UNIVERSIDADE CATÓLICA PORTUGUESA | INSTITUTO DE CIÊNCIAS DA SAÚDE

POLYMER-CERAMIC NANOCOMPOSITES FOR BONE REGENERATION

Dissertação apresentada à Universidade Católica Portuguesa para obtenção do grau de Mestre em Medicina Dentária

Por

Sónia de Lacerda Schickert

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Sob orientação de Professora Doutora Ana Leite Oliveira

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To my sister, Joana Sofia, who I love and miss tremendously.

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Abstract

Bone is a very important structure in the human body. In the craniomaxillofacial complex, trauma and pathological situations such as maxillofacial tumors produce major bone defects that cannot be healed by physiological processes. As a response to this problem, many strategies have been studied throughout the years, with the purpose to find a material that reunites ideal properties for regenerating or substituting the needed bone and therefore guarantee its physiological and structural functions. Among this strategies, transplantation of bone within the same individual (autografts), from another human being (allograft) or from an individual of another species (xenograft) have been studied and are clinically applied nowadays. However, these techniques revealed to have drawbacks such as donor site morbidity, risk of disease infection and immunological reactions. In this context, new solutions are needed.

In the field of biomaterials, nanocomposites are considered a promising material for many outcomes, mainly because their nanometer-scale allows a superior structural performance, when compared to microcomposite materials. In the bone regeneration area, ceramic nanocomposites dispersed in a polymeric matrix are a promising solution, because they reassemble the physiological bone structure, adding unique biocompatible and stimulating properties to the already outstanding mechanical behavior.

The investigation of the different types of ceramic-polymeric nanocomposites, as well as the co-factors that can be added to enhance the biological response and their processing ability for the commercial level, is extremely relevant. This study intends to present a review of the published information about the *in vivo* and *in vitro* studies that have been performed in the last 5 years and their contribution for the development of an ideal nanocomposite material to be used in bone regeneration, particularly in the craniomaxillofacial context.

Key words: Bone; regeneration; nanocomposite; ceramic; polymer

Resumo

O osso é uma estrutura muito importante no corpo humano. No complexo crâniomaxillofacial, situações como trauma e tumores maxillofaciais têm como resultado defeitos ósseos de grandes proporções que não são passíveis de ser curados por processos fisiológicos. Com o intuito de dar resposta a este problema, várias estratégias têm vindo a ser estudadas ao longo do tempo, para encontrar um material que reúna as propriedades ideias para a regeneração ou substituição do osso em défice e assim garantir o restabelecimento as suas funções fisiológicas e estruturais. De entre estas estratégias, a transplantação de osso no mesmo indivíduo (autotransplante), transplantação de osso entre seres humanos (alotransplante) e de osso de indivíduos de outra espécie (xenotransplante) têm sido estudadas e são clinicamente utilizadas hoje em dia. Contudo, estas técnicas têm mostrado possuir alguns inconvenientes como são a morbilidade da zona óssea dadora, risco de doença infeciosa e reações imunológicas. Neste contexto, novas soluções são necessárias.

Na área de biomateriais, os nanocompósitos são considerados um material promissor para vários fins, principalmente porque a sua escala nanométrica permite uma performance estrutural superior, quando comparada com materiais microscópicos. Em regeneração óssea, nanocompósitos cerâmicos dispersos numa matriz polimérica, apresentam-se como uma solução promissora, uma vez que simulam a estrutura fisiológica do osso, adicionando propriedades de biocompatibilidade e estimulação únicas ao já muito bom desempenho mecânico.

A investigação de diferentes tipos de nanocompósitos cerâmico-poliméricos e cofatores que possam ser adicionados para melhorar a resposta biológica, bem como técnicas de produção para uma possível escala comercial, é extremamente relevante. Este estudo tem como objetivo apresentar uma revisão da informação publicada sobre estudos *in vivo* e *in vitro* que têm sido efetuados nos últimos 5 anos e o seu contributo para o desenvolvimento de um material ideal.

Palavras-chave: Osso; regeneração; nanocompósitos; cerâmica; polímeros

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List of Abbreviations

B

BG – Bioactive Glass

BMP – Bone morphogenetic protein

BMP-2 – Bone morphogenetic protein two

BMP-7 – Bone morphogenetic protein seven

BMSc – Bone Mesenchymal stem cells

BSE – Bovine spongiform encephalitis

C

CPC – Calcium phosphate cement

D

DBM – Demineralized bone matrix

DNA – Deoxyribonucleic acid

E

ECM – Extracellular matrix

e-PTFE – polytetrafluoroethylene

F

FDA – Food and Drug Administration

G

Gel – Gelatin

H

HA – Hydroxyapatite

M

MSCs – Mesenchymal stem cells

MWNT – Multi-walled carbon nanotubes

N

NCES – N- carboxyethyl chitosan

nHA – Nano-hydroxyapatite

P

P66 – Aliphatic form of poyamide

PA – Polyamide

PAA – Poly(aspartic acid)

PAs – Peptide amphiphiles

PCG – Polycaprolactone

PCL – Polycaprolactone

PEG - Polyethylene glycol

PGA – Poly (glycolic acid)

PHA – Polyhydroxyalkanoate

PHB – Poly(3-hyroxybutyrate)

PLA – Poly (lactic acid)

PLEOF – Poly (lactide – co – ethylene oxide fumarate)

PLGA – Poly(lactide – co – glycolic acid)

PLLA – Poly(L – lactide acid)

PP – Polypropilene

PU – Polyurethane

PVA – Poly (vinyl alcool)

S

SBF – Stimulated body fluid

SF – Silk fibroin

SR – Silicone Rubber

T

TCP – Tricalcium Phosphate

TGF – Transforming growth factor

THF – Tetrahydrofuran

TiO_2 – Titanium dioxide

U

US – United States (of America)

α-TCP – Alpha tricalcium phosphate

β-TCP – Beta tricalcium phosphate

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I. Introduction

I. Introduction

Natural bone has a great healing capacity. Through times, this capacity has been studied in order to understand what the limitations are and how they could overcome. A well-known limitation of the natural healing process is related to the extension of the bone defect. When the lesion is larger than 2 mm, the bone structure is not capable of regenerating by itself.[1] Situations such as diseases, abnormalities or trauma which have as a consequence major bone defects have been extensively studied in order to find the best therapeutic solutions.[2]

In the craniomaxillofacial skeleton, bone defects can vary from small (few millimeters) periodontal defects, to large segmental defects that are originated mainly in major trauma, followed by surgical excision or cranioplasty.[3] Such structures, have a complex tree-dimensional structural need, which is difficult to fulfill. In cranial vault defects, the underlying brain needs permanent protection. Segmental jaw defects require restoration of mechanical integrity, temporomandibular joint function and inter-maxillary dental occlusion. Maintaining acceptable facial esthetics is another unique consideration in the treatment of facial defects that cannot be underestimated.[3]

Due to the constant need to heal, techniques to replace, restore, or regenerate bone were noticed as a major clinical quest in the fields of orthopedic, spinal, dental, cranial and maxillofacial surgery. The earliest report of a bone grafting procedure appeared in 1682, in a book by Job Janszoo van Meekeren, a surgeon in Amsterdam. In this document, the author reported a case in Russia, where the surgeon restored a cranial defect using a cranial bone graft from a dead dog.[4] In 1881, Sir William MacEwen of Rothesay, from Scotland, published the first case report of successful inter-human transfer of bone grafts.[5] Since then, many changes occurred due to centuries of subsequent research both in academia and industry.

Now-a-days new materials are being investigated, with the purpose of changing the goal of bone grafts from just a replacing role, to the complete bone restauration, allowing for the own bone to grow and regenerate itself. In this context, polymer-ceramic nanocomposites arise as important candidates for this

application, since bone itself is composed by polymer-ceramic nanocomposite structure.

1. The Bone Biology

Bone is a calcified, living, connective tissue that forms the majority of the skeleton. Their basic constitution is an intercellular calcified matrix, containing collagen fibers and several types of cells within the matrix.[6] Actually, bone is the only mineralized (collagen mineralized with calcium phosphate) structure that contains living cell bodies on its structure. Dentin, for example, is composed of collagen mineralized with calcium phosphate but it does not contain cell bodies, only tubular extensions of cells.[7] Other significant tissue mineralized with calcium phosphate is the enamel.[7] However, in this case, there are virtually no cells or cell processes or, indeed, much too organic matrix.

There are two major types of bone: Cancellous (also called spongy bone) and Cortical.[8] Cancellous bone forms the internal pours framework of bones. It contains stem-cells-rich bone marrow which is essential for the growth of new connective tissue (e.g. muscle, cartilage, bone and tendons) and the production of blood cells. Cortical bone surrounds the cancellous bone and forms an outer shell, thus giving the bone shape and form. In load bearing bones, the cortical component is markedly thickened to form a strong shaft.

Bone is responsible for 5 basic functions:[6] (a) to support all the body structures, (b) to protect vital organs, (c) to be a reservoir of calcium and phosphorus, (d) to function as a lever on which muscles act to produce movement and (d) to host blood-producing cells.

In terms of development, all bones are originated in the mesenchyme either by intramembranous ossification, in which mesenchymal models of bone undergo ossification or endochondral ossification, in which cartilaginous models of bones form and undergo ossification.[6] There are five distinct types of cells associated with bone tissue formation and regulation: osteoprogenitor cells, osteoblasts, osteocytes, osteoclasts, and bone-lining cells.[9] Osteoprogenitor cells (or bone precursor cells) have the ability to proliferate and differentiate into bone cells.

Osteoblasts are the cells that are responsible for the formation of new bone tissue. They start with secreting collagen followed by coating non-collagenous proteins, which are similar to a glue that has the ability to bind the minerals, mostly calcium and phosphate, from the bloodstream. Osteocytes are matured cells derived from the osteoblasts that are responsible for the maintenance of the bone tissue. They function as transporting agents of minerals between bone and blood. Osteoclasts are the largest cells found at the surface of bone mineral. They are responsible for the resorption of the bone tissue. They secrete acids or enzymes to dissolve the minerals as well as collagen from the matured bone. The dissolved minerals then re-enter the bloodstream and are carried to different parts of the body. Bone-lining cells are found along the surface of the matured bone and are responsible for regulating the transportation of minerals in and out of the bone tissue. They also respond to hormones by producing proteins that activate the osteoclasts. Together, these five types of cells are responsible for building the bone matrix with hierarchical structure, self-assembly ability and remodeling capability. All these processes must be in equilibrium to ensure a healthy bone tissue.[6-9]

When bone suffers an injury, it possesses natural healing capacity which allows for a successful regeneration. After damage, the bone adjacent structures cause a hematoma around the broken bone ends.[10] Fibroblasts enter the site and form granulation tissue, composed predominantly of type-III collagen. Then, two different processes occur in the proximal and the distal site. In the proximal, chondroblasts arise from the periosteum and produce hyaline cartilage. In the distal site, osteoblasts arise from the periosteum and produce woven bone (majorly constituted by type-I collagen).[10] Both sites combine and form the fracture callus. The woven bone then undergoes substitution and the hyaline cartilage undergoes endochondral ossification. These processes both produce lamellar bone. Osteoblasts and vascular channels penetrate the mineralized matrix and stronger trabecular bone is laid down. From here, final remodeling occurs by the deposition of compact bone by osteoblasts in resorption pits prepared by osteoclasts.[10] This process, known as primary or direct osteonal healing, is just possible if the gap between bone ends is less than 2 mm and absolute stability exists.

1.1. Craniomaxillofacial anatomy and physiology

The skull is divided in two major parts: The neurocranium and the viscerocranium.[11] The neurocranium, constituted by the cranial bones form the cranial cavity which encloses and protects the brain. In addition, the cranial bones stabilize the positions of the brain, blood vessels, lymphatic vessels and nerves through the attachment of their inner surfaces to meninges (membranes). The outer surface of cranial bones provide large areas of attachment for muscles that move various parts of the head. Underneath it, the skull base forms the floor of the cranial cavity and separates the brain from other facial structures.[11,12] Anteriorly attached to the skull is the viscerocranium.

The viscerocranium forms the anterior part of the cranium and consists of facial bones surrounding the mouth, nose, and most of the orbits. They consist on the framework for the face and provide support for the entrances to the digestive and respiratory systems. Therefore, vital functions such as breathing, eating, talking, smelling, seeing and having equilibrium are ensured by this bones. Moreover, it is also an esthetically important region. Therefore, any change to its normal constitution and/or function seriously damages the individual's life quality.[12] In such situations, maxillofacial surgery applies different technics for healing and restoring the normal function.

1.2. The need for maxillofacial surgery

Surgeries concerning the use of bone replacements or bone grafts in the craniomaxillofacial area are requested in two major situations: in case of trauma or when there is a pathological abnormality in the anatomy and physiology of this area (e.g. a congenital and developmental deformity or an acquired condition).

Causes of craniomaxilofacial trauma injuries vary from interpersonal violence to rodoviary accidents.[13] Fractures of the maxilla are the result of considerable force and are often associated with craniocerebral injury. The maxilla offers a high resistance against forces directed upwards but relatively little resistance if the impact is directed horizontally. Fractures may occur at three

levels, Le Fort I, II and III, and may be unilateral or bilateral (Figure 1-A). The palate may also be fractured in the midline. Mandibular fractures, which are the ones that happen more often,[11,13-15] may happen in different sites of the mandible (Figure 1-B). The symptoms and signs of a fractured mandible include pain and swelling over the fracture site as well as trismus and malocclusion.

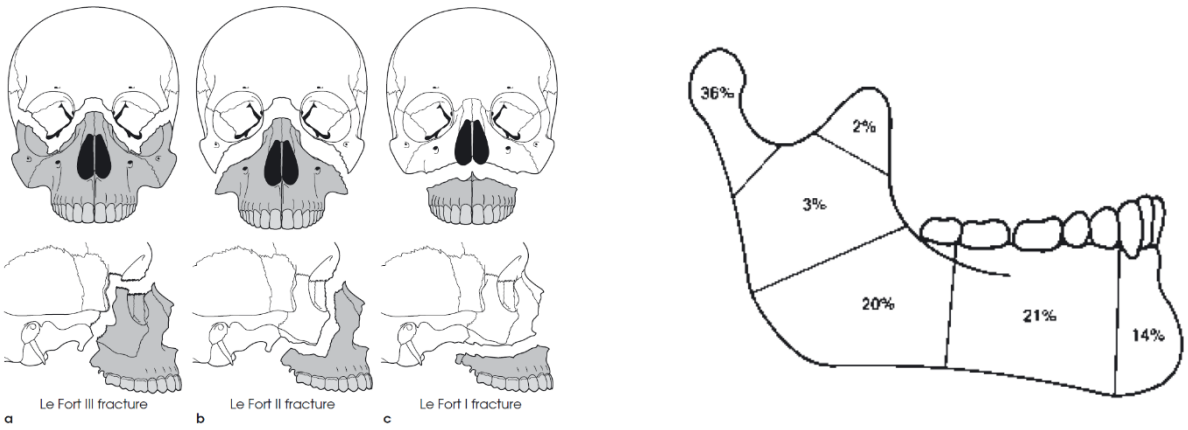


Figure 1 – Maxillofacial Trauma

(A) Mandibular Fractures: Le Fort fracture I, II and III. (B) Incidence of fractures occurring at various sites of the mandible. [15]

Congenital and developmental deformities of the cranial skeleton occur due to errors in the growth process while the fetus is *in utero* and tend to become more evident in the pubertal growth spurt.[15] They have the particularity of being really easy to diagnose, since the facial deformity is obvious, but extremely difficult to categorize. Since the skin drapes over the bony skeleton, most deformities are caused by bone abnormalities (soft tissue abnormalities are rare and when they occur, they are commonly secondary to skeletal abnormalities). According to Booth *et al.,*[15] these abnormalities can be broadly subdivided into craniofacial anomalies, clefting anomalies and orthognathic deformities. This last one involves the dentofacial anomalies that can be corrected by orthognathic surgery, repositioning parts of the facial skeleton to correct the deformity. Craniofacial and clefting anomalies, may require bone grafts to add bone tissue or realign it through complex maxillofacial surgeries.

