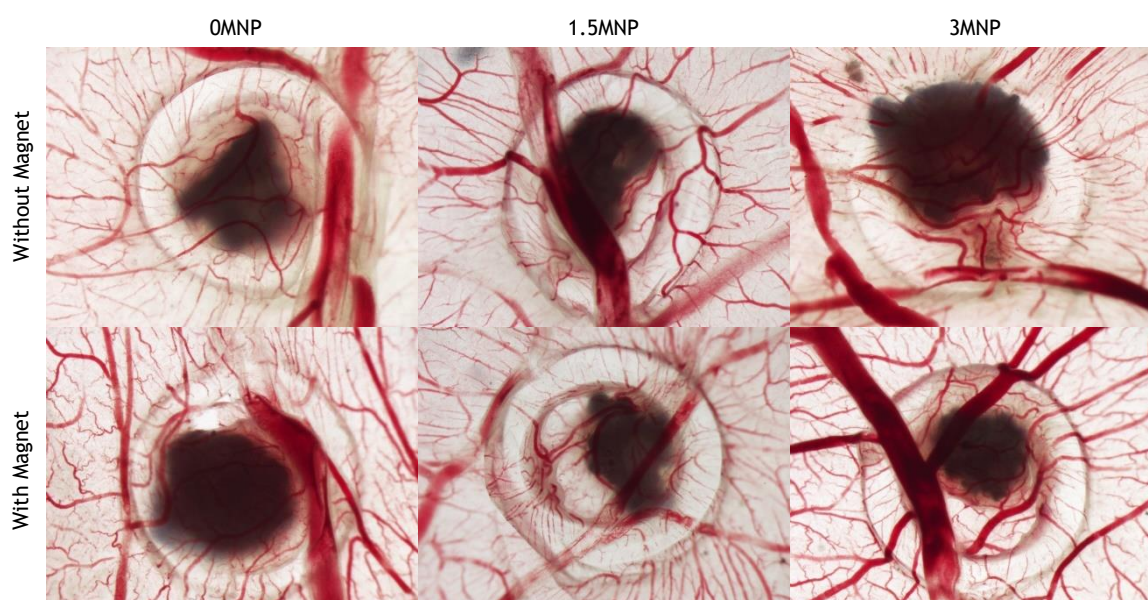


Figure 6.13 - Representative images used to determine the angiogenic response of the materials.

According to the data presented in Table 6.7, it is evident that not all embryos survived the 13-day inoculation period. Additionally, the number of embryos analyzed was lower than the initial count due to the complexity of the process involved in successfully extracting the

embryos from the eggs. This procedure can sometimes be challenging, resulting in unsuccessful attempts.

Observing the images in figure 6.13, we can see that there are several blood vessels that end up surrounding the granule that was inoculated. This suggests that the blood vessels can easily adapt to the surface of the granules. In terms of inflammation, according to the images the granules and the magnetic field do not appear to have provoked the appearance of an inflammatory response, however a histological analysis is necessary.

Since these images are merely qualitative, a quantitative verification of the number of blood vessels was performed, figure 6.14, in order to provide better information on angiogenesis.

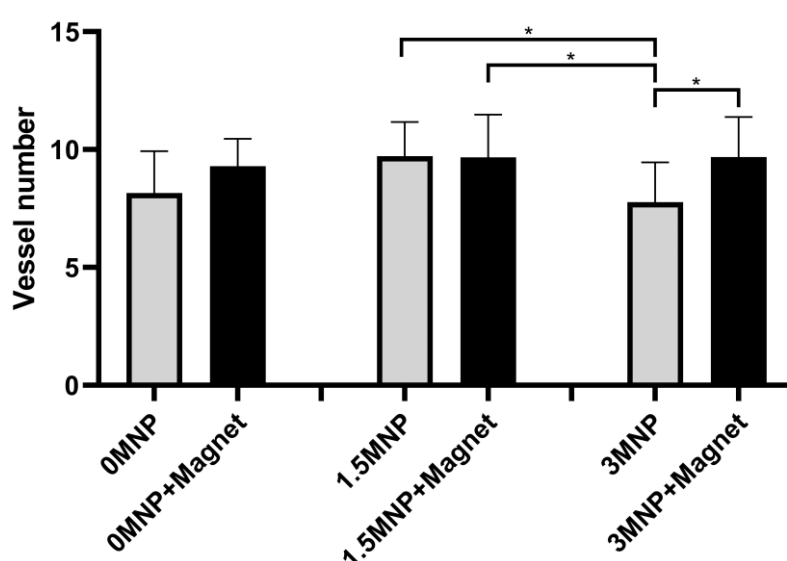


Figure 6.14 - Results obtained quantification of neovessels with the different granules with and without the influence of static magnetic field.

The graph in figure 6.14 shows that the use of magnetic stimulation associated with the presence of MNPs has positive results in the formation of blood vessels. Statistically significant differences were found between 1.5MNP and 3MNP ($p < 0.05$), 3MNP and 1.5MNP+Magnet ($p < 0.05$), 3MNP and 3MNP+Magnet ($p < 0.05$).

Subsequently, a histological analysis of the excised inoculations was performed, in which the images of figure 6.15 were obtained. After microscopic visualisation, a score system was created considering the inflammatory response, in which score 0 corresponds to a CAM excised without any type of inflammation, while score 1 indicates that there is an inflammatory response.