In the craniomaxillofacial complex, a high percentage of diseases affecting the bones are tumoral conditions.[16] Bone tumors of the maxillofacial region may arise from osteogenic, chondrogenic, fibrogenic, vascular, hematopoietic and other elements of the bone.[17] Table 1 shows the World Health Organization classification of benign and malignant bone tumors of the maxillofacial region.[18] As a consequence to tumors excisions, a critical-sized bone defect remains, leading to noticeable deformity and dysfunction. These situations generally require reconstructing strategies such as the replacement with autogeneous or allogenic bone grafts, xenografts or alloplastic materials. The clinical results of these procedures are limited, since the craniomaxillofacial bones have a complex three dimensional structure and the replacement materials do not possess the necessary characteristics to fulfill their goal.[19] Other request for bone augmentation has to do with the loss of alveolar bone after teeth loss. The main purpose of creating new alveolar bone is generate a bone structure to anchor dental implants and dentures and allow the requalification of the orthognathic system.[19]

Table 1 - World Health Organization classification of benign and malignant bone tumors of the maxillofacial region [18]

Cathegory	Benign	Malignant
Osteogenic	Osteoma Osteoid osteoma Osteoblastoma	Osteosarcoma
Chondrogenic	Chondroblastoma Chondromyxoid fibroma Chondroma	Chondrosarcoma
Fibrogenic	Fibrous dysplasia	Fibrosarcoma
Vascular	Hemangioma	Hemangioendothelioma
Hematopoietic	-	Plasmacytoma Lymphoma
Others	Giant cell tumor Aneurysmal bone cyst Meningioma	Chordoma Ewing sarcoma

1.3. Bone as a Nanocomposite

Nanocomposites are defined as a heterogeneous combination of two or more materials in which at least one is at the nanometer-scale.[20] A good example of a nanocomposite is bone tissue: it is composed of two major phases at the nanoscale level, namely the organic (protein) and the inorganic (mineral). These phases have multiple components which consist of, in decreasing proportions: minerals, collagen, water, non-collagenous proteins, lipids, vascular elements, and cells (Table 2).[21] The mineral of bone is mainly composed of HA and the organic part of bone is mainly composed of collagen. Here, collagen acts as a structural framework in which plate-like tiny crystals of HA are embedded to strengthen the bone. Bone collagen has a typical fibrous structure, whose diameter varies from 100 to 2000 nm. Similarly, HA in the bone mineral is in the form of nanocrystals, with dimensions of about 4nm by 50nm by 50nm.[22,23] The primary role of minerals is to provide toughness and rigidity to the bone, whereas collagen provides tensile strength and flexibility needed.[21] In other words, the function of collagen fibers is to provide strength in tension and resistance in bending whereas the apatite crystals embedded between the nanofibers will resist to compression.[24]

Table 2 - Components of bone [21,23]

(This composition can vary slightly from species to species and from bone to bone)

<table>
<tr><th>Inorganic Phase</th><th>Wt %</th><th>Organic Phase</th><th>Wt %</th></tr>
<tr><td>Hydroxyapatite</td><td>≈ 60</td><td>Collagen</td><td>≈ 20</td></tr>
<tr><td>Carbonate</td><td>≈ 4</td><td>Water</td><td>≈ 9</td></tr>
<tr><td>Citrate</td><td>≈ 0,9</td><td rowspan="3">Non-collagenous proteins
(osteocalcin, osteonectin, oeteopontin, thrombospondin, morphogenic proteins, sialoprotein, serum proteins)</td><td rowspan="3">≈ 3</td></tr>
<tr><td>Sodium</td><td>≈ 0,7</td></tr>
<tr><td>Magnesium</td><td>≈ 0,5</td></tr>
<tr><td colspan="2" rowspan="2">Other traces (Cl^- , F^- , K^+ , Sr^{2+}, Pb^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+})</td><td colspan="2">Other traces
(Polysaccharides, lipids, cytokines)</td></tr>
<tr><td colspan="2">Primary bone cells
(Osteoblasts, osteocytes, osteoclasts)</td></tr>
</table>

1.4. Hierarchical structure

Because of its structural function, bone needs to be tough and resistant. The key to this strength is the complex structural hierarchy into which it is organized in a self-assembling mode. Thus, bone can be considered as an assemblage of various levels of hierarchical structural units elegantly designed at different scales, from nano to macro (Figure 2), to meet multiple functions.

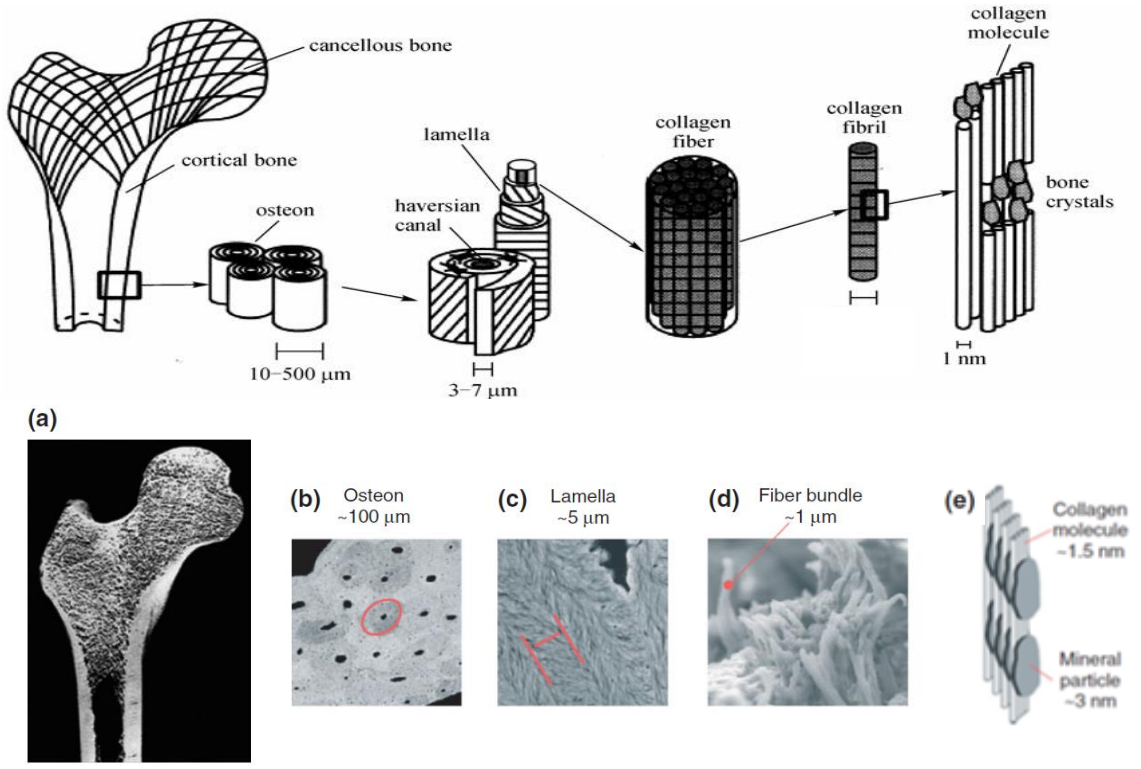


Figure 2 - Bone hierarchical organization

(a) the macrosctructured level: bone is divided in compact and spongy bone; **(b)** the microstructured level: osteons; **(c) and (d)** the mesostructured level: lamellae and its fibrillar composition **(e)** the nanoscopic level: the 2 major components, collagen and HA. [2,8]

At the macrostructure level, matured bone can be divided in two types: spongy bone and compact bone. Structurally they are different because they play different roles in bone. Spongy bone represents approximately 20% of the total amount of bone, whereas compact bone represents the other 80%. Spongy bone is very porous and light and has a high concentration of blood vessels (which are

responsible for the transportation of nutrients, oxygen and body fluids). Compact bone, on the other hand, has a very dense structure and is less porous, not being able to host a large amount of blood vessels. The compact bone functions include supporting bone mechanically in tension, compression and torsion. Spongy bone functions mainly in compression.[8]

At the microstructural level, compact and spongy bone have different structural units. In compact bone, osteons or harvesian systems repeat themselves working as weight-bearing pillars, while spongy bone contains an interconnecting framework or trabeculae.[8] Each osteons consists of concentric layers, or lamellae, that surround a central canal, the haversian canal, that contains nerve and blood supplies. As the body ages, the number of osteons increase, resulting in a tissue completely filled with osteons, which are in direct contact with each other. When bone is compressed, osteons present a unique crack pattern, consisting of stacks of ark-shaped circumferential microcracks that propagate radially from the center of each osteon.[8] As the compression level rises, the radial cracks that exist in the neighboring osteons connect, forming a network of cracks throughout the tissue that redistributes the stress and preventing the catastrophic failure, despite the large amount of energy applied. Furthermore, this unique type of microcracking allows the material to maintain its high strength and resilience even in the inelastic regime of deformation. This example illustrates how the hierarchical organization of bone (in this case in the microstructural level) determines its unique mechanical resilience.[8]

The lamellae are made of one or more parallel fibrillar arrays varying in thickness from a few hundred nanometer to a few microns. The thicknesses and the main fibril direction in neighboring lamellae can vary leading to the formation of unique patterns that are often species specific and can even vary among different bone types in the same species. The spaces between the fibrils contain extrafibrillar mineral crystallites and noncollagenous molecules, such as glycoproteins and glycosaminoglycans that provide a viscoelastic medium isolating the collagen fibrils. In addition, this non-collagenous proteins have other important function: they form a supramolecular network based on the ionic cross-links between their acidic side chains and the calcium ions in the solution as well as the interactions between these acidic moieties and calcium ions on the surface

of mineral crystal. This ions based, reversible and sacrificial networks dissipate large amounts of energy under mechanical stress and reforms quickly when the load is off.[8,23,25] In the hierarchical structure, the mesostructural level is of particular importance because although its basic components are in the nanoscopic level, the chains that they form together have specific mechanical functions when joined. This can be considered as another good example of the benefits of bone's hierarchical organization.

Finally, at the nanostructured level, the two major components (collagen fibers and nanocrystals of HA) are found (Figure 3) in higher proportions among other minor components such as non-collagenous proteins, molecules of water and lipids. The presence of water and lipids is essential for cellular functions: water plays and important role in fluid movement within bone, transports nutrients and waste products and also calcium and cytokine factors, and is an important determinant of the biomechanical behavior of bone while lipids play an important role in regulating the biomineralization process.[2]

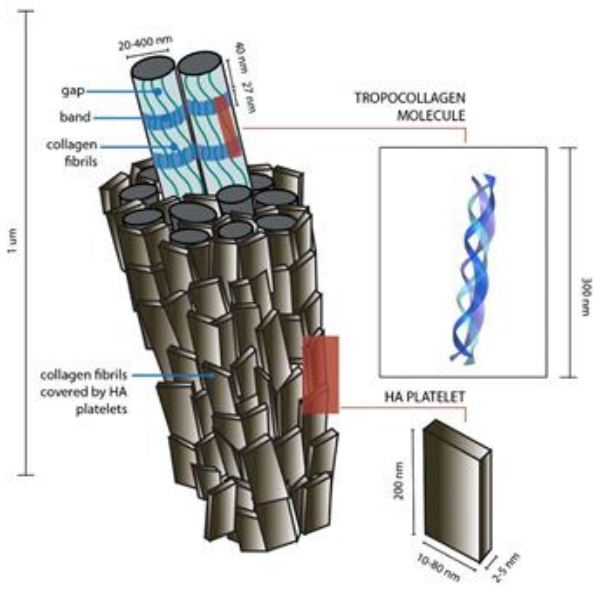


Figure 3

The nanoscopic detail of a fibril [26]

Structurally, a collagen framework embeds plate-like tiny crystals of HA. This conformation is essential because the mechanical properties that are characteristic of the bone tissue exist do to the isolated and combined properties of its nano components. This means that, in the end, all boils down to high level of integration of the mineral and organic phases at atomic and molecular levels.[7] A badly formed collagen has compromising effects on the structural and mechanical characteristic of bone and an example of this is *Osteogenis Imperfecta.* In this disease, a mutation in the genes that are responsible for the production of collagen type I can lead to an abnormal triple helix structure, compromising the whole bone structure.[27] Moreover, Currey *et al.,* [28] preconized that one of the reasons why ionized radiation decreases the bending strength and the work to fracture of bone structures, is due to the radiation specific damaging of the collagen matrix in an irreversible way.

Nonstoichiometric carbonated hydroxyapatite is the specific type of HA found in bone tissues. The fact that it presents itself in plate-like crystallites with very small average dimensions is the reason for the unique mechanical behavior of its structure. In fact, Gao *et al*.[29] demonstrated that crystals this small are extremely tolerant to flaws and that this hydroxyapatite specific crystals have a strength of a perfect crystal. Other favorable characteristic is the high surface and bulk ratio of these crystallites that increase their chemical reactivity and consequently the interactions with the organic molecular components.[30]

2. Bone Grafts

Bone grafting is a surgical procedure which entails replacement or reconstruction of missing or damaged bone with material from either patient's own body, an artificial or natural substitute.[31] In order to accomplish its goal, bone grafts should be osteoconductive, osteoinductive and osteogenetic:[32] (A) Osteoconduction is a term that means that bone grows on a surface. Therefore, an osteoconductive surface is the one that has ideal characteristics to permit bone growth, by stimulating the attachment, survival, migration and distribution of cells that are involved in bone formation such as mesenchymal cells, osteoblasts, osteoclasts, and vasculature; (B) Osteoinduction refers to the

process in which primitive, undifferentiated and pluripotent cells are somehow stimulated to develop into a bone forming cell lineage. It can be considered that osteoinduction is the process by which osteogenesis is induced; (C) Osteogenesis is the formation of bone in general. It can be produced by cells of the host or by living cells included in the graft.[33,34]

2.1. Classification of Bone Grafts

There are several categories of bone grafts which encompass a variety of materials, material sources, and origins.[33] In order to present a more complete classification, one will be used, adapted from Laurencin *et al.*.[35]

a) Harvested bone grafts

Autografts

Allografts constitute 58% of the bone substitutes and are typically tissues harvested from the patient's own bone tissue such as the iliac crest.[2] They are considered the gold standard for bone repair, because they offer minimum immunological rejection, complete histocompatibility and provide the best osteoconductive, osteogenic and osteoinductive properties. Autografts usually contain viable osteogenic cells, bone matrix proteins and support bone growth which are obtained from vascularized and non-vascularized cortical and autologous bone marrow grafts. They offer structural support to implanted devices and ultimately become mechanically efficient structures as they are incorporated into surrounding bone through creeping substitution.[33] However, they are limited in availability and often associated with donor-site morbidity and increased operative blood loss particularly when a large graft is required.[36]

Allografts

Allografts are tissues obtained from banked freeze-dried bones of human cadavers and represent about ≈34% of the bone substitutes. They are

osteoconductive and have fewer limitations on supply.[35] However, allografts are usually not osteoinductive or osteogenic and are associated with risks of immunological reaction or disease transmission. Furthermore they possess insufficient mechanical properties for load-bearing bone applications.[2]

Xenografts

Xenografts are harvested from one individual and transplanted into another individual of a different species. The common available xenografts are derived from coral, porcine, and bovine sources.[37] Xenogenous bone grafts are a theoretically unlimited supply of available material if they could be processed to be safe for transplantation in humans. A major concern with bovine-derived products is the potential transmission of zoonotic diseases and prion infections such as bovine spongiform encephalitis (BSE). Xenografts, similar to allograft, lose their osteogenic and partly osteoinductive properties during the processing to counteract their antigenic properties and prevent transmission of infection.[38] Because xenografts produce poor clinical outcome, new insights in this same field have been investigated. For example, Keskin *et al.,*[39] evaluated the effectiveness of autologous bone marrow on the healing of bone defects filled with bovine-derived xenografts in rabbits. They concluded that when xenografts were combined with autogenous red bone marrow, the drawbacks of xenografts are slightly compensated, but even with this enhancement of biocompatibility, their origin and properties continue to arise unsolvable problems.

b) Bone Graft Substitutes

While harvested bone grafts use pre-existing bone, bone graft substitutes are biomaterials made of a variety of sources and using different strategies to promote bone growth in the host.[35]

Growth factor-based bone graft substitutes

Growth factors are secreted by a wide range of cell types to transmit signals that activate specific developmental programs, controlling cell migration, differentiation and proliferation.[40] Therefore, integrating them in a biomaterial with regeneration purposes is a great strategy for enhancing its biological properties, simulating host cells to adhere to the material and proliferate. Polymeric systems can be successfully used to administer small doses of factors at defined dose rates directly to target cells. In fact, biodegradable polymers are able to provide a controlled release of growth factor delivery through its degradation rate. In the bone regeneration field, osteoinductive growth factors have been reported.[41,42] Table 3 specifies these factors, as well as its origin and specific function.

Table 3 - Influence of growth factors on graft incorporation and bone healing.[43]

Growth factor	Cell Origin	Function
Tumor necrosis factor	Macrophages	Increases bone resorption
Fibroblast growth factor	Inflammatory cells, osteoblasts and chondrocytes	Increases cell replication and collagen formation and has angiogenic purposes
Platelet-derived growth factor	Platelets, monocytes, endothelial cells	Increases cellular proliferation and collagen formation
Insulin-like growth factor	Osteoblasts, chondrocytes	Stimulates chondrocyte formation
Transforming growth factor β	Platelets, osteoblasts and chondrocytes	Increases proteoglycan synthesis and decreases collagen synthesis
Bone morphogenic proteins -2, -4, -7.	Mesenchymal stem cells, osteoblasts	Induces progenitor cells to become bone-forming cells.

Among this group of growth factors, BMPs are a group of high interest. They are members of the β-transforming growth factors (β-TGF) superfamily and have been identified as having different degrees of cellular activity, including cartilage or bone inducing properties. Lee *et al.,* [41] demonstrated that the use of BMP-2 enhanced significantly bone regeneration in a critical size femoral defect rat model in amounts that are one order of magnitude lower than that required for healing in this animal. The presence of more mature bone in the new ossified tissue was noted when a low dose of BMP-2 was delivered using a biomimetic supramolecular system. In human patients, the failure to naturally regenerate bone seems to be related to the genetic changes in the BMP signaling system.[44] The US Food and Drug Administration (FDA) has approved two recombinant proteins that are presently commercially available: recombinant human bone morphogenic proteins rhBMP-2 and rhBMP-7.[31] Clinical orthopedic studies have shown the benefits of these recombinant human BMPs but side effects such as swelling, seroma, and increased cancer risk, have been reported, probably due to high BMP dosage.[42]

Cell-based bone graft substitutes

These bone grafts combine living cells with biomaterial scaffolds *ex vivo* to allow the development of a three-dimensional structure. Autologous cells may be used in this approach via the isolation of a small number of differentiated adult cells or stem cells, followed by in vitro expansion to produce the material that will be reimplanted in the host posteriorly.[40] Mesenchymal stem cells (MSC) are capable of continuously replicate themselves, while a portion becomes committed to mesenchymal cell lineages such as bone, cartilage, tendon, ligament, and muscle, depending on the outer stimuli that they receive. They are considered to be a great tool for regenerative cell therapy and have been used since the 1980s, when Friedenstein *et al.*[45] first reported their effectiveness on developing engineered tissues.

Wong *et al.,*[46] in a clinical trial performed with injectable cultured bone marrow-derived mesenchymal stem cells was able to regenerate cartilaginous and osseous tissues in patients that suffered a high tibial osteotomy procedure.

Though this study, the authors proved the effectiveness of the mesenchymal cell based treatments.

Osteocel ® Plus, a commercially available example of cell-based bone graft is produced by NuVasive ® since 2005. It consists in an allograft cellular bone matrix containing mesenchymal stem cells (MSCs) and osteoprogenitor cells extracted from adult donor tissues, combined with Demineralized bone matrix (DBM) and cancellous bone.

Ceramic-based bone graft substitutes

These bone grafts have in its constitution ceramic based materials, such as calcium phosphate, calcium sulfate, and bioglass used alone or in combination. Bioceramics can be divided in 4 categories: they can be silicate-based, as are wollastonite ($CaSiO_3$), bioglasses and diopside ($Ca_2MgSi_2O_6$), phosphate-based, as are hydroxyapatite and β-TCP, carbonate-based, as are the coral-ceramic ($CaCO_3$) and finally sulfate-based, as is calcium-sulfate ($CaSO_4$). The major component of these ceramics determines its biological behavior towards natural bone.

OsteoGraf ® (DENTSPLY ®) and Osteoset ® (Wright Medical Technology ®) are two commercial examples of these materials. The first, Osteograft ®, uses hydroxyapatite as bone graft material in either a block or a particulate form, whereas Osteoset ® is a calcium sulfate tablet used for bone defect sites.

Polymer-based bone graft substitutes

These are bone grafts that use natural or synthetic, degradable or non-degradable polymers alone or in combination with other materials. Some commercially available examples include CORTOSS ® (Orthovita ®), which is a self-setting glass ceramic polymeric composite engineered specifically to mimic the characteristics of human bone and to provide fixation for vertebral compression fractures, among others.

2.2. Essential properties of bone grafts

The biological process leading to graft incorporation is very similar to that of fracture repair.[3] Bauer *et al.* [47] summarized graft incorporation into five major steps:

I. Hematoma formation, release of bone inducing factors and cellular recruitment;

II. Inflammation and development of fibrovascular tissue, connecting the graft to the adjacent bone

III. Vascular invasion of the graft;

IV. Focal resorption of the graft by recruited osteoclasts;

V. New bone formation, union between the graft and the surrounding bone, and graft remodeling.

In order to accomplish the incorporation stages properly, the bone graft has to have some basic characteristics. The primary requirements of any implant material or device are related to their biocompatibility aspects and *in vivo* function[3,31,32,35,47]. First of all, bone grafts should be biocompatible so that they can integrate with the host tissue without any harmful immune response.[48] In order to enhance this property, modified implant surfaces may regulate the host response, attachment of cells and their functions.[49] Furthermore, bone regeneration strategies should be biodegradable with non-toxic degradation products that can be metabolized and excreted by the body, and with controllable degradation kinetics to match the rate of bone healing process so that the newly formed tissue compensates the mechanical and mass loss of the degraded matrices. Products that may result from corrosion, resorption, hydrolysis and enzymatic reactions, may direct local and systemic immune responses, affecting significantly the implant-host integration.[50]

A complete regeneration and functional restoration may be achieved when the bone graft is well integrated with the host, remodeled and replaced with native bone tissue. To ensure this process, the material should have a porous structure to enable the transport of oxygen and nutrients.[51] Porosity is the percentage of void space within a solid object.[33,51] Although it is known that the ideal pore size for a synthetic bone graft, should be similar to that of spongy bone,[33] there is not

a consensus among researchers about its ideal size. It has been demonstrated that microporosity (pore size <10 μm) allows body fluid circulation whereas macroporosity (pore size >50 μm) provides scaffold (pore size-100-200 μm and porosity-60-65%) for bone-cell colonization.[52] For instances, Gauthier *et al*.[53] reported that the ideal macropore size for bone ingrowth is a pore size diameter of ~600 μm, because it provides better biological responses when compared to a smaller size (~300 μm) whereas Kuhne *et al*.[54] demonstrated in an *in vivo* study performed in rabbits that the optimal size of the pores in a coralline hydroxyapatite bone substitute is ~500 μm. Besides porosity, surface roughness is also very important, since it promotes cellular adhesion, proliferation, and differentiation of anchorage-dependent cells.[51]

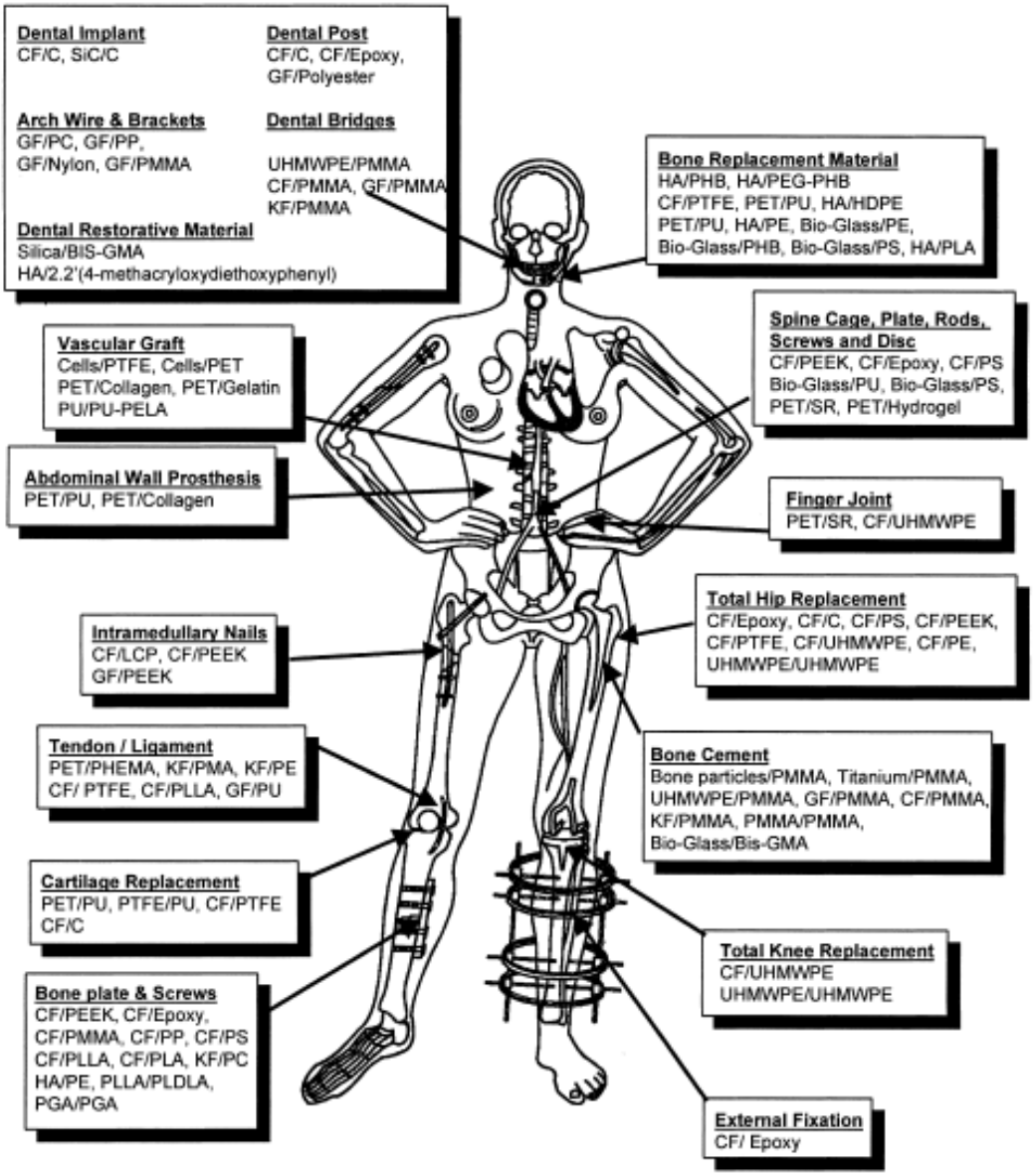
A bone graft substitute should have adequate mechanical properties to support the native forces usually experienced under loading.[19] This is most critical to protect the tissues and transmit the compressive and tensile force and mechanical cues across the defect to the regenerative cells. The degradation profile of the implanted graft should allow the mechanical load to be supported and gradually transferred to the newly forming tissue within the implant.[55]

To enhance the cell infiltration and consequently the regeneration of new bone tissue, osteoconductive materials such as hydroxyapatite, collagen, autogenic and allogenic bone can be used.[1] Other effective solution can be the use of osteoinductive materials, such as demineralized bone matrix (DBM). Kinard *et al.* [56] stands that DBM promotes formation of new bone when applied to defect sites. That has to do with the fact that DBM possesses in its constitution BMPs, which, as already referred, are proteins with a huge osteoinductive capability. By using DBM in a biodegradable hydrogel, the authors were able to promote bone augmentation in rats, correlating directly the DBM dose with the amount of new-formed bone. Other osteoinductive responses may be produced by several growth factors, as referred before, always concerning the best host-response to the graft.[43]

3. Matching bone through nanocomposites

In order to replace the classical solutions to bone replacement, several approaches have been studied throughout the years. Therefore, and regarding the nanostructure of bone with its organic (collagen) and inorganic (HA) phase, research has been conducted to develop therapeutic materials with the same hybrid structure that combine the strength, stiffness and osteoconductivity of an inorganic component with the flexibility, toughness and resorbability of an organic phase. In this context, researchers have developed polymer-ceramic nanocomposites which have the advantages of both polymers (structural stability, strength, biocompatibility and desired shape) and ceramics (bioactivity and osteoconductivity), and are more close to natural bone.[1,57]

Polymers have been used (combined or individually) in different applications (Figure 4).[58] They can be divided into two groups regarding their source: natural and synthetic. Cellulose, collagen, agarose, chitin or hyaluronan form the members of natural polymeric materials or so-called biological polymers.[31] Natural polymers such as collagen have been used for bone tissue engineering purposes. In contrast to natural polymers, synthetic polymers are also used in the bone engineering field. Some examples of these polymers are poly-lactic acid (PLA), poly-glycolic acid (PGA), polyurethane (PU) and polycaprolactone (PCL), from which PLA and PLGA have receives the highest interest because of their biological properties and easy processability.[58]



Legenda

CF: Carbon Fibers; **C:** Carbon; GF: Glass Fibers; **KF:** Kevelar Fibers; **PMMA:** Polymethylmethacrylate; **PS:** Polysuifone; **PP:** Polypropylene; **UHMWPE:** Ultra-high-molecular weight polyethylene; **PLDLA:** poly (L-DL- Lactide); **PGA:** Poliglycolic acid; **PC:** Polycarbonate; **PEEK:** Polyethereutherketone; **HA:** Hydroxyapatite; **PMA:** polymethacrylate; **BIS-GMA:** Bis-phenol A glycidyl methacrylate; **PU:** polyurethane; **PTFE:** Polytetrafluorethylene; **PET:** Polyetylenetephthale; **PEA:** Politethylacrylate; **SR:** Silicone rubber; **PHB:** Polyhydroxybutyrate; **PEG:** Polyethyleneglycol; **PHEMA:** Poly (20hydroxyethyl methacrylate)

Figure 4 - Various applications of composite - polymers throughout the body [58]

Regarding their response when applied to living tissues, polymers can be biodegradable or non-biodegradable. Biodegradable synthetic polymers, like natural polymers, are resorbed by the body. To have the implant being resorbed by the body while the replacing tissue is regenerating is highly advantageous.

These polymers do not require a second surgical event for its removal once it is no longer needed. This last characteristic can be a huge advantage in the bone field, because the bone can develop a phenomenon called “stress shielding”: when a bone fractures and it is fixed with a rigid, non-biodegradable stainless steel implant, it may not heal properly and can eventually re-fracture upon removal of the implant.[59] Bone does not carry sufficient load during the healing process, since this is carried mostly by the rigid stainless steel.[59] However, an implant prepared from a biodegradable polymer can be engineered to degrade at a rate that will allow for the slow transfer of the load to the healing bone.[60] Poly(glycolic acid), poly(lactic acid) (PLA) and their copolymer poly(lactide-co-glycolide) (PLGA), polydioxanone, poly (ethylene oxide) and poly(trimethylene carbonate) are examples of polymers that have been used for this purpose. In addition, poly(ε-caprolactone) (PCL), polyanhydrides, poly(vinyl alcohol) (PVA), polyurethanes and recently polyhydroxykanoates (PHA) which are linear polyesters of microbiological origin, have also been investigated for bone regeneration.[31,57]

At the same time, non-biodegradable polymers have been used in bone tissue engineering due to their better mechanical properties and chemical stability than biodegradable polymers.[57] In the bone field, these materials are mainly used when the tissue cannot be regenerated due to large losses or for elderly patients with a less effective self-healing ability of the tissue. Currently, synthetic nonbiodegradable polymers used are for instances: polyethylene, polypropylene, polytetrafluoroethylene, poly (vinyl chloride), polyamide (PA), poly (methyl methacrylate), polycarbonate, poly (ethylene terephtalate), poly (ether ketone), acrylics and silicones.[57]

Ceramics such as calcium phosphates and calcium sulfates are clinically used as implant coatings or bone-void fillers because of their attractive biodegradable, bioactive and osteoconductive properties, and also because they have a notable ability to bond directly to bone.[57] Calcium phosphates appear in several different forms, being hydroxyapatite ($Ca_{10}(PO_4)_6OH_2$) and tricalcium phosphate ($Ca_3(PO_4)_2$) the most currently used.[1] Hydroxyapatite (HA) is a naturally occurring calcium phosphate that comprises up to 70% of the dry weight of bone. The synthetic form of HA is osteoconductive and can have a crystalline

structure similar to the HA in bone. HA synthesized at high temperatures is highly stable and slower to resorb than its endogenous form and may stay at the site of implantation for many years. Tricalcium phosphate (TCP) is a bioceramic that exists in alpha (α-TCP) and beta cristal (β-TCP) forms.[61] α-TCP is the high-temperature and β-TCP is the low-temperature polymorph of TCP.[62]

β-Tricalcium phosphate (β-TCP) has been successfully used in posterolateral spinal fusions, dental procedures and as a component of bio-resorbable screws.[63] In fact, Ogose *et al.* [63] demonstrated that β-TCP appears to be advantageous in comparison to HA for surgery involving bone tumors and exibits superior osteoconductivity. Calcium sulphate ($CaSO_4$) is resorbed within 6 weeks – which is faster than both HA and TCP. It was already successfully used in tuberculosis. Calcium sulphate pellets have been used as autograft extender for instrumented short segment posterolateral spinal fusions for degenerative disease.[64] Although the biological characteristics are favorable for bone engineering purposes, the main drawbacks of these materials are their brittleness, low fracture strength, low mechanical reliability, lack of resilience and high density which makes them susceptible to catastrophic failure.

3.1. The structural organization of polymer-ceramic nanocomposites

Typically, the polymer-ceramic composites used for bone replacement and regeneration are systems in which a ceramic filler is dispersed within a polymer matrix. In nanocomposites, the dispersed filler material is at the nano dimension. One of the main reasons for using nanomaterials is the large surface to volume ratio, which increases the number of particle-matrix interactions, increasing the effects on the overall material properties.[65,66] The interphase formed around nanoparticles exhibits distinct properties from those of the bulk matrix.[67] It can be a region of altered chemistry, polymer chain mobility, degree of cure or crystallinity Therefore, by controlling the degree of interaction between the polymer and the nanofiller, the properties of the entire matrix can be controlled.[68]

Another important consideration is the dispersion of the filler,[65] since a well dispersed system yields more desirable composite properties. For instance,

particle agglomerates decrease the material performance by the inclusion of voids that act as preferential sites for crack initiation and failure.[65] Particles, especially in the nano range (less than 100 nm) tend to agglomerate, or cluster, due to the dominant intermolecular van der Waals interactions between them.[65] Therefore, when fabricating a polymer-nanocomposite scaffold, the technique to disperse nanoparticles into the polymer matrix is important, because it provides a good mechanical behaviour *in vivo*.