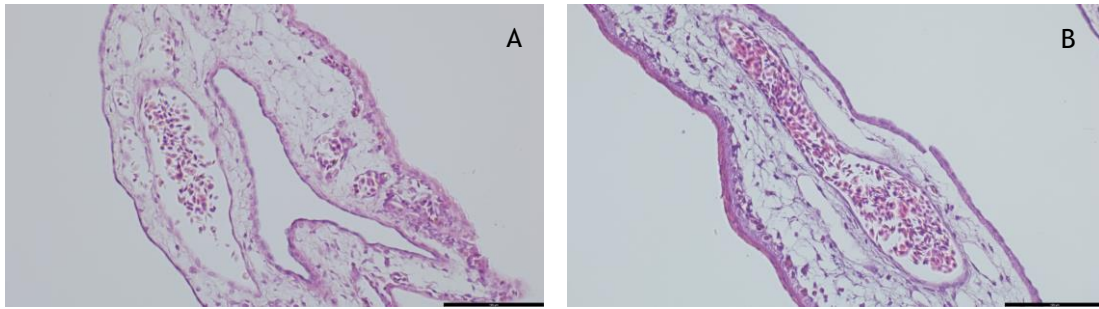


Figure 6.15 - Representative images of histological analysis for inflammation response. (A) Score 0 = no inflammatory reaction. (B) Score 1=with inflammatory reaction.

Through figure 6.15, it was seen that the inflammatory response found is very small, and no severe cases of inflammation were found. However, after checking all the samples it was possible to see that there was an inflammatory response in 51.4% of the samples. Despite this, no significant differences were found between the different concentrations of MNPs nor in the application of SMF, since the results were quite random, not showing any kind of trend. Unlike a study by Coelho et al, where a superior inflammatory response was obtained with Hap/MgO granules[78].

In summary, it was possible to see that the use of SMF together with MNPs had a positive impact on angiogenesis, promoting vascularisation and blood vessel formation. Moreover, despite the appearance of a slight inflammatory response, this did not have an associated cause. These results are in agreement with studies by Nájera-Romero et al. and Manjua et al., both of whom performed CAM assays in which the former used chitosan/hydroxyapatite scaffolds and the latter a magnetic stimulation of 0.08 T, having in both cases an increase in the number of blood vessels, confirming the stimulation of angiogenesis and no toxicity[79], [80].

Chapter 7

Conclusion and Future Perspectives

Bone tissue engineering has attracted much research investment to address diverse issues related to bone injuries. The field has evolved, including the use of external stimuli such as magnetism for improved outcomes.

This study focuses on the application of a static magnetic field together with ceramic granules coated with collagen type I mixed with different concentrations of magnetic nanoparticles (0, 1.5, and 3), and how this can influence bone regeneration.

With this purpose, hydroxyapatite was used since it contains important properties such as biocompatibility, bioactivity and slight biodegradability.

There are several studies showing that the application of external stimuli such as magnetism is favorable, especially when it is used together with magnetic nanoparticles. For this work FeHap^{2+/3+} nanoparticles were synthesised.

A characterisation of the granules was carried out which started with FTIR analysis to verify the chemical composition of the material by identifying the functional groups. It was found that although the granules were different from each other, the same functional groups were always found, and there were no major differences between the spectra.

Mechanical tests were carried out, such as compression test in which it was observed that the increase in MNPs concentration improved the mechanical properties. In addition, friability tests were performed in which all types of granules are below 1% of total mass loss.

The granules were analysed using SEM to better understand the morphology and distribution of collagen and MNPs. Through the EDS spectrum and the analysed spots, the elements that would be expected are found, as well as the fact that there was an increase in iron when the concentration of MNPs increased.

To check for porosity and morphology, the 3D structure of the granules was studied using micro-CT, these were found to have an irregular shape, capable of forming macro-pores when placed together.

The magnetic behaviour of the nanoparticles was analyzed by SQUID and found to be superparamagnetic, which is beneficial for a biomedical application, as they are only stimulated in the presence of a magnetic field.

For the *in vitro* studies, pre-osteoblast cells were used and seeded in the granules. As a first phase, the metabolic activity of the cells was tested at days 7, 14 and 21, showing that on day 14 there were higher values of viability and cell proliferation. Moreover, the use of SMF together with MNPs showed to have a positive impact, especially when osteogenic factors are used.

Subsequently the granules were observed by confocal microscopy, where it was found that the granules exhibited no cytotoxicity, promoting cell adhesion and migration through the whole granule structure.

ALP enzyme activity was quantified at 7, 14 and 21 days. When the medium was supplemented with osteogenic factors, there was greater enzyme activity on day 21. On the contrary, without the addition of osteogenic factors, there was an increase on day 14 which continued until day 21. Overall, in both cases, the presence of SMF with MNPs showed a positive impact on the differentiation of osteoblasts.

The *in vitro* tests ended with the alizarin red assay, with the aim of identifying the mineralisation phase of the cells. After performing the test, it was found that iron binds to alizarin, making the test completely unfeasible. As such, this proved not to be the best test for mineralisation and as such, in the future, the Osteocalcin and Osteopontin tests should be performed.

In vivo tests were also carried out, namely CAM assays. Granules were inoculated for 13 days, and it was found that the application of SMF together with MNPs promotes vascularisation. Moreover, these did not induce an inflammatory response, confirming once again that granules do not present cytotoxicity.

Finally, the use of hydroxyapatite granules with collagen and MNPs impregnation associated with external stimulation through the application of a static magnetic field was shown to have a positive impact on cell proliferation and differentiation. In addition, they have been shown to promote angiogenesis and are non-toxic. Since these granules have a compressive strength similar to trabecular bone, and since they contain superparamagnetic particles, they are excellent candidates for possible replacement of bone autografts. However, in the future, mineralisation tests should be performed, and endothelial cell assays should be initiated to help validate these results.

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