In order to simplify the discussion about nanofillers, Ajayan *et al.*[68] grouped nanofillers into three categories, according to their geometrical shape: fiber-like, plate-like and three-dimensional (Figure 5). This is a convenient way to discuss polymer-based nanocomposites, because the processing methods used and the properties achieved depend strongly on the geometry of the fillers.

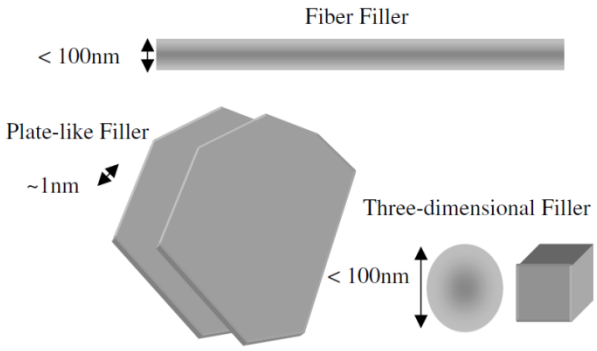


Figure 5

The geometry of nanoscaled fillers [68]

a) Nanosphered and nanopatirculated composites

Nanoshperes or nanoparticles can be dispersed throughout a continuous matrix to induce porosity,[69] to improve mechanical properties of a bulk scaffold as a reinforcement phase[70] or a crosslinking agent[71] and as a drug delivery vehicle.[72] They can also be used as building blocks to establish scaffolds by a bottom-up approach without a surrounding matrix material. Particulate building blocks can also be used to prepare 3D stand-alone scaffolds without surrounding material through rapid prototyping,[73] random packing[74] or directed assembly.[75]

Rapid prototyping uses a computer-aided design to produce 3D constructs with customizable architecture using nanospheres as building blocks.[73]

Nanopaticulated composites can be produced in a random packing technic or in a direct assembly. In a random packing technic, composite gels are putted up together in a structurally random way, which leads to poor integrity of these gel-based scaffolds resulting from weak interparticle interactions and consequently poor mechanical stability.[74] To preserve the agglomeration of nanosphere formulation after implementing these scaffolds, maintaining the particles together, crosslinkers and compatibilization agents have been investigated.[75] On the other hand, directing the assembly by introducing interparticle forces (such as electrostatic forces or hydrophobic interactions), can produce formulations with enhanced structural integrity and mechanical stability without the use of compatibilization agents or crosslinkers.[75] Therefore, this technique allows for a level of structural organization that ensures good mechanical properties. Clinically, these techniques have been used to produce injectable gels which act as scaffolds for bone regeneration in minimally invasive surgery.[76]

When dispersing nanoparticles in a polymeric matrix, two main alternatives can be considered: they can be dispersed in the polymeric matrix during the polymer synthesis or after the polymer synthesis being complete.[65] By blending the nanoparticles in pre-synthesized polymer matrixes, it is possible to have a full control over both the nanoparticles and the matrix. The preparation of most of the nanoparticles and nanomaterials can be divided into four main methods.[65]

I. The wet method, in which nanoparticles are prepared in a medium using a sol-gel technique, emulsion approaches or the intercalation polymerization;

II. The dry method, that includes preparing of nanoparticles using methods such as the abrasive, burning, gas impinging and collision approaches, high-energy ball milling processes;

III. The evaporating method, where nanoparticles are prepared using chemical vapor deposition or the gas deposition approach, like laser gas vapor deposition.

IV. The sedimentation method, for special nanocomposites that possess heavy weight selection particles.

In ceramic-polymer nanocomposite processing, wet and dry methods are used very often, being the sol-gel technique one of the most used. It consists in a series of hydrolysis and condensation reactions that transform a solution, which has colloidal particles dispersed in it, into a gel, where both liquid and solid are dispersed in each other, through low temperature methods.

b) Nanofiber composites

An important class of nanostructured biomaterials on which intensive research has been carried out is nano-fibrous materials, especially biodegradable polymer nanofibers. In this specific material, nanofibers are dispersed in a biodegradable polymer matrix. The large surface-area-to-volume ratio of nanofibers combined with their porous structures favours cell adhesion, proliferation, migration, and differentiation, as it was already discussed. There are three main techniques to produce nanofibers: phase separation, self-assembly and electrospinning.[77] Nanofiber mats can be morphologically similar to extracellular matrix (ECM) of the natural tissue, which is characterized by a wide range of pore diameter distribution, high porosity, effective mechanical properties, and specific biochemical properties.[78]

Phase separation, for instance, is a technique often used to produce nanofibrous scaffolds.[77] This process is based on thermodynamic demixing of a homogenous polymer solvent solution into a polymer-rich phase and a polymer-poor phase. Cooling the solution to a specific point causes this separation. However, usually there is lacking of interconnected macropores, so this technique is often combined with other scaffold fabrication techniques (e.g. particle leaching) to allow broader control over the scaffold architecture.[79,80] With this technique, nanofibers with a range of diameters of 50 to 500 nm are produced. Ma *et al.*[81] have used this technique to produce a PLLA fibrous structure from a PLLA/THF solution to be used in regeneration scaffolding materials that provide a better environment for cell attachment and function.

Self-assembly is the most complex technique and it allows the creation of nanofibers with very small diameters (a few to 100 nm). It basically consists on an autonomous organization of components that are able to assemble at the

molecular level.[77,82] Being a natural process for several essential biological components including nucleic acid or protein synthesis, self-assembly technology usually incorporates some specific biological components of the extracellular matrix (ECM), closely mimicking the ECM assembly process.[80] In this process, molecules require some specific configurations to be assembled into nanofibers. Molecules that meet this requirements are peptide-amphiphiles (PAs),[80] oligopeptides, synthetic diblock/triblock copolymers[83] and dendrimers.[84] Both self-assembly and phase separation have the disadvantage to create only short strands of nanofiber.[80]

Another example is electrospinning, which in contrast, represents the most reliable method to simply fabricate long continuous strands of nanofibers with a diameter ranging from nanometers to microns (50–1,000 nm). Besides being simple, it is relatively quick and cost-effective.[85] This process involves subjecting a polymeric solution to a high electric field, which overcomes the surface tension of the solution and drives the ejection of a polymer jet. The charged polymer solution or melt is ejected, dried and solidified onto a grounded substrate. The ejected polymer solutions repel each other during the travel to the grounded collector, which forms thin fibers after solvent evaporation. By controlling the spinning conditions, the resulting fibers can range from about 0.02 μm to about 20 μm.[86] Many electrospun nanofibrous biomaterials have been investigated as tissue regeneration scaffolds.[78,86] Materials used in electrospinning can be natural macromolecules such as collagen, chitosan, silk fibroin; synthetic biodegradable polymers such as PGA, PLGA, PLLA, PCL; and combinations of these natural and synthetic polymers. In addition, various substances (proteins, growth factors, and hydroxyapatite) can be incorporated into nanofibrous materials during electrospinning. Therefore, electrospun nanofibrous biomaterials have been explored to engineer various tissues. However, significant challenges still exist in using this technique to fabricate complex 3D scaffold shapes or to generate designed internal pore structures, limiting its potential for many tissue engineering applications.[80]

II. Material and Methods

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Search Strategies

In order to perform a bibliographic review as complete as possible, data was extracted and selected from scientifically relevant databases, in this case ScienceDirect and PubMed.

In a first stage, the following combination of words was used in both online data-bases: “Bone AND Regeneration AND Nanocomposite AND Polymer AND maxillofacial AND NOT review”. In the PubMed database, 5 results were found and in ScienceDirect, 33. It was then realized that this combination of words restricted too much the online search too much, so other MeSH terms were selected and integrated in the combination. The word “cranial” was selected (replacing the word “maxillofacial”), to provide articles about bone regenerative measures using nanocomposites among the whole cranial complex and not just the maxillofacial area. The MeSH term “augmentation” was added as well, in order to include articles about the use of regenerative techniques in alveolar bone augmentation. The different MeSH words and combinations, as well as the obtained results are included in results diagram (Annex 1) and can be summarized as follows:

- “Bone AND Maxillofacial AND Regeneration AND nano composite AND polymer NOT review”, with the particularity of having the nanocomposite written separately (nano composite), obtained 5 results in PubMed and 73 in ScienceDirect;
- “Bone AND Cranial AND Regeneration AND nanocomposite AND Polymer NOT review”, in which the word “Maxillofacial” was replaced by the word “cranial”, obtained 9 results in PubMed and 27 in Science Direct;
- “Bone AND cranial AND regeneration AND nano composite AND Polymer NOT review”, the same search performed before, with nanocomposite written separately (nano composite), obtained 13 results in PubMed and 60 in ScienceDirect;

- "Bone AND maxillofacial AND augmentation AND nanocomposite AND polymer NOT review", in which the word regeneration was replaced with the word "augmentation", obtained 0 results in PubMed and 6 in ScienceDirect;
- "Bone AND maxillofacial AND augmentation AND nano composite AND polymer NOT review", the same search performed before, with the word nanocomposite written separately (nano composite), obtained 0 results in PubMed and 23 in ScienceDirect.

Moreover, relevant online databases providing information about clinical trials in progress (www.clinicaltrials.gov, www.centerwatch.com/clinicaltrials, www.clinicalconnection.com) were initially considered and checked, using the combination of words: "Bone AND Regeneration AND Nanocomposites". However, no results were found.

Study Selection

The total number of results from the all the different online searches is 255. From these, duplications were found and excluded as well as studies that were published before 2009. 150 articles remained for individual selection based on the inclusion and exclusion criteria. Titles and abstracts of all remaining reports were read to make sure that the exclusion criteria were applied properly.

Inclusion and Exclusion Criteria

Eligibility criteria included *in vitro* studies that evaluated the biocompatible and stimulating capacity of materials composed of nano-ceramic particles dispersed in a polymeric matrix with bone-related cells, *in vivo* studies that comprehended the investigation of the mechanical, structural and bioactive behavior of polymer-ceramic nanocomposites, clinical trials performed to evaluate the overall performance of nanocomposites in humans, specifically in the craniomaxillofacial region and pshysicochemical studies of novel regeneration systems including polymers and ceramic nano-particles.

The exclusion criteria were: case reports and case studies, book chapters, conference proceedings, papers written in other language that not English, articles that are published in open access magazines, *in vivo* studies with a small amount of samples (less than 5), *in vitro* studies using cells that are not bone-related, studies that use micro-sized composites and finally articles that state about nanocomposites without a both ceramic and polymeric constitution.

Data extraction and meta-analysis

From the studies included in the final analysis, the following data was extracted: year of publication, type of study (*in vitro* or *in vivo,* since no relevant clinical trials were found) and country of origin of the research.

In a second stage, the *in vitro* and *in vivo* studies were separated and different information was analyzed within each group. In the *in vitro* group, the selected information was: polymeric matrix, nano-filler, nanocomposite specifications, type of cell used for the investigation, type of in vitro evaluation and final outcome. In the *in vivo* group, the selected information was: polymeric matrix, nano-filler, nanocomposite specifications, type of animal used to perform the study and its characteristics (age and weight), site of implementation of the evaluated material, observation period, method of *in vivo* evaluation and outcome.

III. Results

III. Results

In the present analysis 150 studies were considered for evaluation, as result of the performed searching process. Out of these, 30 met the inclusion criteria.

1. Year of publication

Figure 6 represents the amount of relevant papers published between 2009 and 2013.

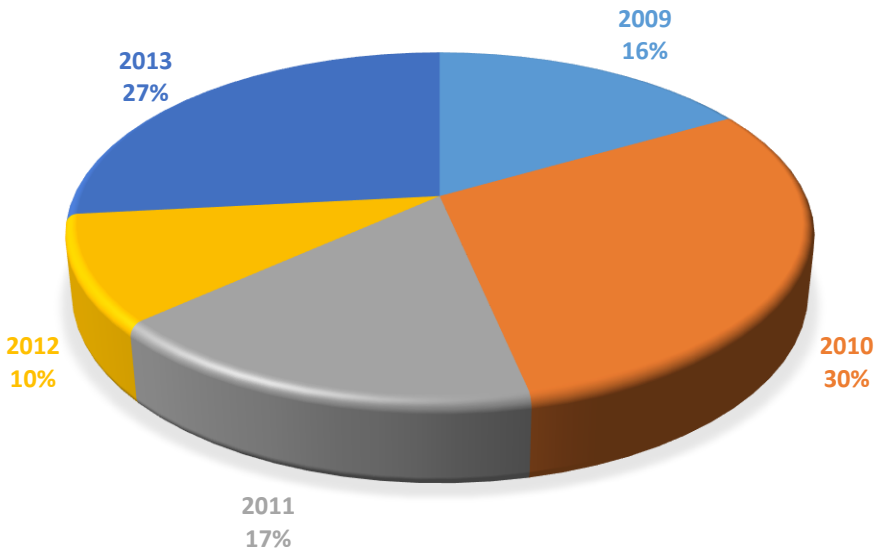

Figure 6

Relevant papers published *per year* between January of 2009 and 2014

It is noted that the majority of papers concerning the aim of this study, were published in 2010 [34,87-93] and 2013 [94-102].

2. Study design

Among the 30 articles considered for this thesis, three types were found: *in vivo* studies, *in vitro* studies and both *in vitro* and *in vivo* studies. In order to simplify, articles including both *in vivo* and *in vitro* studies were divided and the studies were considered separately.

Figure 7 represents the percentage of studies included for this thesis, in which 63 % are *in-vitro* studies, 37% are *in vivo* studies. The majority of studies are *in vitro* assays.

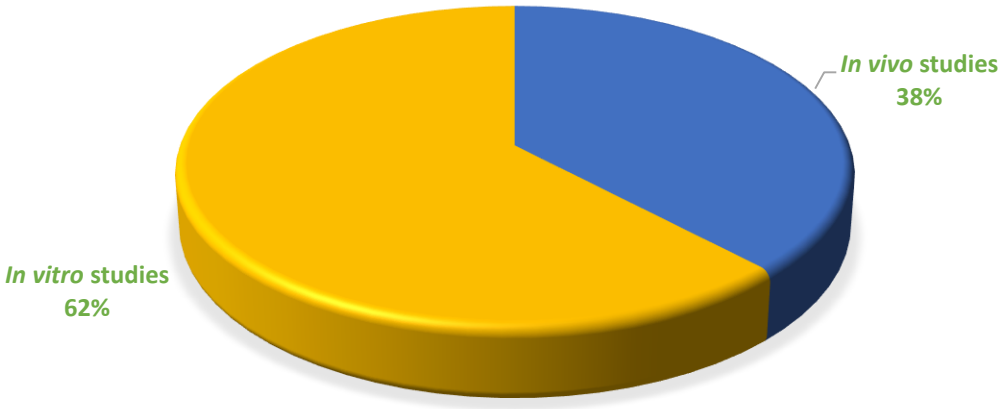

Figure 7

Amount of *in vitro* and *in vivo* studies.

3. Country of Origin

The analyzed articles are originated from different parts of the world. By extracting the country of origin of these articles, it was possible to conclude the ones that provide a higher contribution in the research of polymer-ceramic nanocomposites applied to bone regeneration. Figure 8 presents the distribution of articles according to their country of origin. In articles that resulted from a collaboration of different countries, the country of the corresponding author was de one taken in consideration.

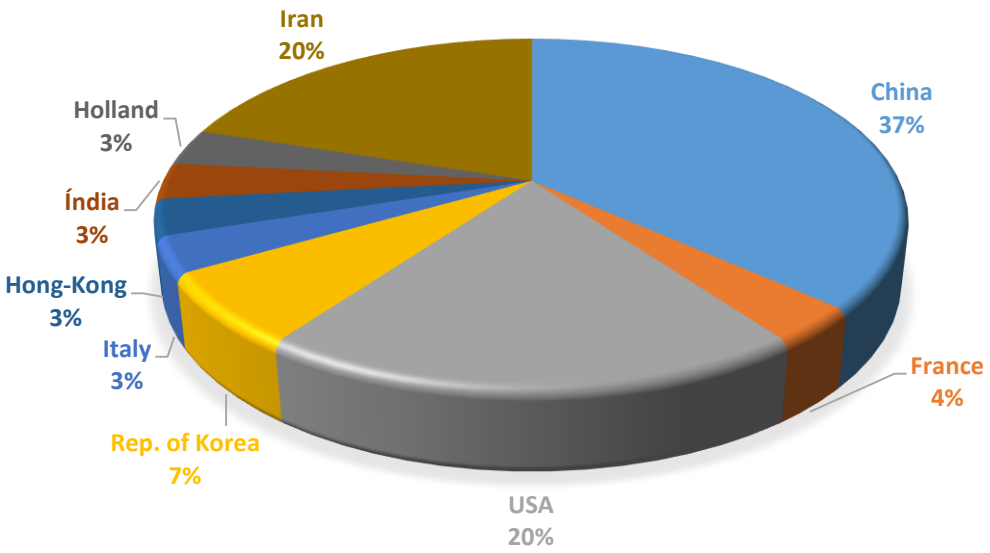

Figure 8

Different counties of origin of the authors of the included studies

After analyzing the above figure, it is possible to conclude that China, USA, Iran and Republic of Korea are the countries that published the highest amount of articles about this theme, since 2009 until now.

4. Publishing Journal

Figure 9 represents the journals that include the articles used for the development of this bibliographic review.

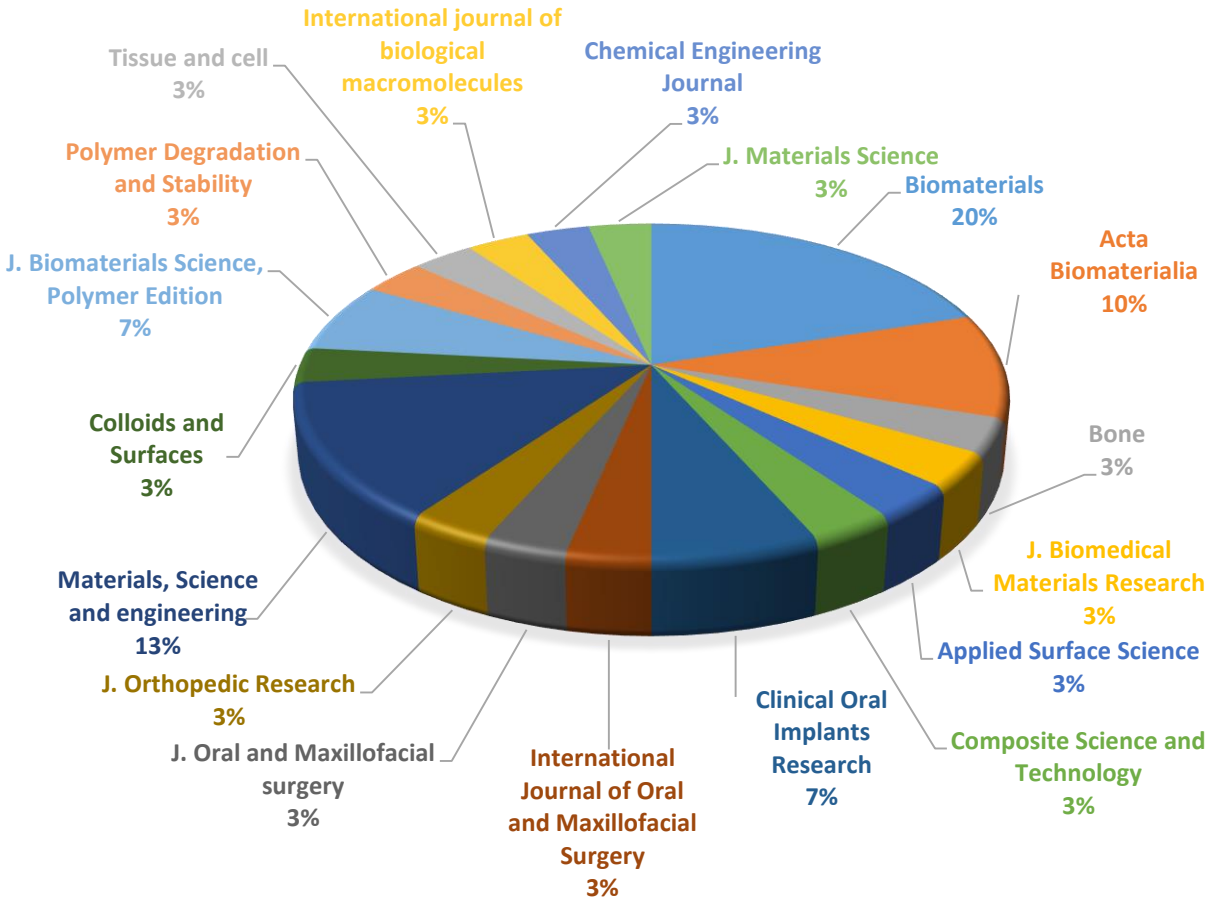

Figure 9

Journals with representation in the present thesis

Among them, 20% of the articles were extracted from *Biomaterials* (with a 5-year impact factor of 8,911), 13% from *Materials, Science and Engineering* (with a 5-year impact factor of 3,076) and 10% from *Acta Biomaterialia* (with a 5-year impact factor of 6,191). There are two journals that possess a representation of 7% in this dissertation: *Journal of Biomaterials Science*, *polymer edition* (with

an impact factor of 2,158) and *Clinical Oral Implant Research* (with an impact factor of 3,123). All the other journals have a representation below this percentages.

This journals can be grouped into three major categories: (a) journals that are more generalist in the biomaterial area, focusing on the publication of articles that study biomaterials characterization and implementation for the generalized medical area (eg. *Biomaterials, Acta Biomaterialia* and *Journal of Biomaterials Science*, *polymer edition*), (b) journals that publish articles regarding the use of biomaterials in the orthopedic area, which includes innovations concerning bone tissue, or specifically bone tissue investigation and (c) journals that publish specific innovations in the oral and maxillofacial area, including the research of novel materials developed specifically for this area (eg. *Clinical Oral Implant Research, International Journal of Oral and Maxillofacial Surgery, J. Oral and Maxillofacial surgery*). In order to understand which type of journal published the most articles in the area aimed in this review, a graphic was constructed (Figure 10).

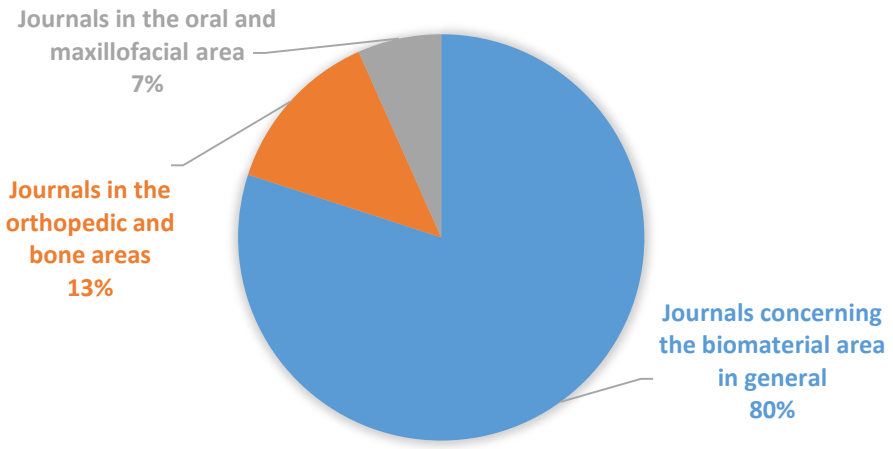

Figure 10

Types of journals publishing more articles about polymer-ceramic nanocomposites in the maxillofacial area

It is explicit that 80% of the journals that include the articles used in this dissertation belong to the biomaterials area. Just 13% are journals regarding the maxillofacial area.

5. Materials Selection

a) Polymers

As referred in the introductory section, polymers can be biodegradable or non-biodegradable and natural or synthetic. Furthermore, the origin of the used polymers was analyzed, in the attempt to establish a tendency of which polymers, natural or synthetic, are used the most in the polymer-ceramic nanocomposite field directed for bone regeneration, in the last 5 years. Figure 11 demonstrates the analysis performed.

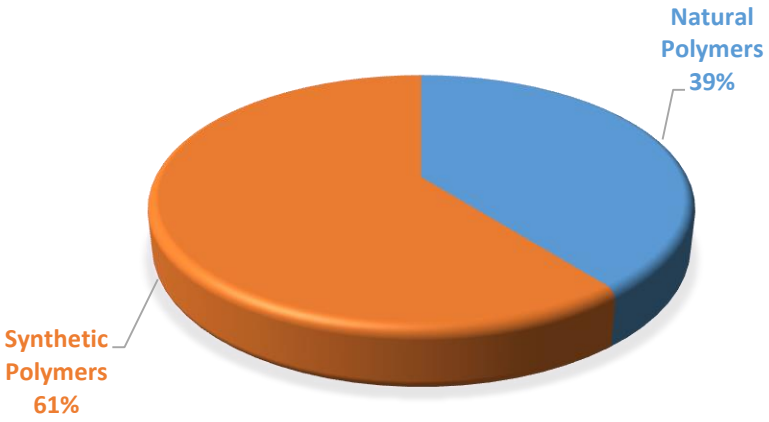

Figure 11

Origin of polymers used in the cosidered articles

It was concluded that among the polymers chosen by the authors of the 30 articles considered for evaluation, 61% have a synthetic origin, while 39% are

extracted from natural sources. The polymeric constitution of nanocomposites in the *in vivo* and *in vitro* assays, specifically, will be evaluated later in this review.

b) Nanofillers

In order to understand which nanofillers are used the most in the considered articles, Figure 12 was created. It is however important to refer that some of the articles considered include non-ceramic nanofillers as a second or third nanofiller, that joins forces with the ceramic component, with the purpose of attributing specific structural or biological properties. These components were considered as well.

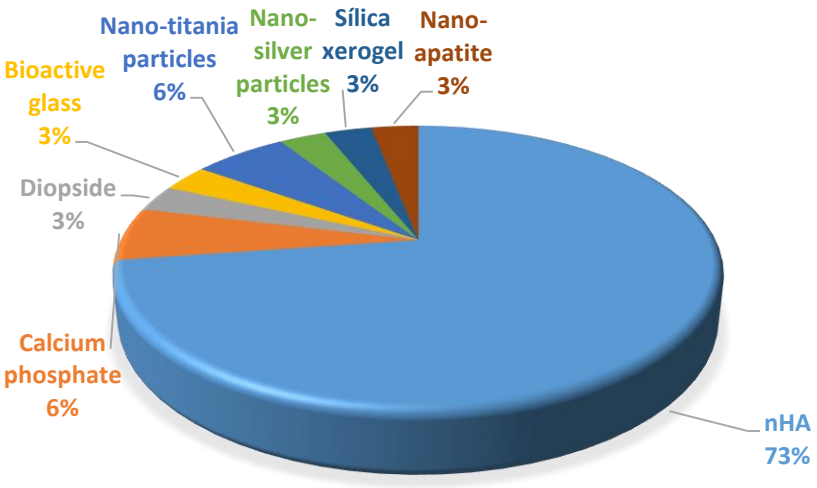

Figure 12

Nanofillers that constitute the nancomposites studied in the considered articles

It is evident in Figure 12, that the majority of the articles use nano-hydroxyapatite as the ceramic nanofiller of the investigated nanocomposites. Moreover, additional non-ceramic fillers (nano-titanium[92,103,104] and nano-silver[92,104] particles) constitute 9% of the nanofillers used.

The nanofillers chosen specifically in the *in vivo* and *in vitro* assays will be analyzed later in this review.

6. *In vivo* Studies

The *in vivo* studies discussed in this dissertation can be divided in two major groups: (a) The ones that aim to evaluate the *in vivo* biocompatibility of the scaffolds by implanting them in a non-osseous environment (sub-cutaneous or muscular) and evaluating the body's immunity response towards the scaffold and (b) the ones that aim to analyze the bone regenerative capacity of the scaffolds by implanting them in artificially created bone defects and evaluating their capacity of stimulating bone regeneration, by replacement of the scaffold for new-formed bone. Table 4 and 5 address the two distinct groups separately. They explore the different articles analyzing the following parameters: Composition and specifications of the used nanocomposites, animal characteristics and site of implantation, observation period, method of evaluation and outcome.

Table 4 – *In vivo* studies: osseous implantation

Ref.	Polymeric Matrix	Nano-filler	Nanocomposite specifications	Type of animal used and its characteristics	Site of implantation	Observation period	Method of *in vivo* evaluation	Outcome
Kweon *et al.*[105]	Silk Fibroin (SF)	nHA derived from egg-shells	nHa was extracted from egg shells that underwent a calcination procedure. It was then incorporated in the SF scaffold by dipping the scaffold in a nHA supersaturated solution.	Sixteen 4 month-old New Zealand white rabbits. Weight: 2,5 to 3,0Kg	8mm-diameter defects were created in each side of the cranial midline	4 and 8 weeks	Different scaffolds were implanted in the animals: pure nHA, pure SF and nHA/SF. The samples were fixed, sectioned and evaluated histomorphometrically.	The nHA was evenly distributed in the SF web shaped scaffold. The scaffold that presented a higher amount of bone formation was the pure nHA at 8 weeks. There was a low bone regeneration in the SF/nHA scaffold, attributed to the slow degradation of the SF.
Fu *et al.*[106]	Triblock PEG-PCL-PEG copolymer (PECE) and collagen	nHA	The PECE was freeze-dried and reduced to powder. This powder was mixed with nHA, and collagen and an injectable hydrogel composite was obtained, with 60% of PECE, 10% of collagen and 30% of nHA.	9 adult New Zealand White rabbits Weight: 2,5 to 3 Kg	Two 10 x 5 x 2 rectangular defects were created in both right and left parietal bones	4 and 20 weeks	The created bone defects were injected with the nanocomposite, and its bone regeneration capacity was assessed though radiographic and histological studies.	The filled bone defects showed a high-density tissue in the middle space at the 4th week, that grew until the defect was completely closed by new formed bone, at the 20th week.
Fricain *et al.*[95]	Pullulan/ Dextran polysaccha-ride	nHA	75/25 Pullan/Dextran macroporous scaffolds were synthesized. nHA was incorporated and the scaffold was freeze-dried.	4-year-old adult goats Weight: 70 ± 15 Kg	1 cm in diameter and 8mm depth mandibular defects	1 and 6 months	Defects were filled with Matrix + nHA or left empty. Histological evaluation was performed.	Mineralization of the bone defect started at 1 month and was clearly evidenced at 6 months. The mineralized tissue completely filled the critical size bone defect after 6 months of implantation.
Li *et al.*[107]	Collagen and poly (L-lactide acid) (PLLA)	nHA	3 different scaffolds were produces: nHA/PLLA scaffold alone, nHA/PLLA scaffold combined with rhBMP-2 and nHA/PLLA combined with P24, a synthetic BMP – 2-related peptide.	Thirty 8-week-old male Sprague-Dawley rats. Weight: 200 to 250g	5mm-diameter defects were created at the center of the right parietal bone.	6 and 12 weeks	The 3 different scaffolds were implanted. The samples were evaluated while the animals were alive, by radiographic examination. Histological evaluation was performed to the fixed harvested *calvariae* samples.	At 6 weeks, the rats implanted with scaffolds containing rhBMP or P24 showed new bone mineralization, while the ones implanted with nHA/PLLA scaffolds didn't exhibit bone formation at all. With 12 weeks, all scaffolds demonstrated bone formation, but the ones with groth factors showed a bigger amount of bone formation.

Table 4 – In vivo studies: osseous implantation (continued)

Ref.	Polymeric Matrix	Nano-filler	Nanocomposite specifications	Type of animal used and its characteristics	Site of implantation	Observation period	Method of *in vivo* evaluation	Outcome
Zhang *et al.*[91]	Polyamide (PA66)	nHA	nHA/ PA66 membranes were bought, as well as the e-PTFE membranes that were used as a comparison model.	36 male Sprague-Dawley albino rats, 12 weeks old. Weight: 208 to 320g	Two calvarial defects, 5mm in diameter were created in the bilateral sides in the midline	1, 4 and 8 weeks.	The rats were divided in 3 groups: bone defect covered with a nHA/PA66 membrane with a e-PTFE membrane and with a PA66 membrane. After the rats were killed, the harvested bone were analyzed and serological tests were performed.	In defects covered with nHA/PA66 and e-PTFE, the defects started with a small amount of bone-like particles in the first week. In the 4th, the density increased remarkably and at the 8th week, the bone defects were closed by new developed bone. The defect covered by a PA66 membrane did not show bone growth.
Liu *et al.*[100]	Chitosan	nHA	nHA/ Chitosan scaffolds were produced by a coprecipitation process.	25 female two-month-old Sprague-Dawley rats	Two full - thickness rectangular defects of 5 x 5 mm in the sagittal suture (cranial defects)	10 and 20 weeks	One of the defects was implanted with nHA/chitosan scaffolds seeded with BMSC cells and the other was implanted with the scaffold without cells. Blank groups were left untreated.	After 10 weeks, the bone defects that were implanted with nHA/chitosan/BMSC scaffolds presented regenerated bone in the central part of the repaired area. The other two just presented fibrous tissue. After 20 weeks, the first defect was almost completely repaired, whereas the other two remained with non-mineralized spaces.
Zhang *et al.*[93]	Polyamide	nHA	Rectangular nHA/PA blocks were produced with a porous structure of 65-70%	15 New Zealand white rabbits Weight: About 3,5 Kg.	15mm x 10 mm bone defect created in the both sides of body of the mandible.	4, 12 and 24 weeks.	The defects in one side of the mandibles were implanted with nHA/PA blocks and the other side was kept empty, for comparison purposes. Radiographic examinations were performed before the animals were sacrificed. Mandibles were removed, cleaned and prepares for histologic testing.	There was no inflammation or rejection of the scaffolds. After 4 weeks of implementation, a beginning stage of mineralization was found around the nHA/PA blocks. The mineralization progressed continuously and at the 24th week, the density of bone formed in the defect filled with the nanocomposite was similar do the host's bone. The control group showed a reduction of the defect size, but it was not regenerated.

Table 4 – *In vivo* studies: osseous implantation (continued)

Ref.	Polymeric Matrix	Nano-filler	Nanocomposite specifications	Type of animal used and its characteristics	Site of implantation	Observation period	Method of *in vivo* evaluation	Outcome
Chiu *et al.* [103]	Gelatin from porcine skin	nHa	A so-called GEMOSIL porous scaffold derived from a formable paste by a new powder process consisting of coating, kneading and hardening. A buffer solution and an enTMOS coating were added. Nanocrystals were interwoven inside the aminosilica matrix after hardening. TiO_2 was incorporated to a part of this powder.	6 Sprague-Dawley rats, 11 to 13 week old. Weight is not specified.	8 mm-diameter critical size defects in the rat's calvaria	8 weeks	Nanocomposites with and without TiO_2 were implanted in the bone defects.	Both nanocomposites revealed new bone formation 8 weeks after implantation. However, the scaffold with titanium additives had a higher level of bone density. This suggests that titanium increases bone ingrowth.
Li *et al.* [104]	Polyamide 66 (PA66)	Silver ion-substituted nHA and titania nano-particles	0,3mm tick membranes were produced. The silver concentration is 0,5 wt% and the titania concentration is 2.5 wt%. nHA was incorporated though a precipitation reaction. A PA66 pure membrane was produced as a control membrane	27 Sprague-Dawley rats Weight is not specified	Two full-thickness circular defects (5mm in diameter) were created in the skull of each rat	1, 4 and 8 weeks	The total amount of rats was divided in three groups: the first received Ag-nHA-TiO_2 – PA66 membranes, the second received pure PA66 membranes and the third group had its defect left empty for control. Radiographic and histological tests were performed.	The first group showed some bone growth in the edges of the defect at the 4^{th} week. At the 8^{th} week, these defects looked similar to the surrounding bone, which means that the defect was regenerated. The defects covered with pure PA66 membrane and the ones left empty did not show healing of the defect.
Tan *et al.* [108]	Calcium Alginate hydrogel and collagen particles	nHA	Injectable bone regeneration nanocomposites were produced by varying the amounts of each component: nanocomposites with and without chitosan were developed.	24 male Sprague-Dawley rats Weight: 320-350g	5mm defects were performed in the parietal bones	4 and 8 weeks	The animals were divided in tree groups: In one, the bone defects were filled with the injectable nanocomposite having a nHA/chitosan composition, in the second, nanocomposites were composed only by HA and in the third defects were left empty.	After 4 weeks, both composites were divided in islands and some new formed bone is detectable, mainly in the edges. After 8 weeks, histology showed more regenerated bone in the nanocomposite containing nHA/ chitosan, when compared to the HA nanocomposite.

Table 4 – *In vivo* studies: osseous implantation (continued)

Ref.	Polymeric Matrix	Nano-filler	Nanocomposite specifications	Type of animal used and its characteristics	Site of implantation	Observation period	Method of *in vivo* evaluation	Outcome
Zhang *et al.*[92]	Polyamide (P66)	nHa, nAg and TiO_2 parti-cles	nAg – HA – TiO_2 membranes were produced with ≈ 0,2 mm in thickness and 10mm in diameter. e-PTFE membranes were produced as well, for comparison purposes.	27 male Sprague-Dawley rats Weight is not specified.	5mm diameter calvarial defects	1,4 and 8 weeks	The nAg – Ha – TiO_2 membranes were implanted in the defects and fixed by a medical adhesive. E-PTFE membranes were placed in the other defects for control and some defects were left empty. Rats were killed and block sections of the defects were removed and analyzed by radiographic and histological techniques.	In defects implanted with the nAg – Ha – TiO_2 membranes showed bone trabecula network at the 1st week. At the 4th week, the density of the trabecula increased and at the 8th week, the entire defects were occupied by new bone (very similar to the surrounding bone). In e-PTFE implanted defects, the trabecula network just appeared at the 4th week, but the defect was closed at the 8th week as well. Empty defects remained unmineralized.
Zhao *et al.*[109]	Silk Fibroin (SF) and polyas-partic acid (PAA)	Nano-apatite	80/20 w% SF/PAA porous scaffolds were produced and alternate soaking process was used to grow apatite nanocristals on the silk fibers surface. BMSCs were cultured *in vitro* to generate a cell material complex for *in vivo* testing.	17 adult male Mongrel dogs, aged 18 months Weight: 15 to 20 kg	20mm x 10mm bilateral inferior mandibular border full thickness defects	1, 3, 6 and 12 months	The animals were divided in five groups, in which each group had its bone defect filled with: (A) SF/PAA membranes; (B) SF/PAA/nano-apatite membranes; (C) SF/PAA membranes with seeded BMSCs; (D) SF/PAA/nano-apatite membranes with BMSCs; (E) Autogenous bone. Radiographic and histological assays were performed.	The group with best results was group (D): Callus was observed until the 3 months pos-operation; at 6 months, the volume of new formed bone increased very much and at 12 months, the mandibular defect was complete and no differences were seen to the proximal normal bone. Group (C) showed increased bone formation but failed to fill completely the defect after 12 months. Other groups failed to regenerate the bone defect
Lee *et al.*[110]	Chitosan	Silica xerogel	70/30 wt% Chitosan-Silica xerogel hybrid membranes were fabricated through a sol-gel technique, followed by freeze drying. Pure chitosan membranes were produced.	15 male Sprague–Dawley albino rats, aging 13 weeks Weight: 300 g	Two 5mm diameter calvarial defects were created symmetrically	3 weeks	In each animal, one defect was covered with a pure chitosan membrane for control and the other was covered with the 70/30 wt% Chitosan-Silica xerogel hybrid membranes. Animals were sacrificed and the implantation site was histologically analyzed	No significant inflammation reaction was observed around either membranes. The collagen membrane was partially, but not completely degraded and substituted by new formed bone, while the hybrid membrane was completely degraded and the defect site was replaced by new bone and some collagen fibers.

Table 4 – *In vivo* studies: osseous implantation (continued)

Ref.	Polymeric Matrix	Nano – filler	Nanocomposite specifications	Type of animal used and its characteristics	Site of implantation	Observation period	Method of *in vivo* evaluation	Outcome
Li *et al.*[34]	Polyamide	nHA	nHA produced by a wet synthesis and a hydrothermal treatment was gradually added into the PA matrix and the mixture was set in room temperature for coprecipiatation to occur.	54 New Zealand white rabbits. Weight: about 3 Kg.	3cm incision on the jaw of the rabbits; 12 x 8 mm^2 critical size defects in the bucco-lingual part, in both sides of the mandible.	4, 8 and 16 weeks.	The rabbits were divided in 3 groups, regarding the implant that they would receive: 1- Scaffolds seeded with BMP-7 traduced MSCs; 2- Scaffolds seeded with osteogenically cultured MSCs alone and 3- Pure nHA/PA scaffolds.	After 4 weeks, new bone was observed in all mandibles, but the one treated with scaffolds seeded with BMP-7 traduced MSCs, had a bigger bone density than the others. At 8 weeks, the situation remained the same. At 16 weeks, all mandibles had a comparable osteointegration scenario.

Table 5 – *In vivo* studies: non-osseous implantation

Ref.	Polymeric Matrix	Nano–filler	Nanocomposite specifications	Type of animal used and its characteristics	Site of implantation	Observation period	Method of *in vivo* evaluation	Outcome
Fricain *et al.*[95]	Pullulan/ Dextran polysac-charide	nHA	75/25 Pullan/Dextran macroporous scaffolds were synthesized. nHA was incorporated and the scaffold was freeze-dried.	Balb/c mice, 12 week old Weight: 25-30g	Subcutaneous pockets in the dorsum of the rats	15, 30 and 60 days	Matrix and matrix + nHA were inserted into the animals and analyzed histologically. Mineral content was evaluated.	Matrixes with nHA revealed a high degree of mineralization through time. In contrast, no mineralization occurred in the matrixes implanted alone.
Zhang *et al.*[92]	Polyamide (P66)	nHA and nano-silver	nAg – HA – Tio_2 membranes were produced with ≈ 0,2 mm in thickness and 10mm in diameter. e-PTFE membranes were produced as well, for comparison purposes.	Nine 12-week old male Sprague-Dawley rats Weight: About 300g	Two subcutaneous pockets in the neck region	4 and 8 weeks	NAg-HA-Tio_2/ PA66 membranes and e-PTFE membranes were inserted in subcutaneous spots and its biocompatibility *in vivo* was assessed, using the collected membranes and adjacent tissue in histological observation.	No obvious inflammatory reaction was observed in either membranes after 8 weeks of implantation. Histologically, both membranes were covered with granulation tissue, densely populated by cell nuclei. At the 8^{th} week, the NAg-HA-Tio_2/ PA66 membranes showed less granulation tissue that the e-PTFE membranes. Therefore, NAg-HA-Tio_2/ PA66 membranes were better tolerated after implantation.
Fu *et al.*[106]	Triblock PEG-PCL-PEG copolymer (PECE) and collagen	nHA	The PECE was freeze-dried and reduced to powder. This powder was mixed with nHA, and collagen and an injectable hydrogel composite was obtained, with 60% of PECE, 10% of collagen and 30% of nHA.	12 healthy Wistar rats Weight: 120 to 150g	Pouch in the dorsal muscle	3, 7 and 14 days	The *in vivo* biocompatibility was accessed by injecting 0,5ml of the composite into each muscle pouch. The rats were then sacrificed and the implants as well as surrounding tissue were analyzed.	After 7 days, a slight inflammatory reaction appeared due to the implant's degradation. It was proven that the pECE/nHA/collagen injectable nanocomposite is biocompatible and biodegradable.
Fricain *et al.*[95]	Pullulan/ Dextran polysac-charide	nHA	75/25 Pullan/Dextran macroporous scaffolds were synthesized. nHA was incorporated and the scaffold was freeze-dried.	4-year-old goats Weight: 70 ± 15 Kg.	Intramuscular sites	1 and 6 months	Matrix and matrix + nHA were inserted into the animals and analyzed histologically. Mineral quantification was performed.	Matrixes with nHA revealed a high degree of mineralization starting from the periphery to the center. In contrast, no mineralization occurred in the Matrixes implanted alone.

Table 5 – *In vivo* studies: non-osseous implantation (continued)

Ref.	Polymeric Matrix	Nano – filler	Nanocomposite specifications	Type of animal used and its characteristics	Site of implantation	Observation period	Method of *in vivo* evaluation	Outcome
Tan *et al.*[108]	Calcium Alginate hydrogel and collagen particles	nHA	Injectable bone regeneration nanocomposites were produced by varying the amounts of each component: nanocomposites with and without chitosan were developed.	12 male Sprague-Dawley rats Weight: 320 - 350 g	Dorsum muscle of the spinal column	Rats were grossly observed every day and the tissue was removed after 1, 2, 4 and 6 weeks	The nanocomposite was injected in the rat's muscle and fixed *in situ.* After the incubation period and through a surgical procedure, the nanocomposite with the surrounding muscle tissue was removed and it's biocompatibility as assessed.	There were no obvious inflammatory responses in the daily observations. Histological observation shows that the nanocomposite contained immunological cells after 1 week and that these cells increased through time, starting to divide the nanocomposite after 6 weeks. No fibrous capsule was observed.

a) The choice of animal models among *in vivo* studies

Figure 13 represents the percentage of laboratory animals used in the *in vivo* studies. Figure 13 – A represents the laboratory animals used in the osseous implantations, while Figure 13 – B represents the laboratory animals used in the non-osseous implantations.

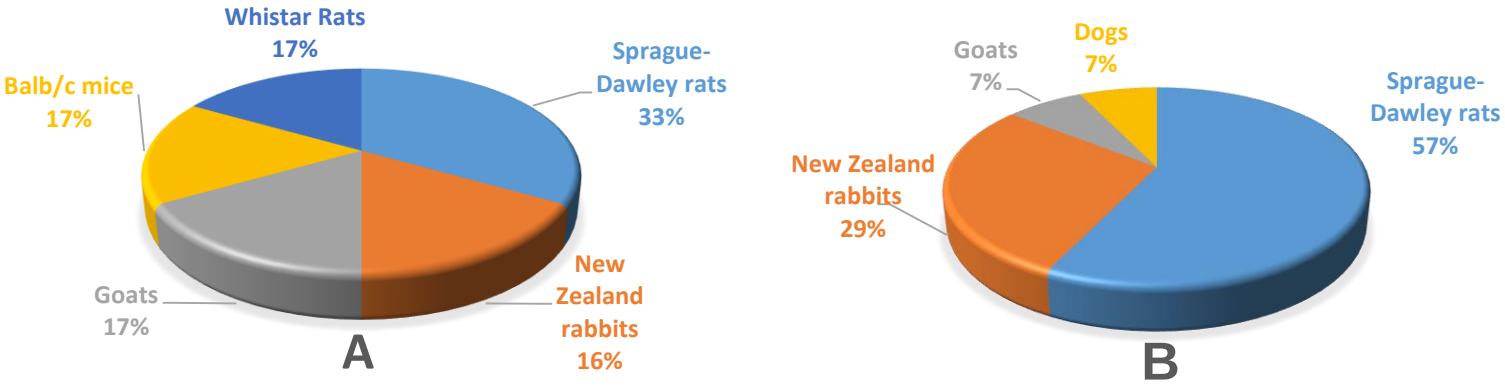

Figure 13

Laboratory animals used in the *in vivo* studies

When analyzing the type of laboratory animals, Sprague-Dawley rats [91,92,100,103,104,107,108,110] are the most commonly used in these studies, followed by New Zealand rabbits [34,93,105,106]. In a smaller proportion, Goats [95], Balb/c mice [95], Whistar rats [106] and Dogs [109] were used as well.

b) The choice of Polymers

In order to understand which polymers were used the most as matrixes of the studied nanocomposites in the *in vivo* studies, an accounting was performed. Figure 14 shows the percentage of polymers used: Figure 14 – A shows the polymers chosen for the osseous implantation and Figure 14 – B shows the polymers chosen for the non-osseous implantation.

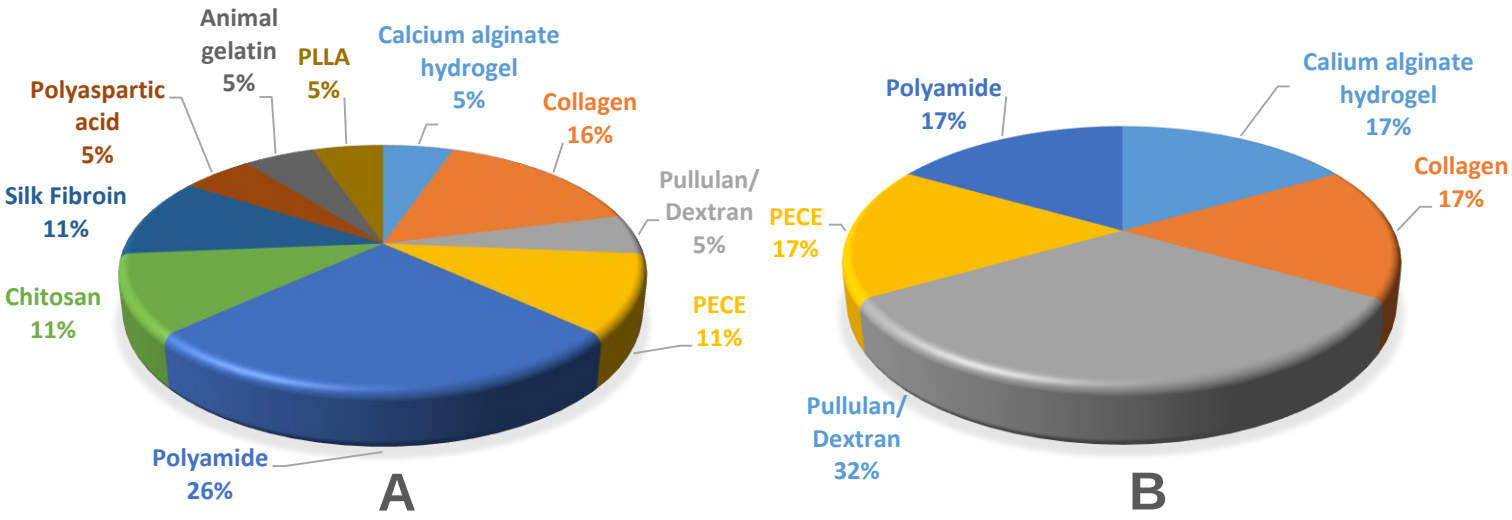

Figure 14

Polymers chosen to integrate the nanocomposites in (A) osseous implantation and (B) non-osseous implantation

When analyzing the osseous implantation, polyamide (in its normal or in its aliphatic state – polyamide 66) [34,91-93,104] stands out as the most chosen polymer to integrate the matrix of the nanocomposites, followed by collagen [106-108], that was used in 16% of the studies. Chitosan [100,110], Silk Fibroin [105,109] and PECE – a triblock copolymer composed of poly(ethylene glycol) - poly(ε-caprolactone) - poly(ethylene glycol) [106] have a representation of 11%.

In the non-osseous implantation, the innovative Pullulan/ Dextran [95] polymer has a bigger representation, since it was subjected to 2 studies of non-osseous biocompatibility in different animals. The other polymers have the same representation, 17%.

c) The choice of nanofillers

Figure 15 represents the percentage of nanofillers used in the studied nanocomposites in both osseous and non-osseous implantation.

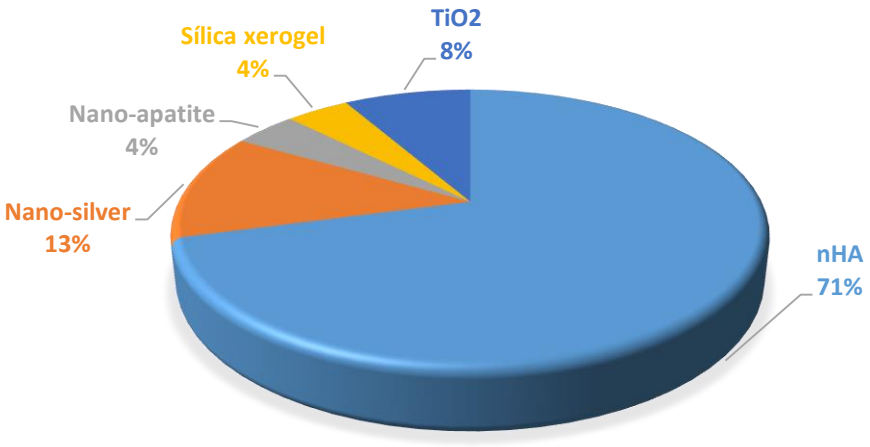

Figure 15

Nanofillers that integrate the nanocomposites studied in both osseous and non-osseous studies

Regarding the most used nanofillers, nano-hydroxiapatite (nHA) is the most selected nano-ceramic , being used in almost all the *in vivo* studies contemplated in this review. Nano-silver particles are integrated as well in 13% of the studies considered.

The 24 *in vitro* studies considered for this review can be divided in 3 major groups based on their model to evaluate the *in vitro* response to the biomaterial in study: (a) the ones that use stem cells to evaluate if the nanocomposite is able to stimulate their differentiation in a osteogenic cell line, by having the right stimulating constitution (b) the ones that use already differentiated osteogenic cells and (c) the ones that use stimulated body fluid, to assess whether the nanocomposite is capable of producing surface apatite particles, when immerged in this solution that replicates *in vivo* fluids, or not. Figure 16 demonstrates the percentage of studies that call upon each of this strategies and Table 6 reproduces their specifications.

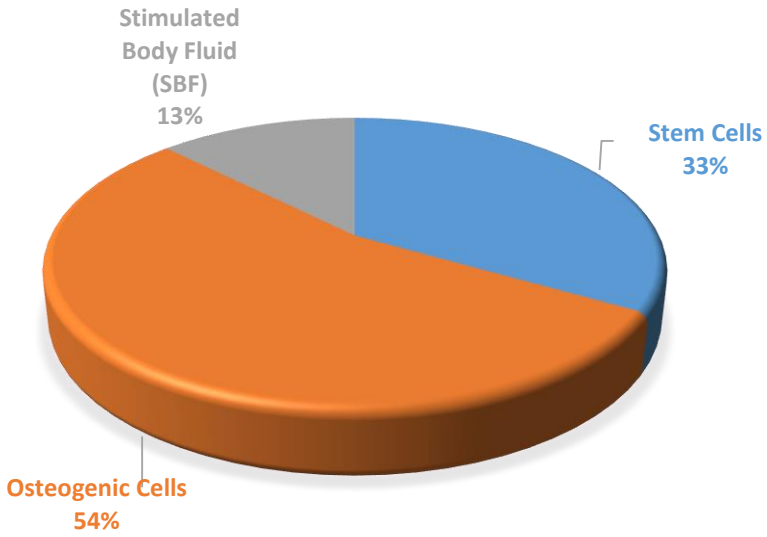

Figure 16

Studies using stem cells, osteogenic cells and Stimulated Body Fluid

The processing techniques used to produce the final nanocomposites can vary according to the involved material and their specifications. To detail these complex techniques and biochemical effect elicited by the materials on the tissue is not the goal of this dissertation. Moreover, the technical procedures for the chemical and physical characterization used in the analyzed studies were

relevant but not exhaustively described since this was not the goal of the present thesis, as well.

In Table 6 are presented, in a comparative way, the different aspects about the retrieved *in vitro* studies including: the polymeric and nanofiller composition of the nanocomposites, their specifications, type of cells used to perform the study, method of *in vitro* evaluation and the final outcome.

Table 6 – Results obtained regarding the *in vitro* studies of nanocomposites to be used in bone applications in the maxillofacial context

Ref.	Polymeric Matrix	Nano – filler	Nanocomposite specifications	Type of cell used	Specifications of the cell	Method of *in vitro* evaluation	Outcome
Liu *et al.*[100]	Chitosan	nHA	3 different scaffolds were produced for comparison: nHA/ Chitosan, produced by a coprecipitation process; Electrospun nHA/ chitosan nanofibers produced by electrosppining and membranous HA/chitosan scaffold were prepared via a simple solution casting method.	Stem Cells	Bone mesenchymal stem cells were isolated from femurs and tibias of Sprague-Dawley rats.	The morphology of the 3 different scaffolds was evaluated. For the cell culture, cells were seeded on top of the 3 different scaffolds and their proliferation and characterization was performed.	BMSC showed a different cell-morphology on the nHA/chitosan scaffold, when compared to its shape in the other 2 scaffolds. The cells differentiated and proliferated better on the nHA/chatiosan scaffold. Is was suggested that nHAP/ Chitosan induces the activation of integrin, which activates bone morphogenic proteins and consequently osteogenic differentiation.
Tan *et al.*[108]	Calcium alginate hydrogel and collagen particles	nHA	Injectable bone regeneration nanocomposites were produced though a mixture of colloidal suspensions. Various concentrations of polymer were used to establish comparison.	Stem Cells	Rat bone mesenchymal cells	The structural properties of this injectable material were assessed and an *in vitro* cell culture was performed to analyze its biocompatibility, for 4h, 1, 2, 4 and 7 days.	The alginate concentrations didn't show significant influence in the nanocomposite homogeneity and in the average pores size. The molar concentration of the colloidal solutions can make this properties vary. As for the cell culture test, the cells adhered, spread and survived very well on the nanocomposite surface.
Fricain *et al.*[95]	Pullan/ Dextran polysaccharide	nHA	75/25 Pullan/Dextran macroporous scaffolds were synthesized. nHA was incorporated and the scaffold was freeze-dried.	Stem Cells	Human bone marrow stromal cells (HBMSc) extracted from the femoral diaphysis or iliac bone of 30-80 aged patients.	The scaffold was analyzed to verify the quality of dispersion of the nHA in the matrix. The behavior of HBMSc with the scaffold was assessed, with and without addition of nHA.	It was possible to find multicellular aggregates in both scaffolds, with and without nHA. However, the supplementation with nHA increased the proliferation of cells.

Table 6 – Results obtained regarding the *in vitro* studies of nanocomposites to be used in bone applications in the maxillofacial context (continued)

Ref.	Polymeric Matrix	Nano – filler	Nanocomposite specifications	Type of cell used	Specifications of the cell	Method of *in vitro* evaluation	Outcome
Zhao *et al.*[109]	Silk Fibroin (SF) and polyaspartic acid (PAA)	Nano-apatite	80/20 w% SF/PAA porous scaffolds were produced and alternate soaking process was used to grow apatite nanocristals on the silk fibers. Mineralized scaffolds were freeze-dried.	Stem cells	Bone marrow stromal cells extracted from mongrel dogs, 18 months old and weighing from 15 to 20 Kg.	Scaffolds were seeded with bMSCs and incubated for 14 days.	Cell clones were formed 5-7 days after initial seeding and after two-weeks, bMSCs spread and differentiated and in the end of the incubation period, osteoblastic cells were positively identified in the scaffolds. It was possible to find cells attached to the porous structure, as well as fibril network of extracellular matrix and mineralized nodules.
Wang *et al.*[89]	polycaprolactone (PCL)	nHA	Scaffolds were fabricated with different ratios of nHA/PCL by a melt-molding and a leaching technique.	Stem Cells	Bone marrow stromal cells (BMSc) extracted from Dogs.	Scaffolds were characterized before and after a 6 months *in vitro* degradation. Cell culture was performed to evaluate the cytocompatible properties of the scaffolds.	The degradation test showed that this scaffold can retain relatively stable architecture and mechanical properties for at least six months. The porosity level of the nHA/PCL nanocomposites is around 70%.They have good cytocompatibility
Xu *et al.*[90]	Poly(lactide-co-ethylene oxide fumarte) (PLEOF)	nHA	A multi layered fiber reinforced 10 wt% hydrogel/apatite nanocomposite was produced by electrosppining, adding of 1% of a hydrogel precursor solution and pressure and crosslinking.	Stem Cells	Bone marrow stromal cells (BMSc), extracted from Winstar rats	The laminated composites were characterized, its degradation characteristics were assessed and the BMS cells were seeded and analyzed at 7, 14 and 21 days.	This scaffold proved to have a slow fiber degradation rate for structural stability and a fast hydrogel degradation rate to increase the space for cell migration and ECM production. The HA nanocrystals suspended in the hydrogel phase proven to have an osteoconductive potential, promoting the differentiation and mineralization of the BMS cells.

Table 6 – Results obtained regarding the *in vitro* studies of nanocomposites to be used in bone applications in the maxillofacial context (continued)

Ref.	Polymeric Matrix	Nano – filler	Nanocomposite specifications	Type of cell used	Specifications of the cell	Method of *in vitro* evaluation	Outcome
Li *et al.*[104]	Polyamide 66 (PA66)	Silver ion-substituted nHA and titanium nano-particles	0,3mm tick membranes were produced. The silver concentration is 0,5 wt% and the titanium concentration is 2.5 wt%. nHA was incorporated though a precipitation reaction.	Osteogenic cells	Human osteosarcoma cell line - MG-63 osteoblast	The membrane was characterized, its morphology was examined and mechanic tests were performed. Cytocompatibility was assessed though cell culture in top of the membrane, followed by cell morphology examination, viability evaluation and cell differentiation assays.	Characterization of the membrane confirmed well dispersed spherical titanium nano-particles in between needle-like crystals, among the matrix. The addition of silver and titanium did not change the mechanical properties of the nHA/PA66 membrane. In the cell culture evaluation, cells adhered and spread well on the membranes. The addition of silver and titanium decreased the cell viability, but did not affect the differentiation of MG63 cells.
Chiu *et al.*[103]	Gelatin from porcine skin	nHA	A so-called GEMOSIL porous scaffold derived from a formable paste by a new powder process consisting of coating, kneading and hardening. A buffer solution and an enTMOS coating were added. Nanocrystals were interwoven inside the aminosilica matrix after hardening. To a part of this powder, TiO2 was incorporated. All components were added in different concentrations to create different scaffolds.	Osteogenic cells	Murine osteoblast-like cells (MC3T3–E1 cells)	All the created scaffold were characterized and mechanically tested to assess which one possessed better characteristics for *in vitro* assessment. Cells were seeded on the scaffold to access its biocompatibility and to determine the cellular metabolic activity. Cell differentiation was evaluated as well.	The amount of enTMOS coating did not affect the properties of the nanocomposites. However, the amount of TiO2 decreased the hydrophilicity of the scaffolds, promoting cell adhesion. The scaffolds with TIO2 were the ones that promoted more cell differentiation and proliferation in the *in vitro* studies. This scaffolds are not cytotoxic.

Table 6 – Results obtained regarding the *in vitro* studies of nanocomposites to be used in bone applications in the maxillofacial context (continued)

Ref.	Polymeric Matrix	Nano – filler	Nanocomposite specifications	Type of cell used	Specifications of the cell	Method of *in vitro* evaluation	Outcome
Poursamar *et al.*[111]	Polyvinyl Alcohol (PVA)	nHA	Colloidal nHA was synthesized via co-precipitation method inside the PVA aqueous solution and then freeze dried, producing a nanocomposite. Scaffolds with different amounts of both components and in different pH values were produced for comparison.	Osteogenic cells	Sarcoma osteogenic cell line (SaOS-2 cell line)	The scaffolds were characterized and analyzed regarding its mechanical properties. The *in vitro* cell-material interaction study was performed seeding the cells on top of the scaffolds for 4 days at 37 °C.	For producing this scaffold, the optimal pH value that permits the best interaction between both components and allows the precipitation of nHA in the structure is 9. It was proved that increasing the polymer phase in the scaffolds enhanced mechanical properties such as toughness and the elastic modulus. The cell culture test showed that this scaffold allows cell to spread and attach to the porous walls
Azami *et al.*[94]	Gel solution	Calcium phosphate	The gel components were prepared and the nanofillers were dispersed. The scaffold was then freeze dried to create a porous structure through solvent sublimation.	Osteogenic cells	*In vitro* differentiated osteoblasts from Human Endometrial Stem cells	The scaffold was characterized and its mechanical properties were assessed though compression tests. Cell attachment, viability and proliferation was studied using differentiated osteoblast cells.	The authors were able to differentiate human endometrial stem cells in osteoblasts successfully. The diffusion of the nanofillers in the gel matrix was successful. In the cultures cell test, the scaffold proved not to be cytotoxic, but cells did not proliferate.
Ghorbanian *et al.*[96]	Silk fibroin (SF)	Diopside nanocristals	Different SF/Diopside nanocomposites weight ratios were mixed, ultrasonicated and freeze-dried. A pure polymer scaffold was freeze-dried to provide comparison.	Osteogenic cells	Murine osteoblast-like cells (MC3T3– E1 cells)	The scaffolds were characterized, it pores were measured and its surface hydrophilicity was evaluated. Mechanical properties were assessed as well. Finally, an *in vitro* evaluation of its cytotoxicity was performed for 1, 3 and 7 days of culture.	The produced scaffolds possess interconnected pores and almost sufficient porosity (70%) to provide good conditions for cell proliferation. Higher levels of diopside, decrease the amount of pores but improve the mechanical behavior. Moreover, adding diopside improves the hydrophilicity of the scaffold. The biocompatibility of the scaffold is excellent.

Table 6 – Results obtained regarding the *in vitro* studies of nanocomposites to be used in bone applications in the maxillofacial context (continued)

Ref.	Polymeric Matrix	Nano – filler	Nanocomposite specifications	Type of cell used	Specifications of the cell	Method of *in vitro* evaluation	Outcome
Liu *et al.*[112]	Nanofibrous Gelatin (Type B, from bovine skin)	Partially carbonated nHA	A 3D nanofibrous gelatin scaffold was produced by thermally induced phase separation and leaching techniques. nHa was incorporated by SBF incubation.	Osteogenic cells	Murine osteoblast-like cells (MC3T3– E1 cells)	Mechanical tests were performed in the scaffold with and without the nano-apatite component. Cells were seeded in both scaffolds to verify its biocompatibility and analyze the differences in their behavior.	The biomimetic scaffold (mimicking chemical and physical architecture of natural bone ECM) proved to have good porous interconnectivity. Its biological and mechanical characteristics were proven to be excellent. The addition of apatite enhanced osteoblastic cell differentiation as well as mechanical strength of the scaffold.
Thein-Han *et al.*[113]	Silicon Rubber (SR)	nHA	A biomimetic 5 wt% SR/nHA nanocomposite was obtained through a uniform dispersion of nHA to the silicone rubber by a high-pressure solidification.	Osteogenic cells	Murine osteoblast-like cells (MC3T3– E1 cells)	The structural morphology, dispersibility of nHA into SR and mechanical properties were studied. Cytocompatibility and cell viability were assayed. Cellular test were performed in SR constructs and in SR-nHA constructs during 14 days and the cellular response was analysed.	When nHA is added to the SR structure and a good dispersion is obtained, the biological response is favorable, including cell attachment, higher viability and proliferation, and well-spread morphology, compared to pure SR.
Jaiswal *et al.*[97]	Polycaprolactone – gelatin (PCG)	nHA	Two scaffolds were prepared: first nHA was deposited on the surface of electrospun PCG fibers via alternate soaking process and the second had HA powders blended in the electrospinning solution.	Osteogenic cells	Human osteosarcoma cells (MG-63)	Both scaffolds were compared regarding their microstructure, through characterization techniques. Physical, chemical and biological properties were assayed as well. Biological properties such as cell proliferation, cell attachment were determined by growing the chosen cells over the scaffolds.	The alternate soaking process demonstrated to provide better mechanical strength, biocompatibility properties, since it allowed surface precipitation of nHA over the scaffold. The proliferation and cellular adhesion were also observed better on the scaffold that had nHA deposited on the already electrospun polymer. A good osteoestimulation capacity was also found.

Table 6 – Results obtained regarding the *in vitro* studies of nanocomposites to be used in bone applications in the maxillofacial context (continued)

Ref.	Polymeric Matrix	Nano – filler	Nanocomposite specifications	Type of cell used	Specifications of the cell	Method of *in vitro* evaluation	Outcome
Liao *et al.*[99]	Polypropylene	HA nanorods (nHAs) and multi-walled carbon nanotubes (MWNTs)	The materials of PP/nHA and PP/MWNT/nHA nanocomposites were placed together by a melt-blending process and injection modeled into plaques.	Osteogenic cells	Human osteoblast cell line (Saos-2)	Structure and morphology of the scaffolds was analyzed. Thermal and mechanical assays were performed for characterization. In vitro cytocompatibility was tested by culturing the nanocomposites in a cell suspension at 37 °C for 24 and 72 hours. All the assays were done in samples with and without MWNT and in different amounts.	Mechanical and thermal properties are improved by adding a low loadings of MWNT with nHA in a PP matrix. Regarding the cell culture, it was found that in samples with 0,3% MWNT-20% nHA, cells adhere and proliferate better than in samples without MWNT.
Lu *et al.*[87]	N-carboxyethylchitosan (NCECS)	nHA	Materials were synthetized to produce a 5 and a 10 wt% NCECS/n-HA composite film.	Osteogenic cells	Rabbit tracheal cartilage cells obtained from a 2-month-old New Zealand rabbit.	Characterization of the films was performed. Mechanical properties were assessed and *in vitro* cytocompatibility was evaluated in a 9 day assay.	nHA integrates well in a NCECS matrix, producing an uniform film. Cell adhesion, growth and morphology are higher in the film that has a bigger nHA concentration (10 wt%), suggesting that the viability increases with the amount of nHA.
Song *et al.*[102]	Poly (L-lactide) PLLA and poly (L-lactide-co-glycolide) (PLGA)	nHA	Hydroxyapatite grafted poly(L-lactide) (HA-g PLLA) nanoparticles and poly (L-lactide-co-glycolide) (PLGA) scaffolds were prepared by electrosppining at a concentration of 0, 5, 10 and 20 wt%.A HA/PLA membrane was produced as well.	Osteogenic cells	Murine osteoblast-like cells (MC3T3–E1 cells)	Chemical and physical characterization of the electrospun fiber membranes was performed, as well as its *in vitro* degradation rate. The bioactivity was assessed by evaluating their ability to form apatite on their surfaces in simulated body fluid (SBF). The cell adhesion test was performed for a period of 12, 24 and 48 hours.	nHa-g-PLLA fibrous membranes exhibited better mechanics properties than control pristine PLGA and corresponding HA/PLA fiber membranes, which indicates that the grafting technique used to produce the nHA-PLLA membrane enhances its properties. The cells adhere well to the membrane, spreading and proliferation though time.

Table 6 – Results obtained regarding the *in vitro* studies of nanocomposites to be used in bone applications in the maxillofacial context (continued)

Ref.	Polymeric Matrix	Nano – filler	Nanocomposite specifications	Type of cell used	Specifications of the cell	Method of *in vitro* evaluation	Outcome
Lee *et al.*[110]	Chitosan	Silica xerogel	70/30 wt% Chitosan-Silica xerogel hybrid membranes were fabricated through a sol-gel technique, followed by freeze drying. Pure chitosan membranes were produced to provide comparison.	Osteogenic cells	Murine osteoblast-like cells (MC3T3– E1 cells)	The membranes were characterized, their mechanical properties were assayed as well as its biodegradation rate and mineralization properties. The *in vitro* cell responses were evaluated in terms of initial attachment, proliferation and osteoblastic differentiation, after a 10 day culturing.	It was proven that the sol-gel process provides a highly efficient way of producing this scaffolds with a porous and uniform structure. The incorporation of the silica xerogel stimulated the production of a layer of apatite on the scaffolds surface, when immerged in SBF and affected the cells viability positively. Results show that the cells attached and differentiated very well.
Raucci *et al.*[88]	PCL (polycaprolactone)	nHA	Ha/ PCL nanocomposite scaffold (40/ 60 wt %) prepared though a combined phase inversion/ salt leaching technique.	Stem Cells	Bone marrow derived human Mesenchymal stem cells.	Morphological characterization of the scaffolds was performed. Cytocompatibility was assessed by a 15 day cell culture. The cells structure and biological behavior was analyzed and characterized.	The method of preparation allowed highly porous scaffolds. Morphological analysis of the composite confirmed that the HA particles were distributed homogeneously within the PCL matrix. Biologically, cells spread and attached well to the scaffold's porous structure and the mesenchymal cells differentiated into osteoblasts and proliferated.
Moreau *et al.*[114]	Chitosan	Calcium Phosphate cement (CPC)	A high-strength injectable nanocomposite scaffold was produced.	Stem Cells	Bone marrow mesenchymal cells (MSCs), harvested from Wistar Hannover male rats weighting 101-125 g.	Mechanical tests were performed. The proliferation and osteogenic differentiation of the cells on high-strength CPC-chitosan was investigated.	By adding chitosan to CPC, it was possible to strengthen it and enhance its mechanical properties. The MSCs growth on the scaffold was evident and increased by an order of magnitude from day 1 to day 14. MSCs differentiated down the osteogenic lineage and proliferated considerably.

Table 6 – Results obtained regarding the *in vitro* studies of nanocomposites to be used in bone applications in the maxillofacial context (continued)

Ref.	Polymeric Matrix	Nano – filler	Nanocomposite specifications	Type of cell used	Specifications of the cell	Method of *in vitro* evaluation	Outcome
Hayati *et al.*[115]	Poly(3-hydroxybutyrate) (PHB)	nHA	PHB/nHA scaffolds were produced using organic solvents at different mass percentages of nHA.	Osteogenic cells	Human osteosarcoma cell line - MG-63 osteoblast	The scaffolds were characterized, the porosity was measured, and mechanical tests like compression were performed. The biocompatibility *in vitro* was assessed by cell culture.	After characterization, it was concluded that this scaffolds have an interconnected porous architecture with rather high porosity and that this characteristic is not affected by the amount of nHA. The dispersion of the HA nanocristals is affected by its amount: above a concentration of 15 wt%, agglomeration would occur, not allowing a good dispersion of the nanofiller. It was demonstrated that the scaffolds were biocompatible, though the observation of an appropriated cell-scaffold interaction

Table 3 – Results obtained regarding the *in vitro* studies of nanocomposites to be used in bone applications in the maxillofacial context (continued)

Ref.	Polymeric Matrix	Nano – filler	Nanocomposite specifications	Type of solution	Method of *in vitro* evaluation	Outcome
Rezaei *et al.*[101]	Polycaprolactone (PCL)	nHA	20/80 wt% nHA/PCL scaffolds were prepared though 2 stages: an in situ sol-gel process and a salt leaching technique.	Supersaturated stimulated body fluid (SBF)	The dispersion of the nHA on the PCL matrix was analyzed. The mechanical behavior of the scaffolds was compared to the characteristics of a pure PCL scaffold. The scaffold biocompatibility was evaluated by exposing it to SBF.	The characterization of the scaffold revealed that the nano-ceramic particles were homogenously dispersed in the polymeric matrix. The adding of nHA improved the mechanical behavior of the scaffold. By exposing it to SBF, the scaffolds proved to have an excellent ability to precipitate bone like minerals at the surface.
Kouhi *et al.*[98]	Poly (ε-caprolactone) (PCL)	Bioactive glass (BG) nanoparticules	PCL solutions with different percentages of nanoparticules and simvastatin drug were electrosppined, forming a nanofibrous web.	Stimulated body fluid (SBF)	The fibers morphology and mechanical characterization was assed, as well as degradation measurements and *in vitro* drug releases studies. The *in vitro* bioactivity evaluation was carried out in SBF.	The amount of nanoparticles added in the spinning solution, influenced the viscosity of the solution and the ability of spinning it. Moreover, the presence of nBG increased the mechanical properties and affected the nanofiber degradation and simvastatin release. The nanofibers showed excellent bioactivity, inducing the precipitation of bone like apatite mineral, when exposed to SBF.
Lee *et al.*[110]	Chitosan	Silica xerogel	70/30 wt% Chitosan-Silica xerogel hybrid membranes were fabricated through a sol-gel technique, followed by freeze drying. Pure chitosan membranes were produced to provide comparison.	Stimulated body fluid (SBF)	The membranes were immersed in the SBF solution and incubated for 14 days to observe their mineralization. A membrane of pure chitosan was subjected to the same procedure for comparison puroposes.	It was proven that the sol-gel process provides a highly efficient way of producing this scaffolds with a porous and uniform structure. After 1 days, apatite particles were visualized in the hybrid membrane. After 7 days, the surface was almost completely covered with a thin layer of apatite. The density of this layer increased until the 14th day, The chitosan membrane did not show any apatite particle after 14 days.

a) The choice of polymers

In order to understand which polymers are used as matrixes in the *in vitro* studies considered for this review, an accounting was performed. It is important to refer that when a nanocomposite was composed by more than one polymer, they were accounted separately. Figure 17 represents the percentage of polymers used in the *in vitro* studies.

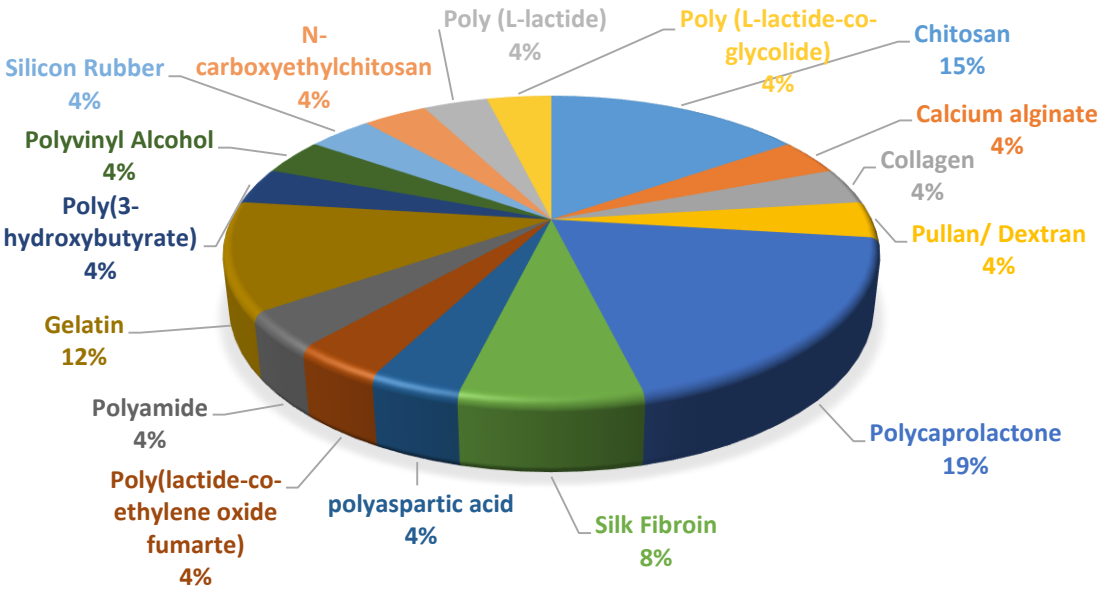

Figure 17

Polymers used as matrixes in the nanocomposites subjected to *in vitro* studies

It is possible to understand that a variety of different polymers was used as matrixes of the nanocomposites subjected to *in vitro* studies. Among them, polycaprolactone stands out with a percentage of 19% [88,89,97,98,101], followed by chitosan [100] that has a representation of 15%. Gelatin was used in 12% of the cases [94,103,108] and silk fibroin in 8%. All the others polymers have a representation of 4%.

b) The choice of nanofillers

The nanofillers used in the *in vitro* studied nanocomposites were considered for accounting to understand which ones were requested the most. Figure 18 demonstrates the accounting performed.

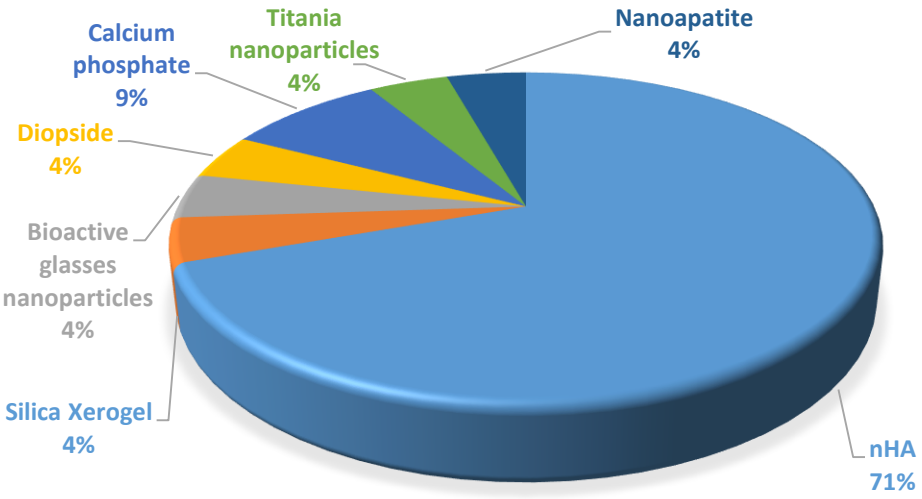

Figure 18

Nanofillers used in the nanocomposites subjected to *in vitro* studies

Nano-hydroxyapatite stands out as having a 71% representation among the *in vitro* investigated nanocomposites [87-90,95,97,99-104,108,111-113,115]. Calcium phosphate follows with a 9% representation [94,114] and all the other nanofillers have a representation of 4% [96,98,104,109,110].

IV. Discussion

IV. Discussion

Nanocomposites have clearly proved their value for bone replacement, augmentation and regeneration.[23,57,77,85] However, the variety of materials that can be combined and its properties and specifications is vast. Therefore, to understand which material combinations provide the best biological, structural and mechanical response is highly important. Polymer-ceramic nanocomposite systems are particularly interesting as they can be closer to bone in terms of its constitution which is mostly an intricate combination of two phases at the nano-level: hydroxyapatite and collagen. The present dissertation has as its main goal to analyze the most recent reported studies based on polymer-ceramic nanocomposites produced for bone replacement and regeneration, focusing on those that are suitable for craniomaxillofacial bone applications. This particular area has been in the forefront of biomedical technology as compared with the orthopedic field due to the less interventional nature of its applications as compared for instance with the orthopedic field.

Materials Selection

In Tables 4, 5 and 6, it is possible to identify all the constituting materials of the nanocomposites studied in the selected articles. Moreover, Figures 11, 15 and 17 analyze the polymers used generally, in *in vivo* and in *in vitro* cases, respectively, while Figures 12, 15 and 18 make the same evaluation, regarding the nanofiller constitution.

a) The choice of polymers

Regarding their origin, polymers can be synthetic or derive from natural sources. As reported in Figure 11 of the results section, the majority of polymers contained in the articles analyzed in this review are from synthetic origin.

Polyamide, the most used polymer in the *in vivo* assays (Figure 15), has excellent mechanical and corrosion-resisting properties, resulting from the strong hydrogen bonds between its own macromolecules and its high affinity to form hydrogen bonds with nano-sized apatites[116,117]. Moreover, it presents a good biocompatibility since it reassembles in some extent the collagen protein both in chemical structure and active groups. Zhang *et al.*., in his published articles[91-93] used polyamide as the polymeric matrix component. In one of his studies,[92] he blended polyamide with nHA, nAg and TiO_2, producing a successful antibacterial scaffold. In other two, he joined polyamide just with nHA and evaluated its *in vivo* performance on rabbits[93] and rats[91] In this last study, he used a polyamide with an aliphatic conformation (Polyamide 66). Li *et al.*[34] produced a nHA/Polyamide scaffold as well and evaluated the same parameters. All these works demonstrated that this material provides great mechanical behavior allied to great biocompatibility.

Polycaprolactone, the most used polymer in *in vitro* assays (Figure 17), is a biodegradable synthetic polymer that possesses a slow degradation rate. It belongs to a group of synthetic polymers with an amazing characteristic: by fine controlling their chemical synthesis and processing, mechanical and physical characteristics such as tensile strength, elastic modulus and degradation rate can be predicted and reproduced with precision. Polymers like poly(lactid acid) and poly(glycolic acid) belong to this group as well. Moreover, these polymers allow for creating scaffolds with different shapes according to the final outcome. These polymers however present a major drawback that is the inability to stimulate cell adhesion or bone growth (they are bioinert). By binding with nano-ceramic fillers, this drawback can be overcome. Raucci *et al.*[88] were able to create a scaffold with excellent mechanical performance, while possessing bioactive characteristics capable of stimulating osteoblast differentiation from human mesenchymal cells, though homogeneous distribution of HA nanoparticles in the polycaprolactone matrix,. Wang *et al.*,[89] used a nanocomposite with the same composition, obtaining similar results, namely the osteoblastic differentiation but, this time, using bone marrow stromal cells extracted from dogs. A study by Jaiswal *et al.*,[97] differed from the previous two by using gelatin in combination with polycaprolactone as a polymeric matrix, in order to produce an electrospun

fibrous polymeric scaffolds that were posteriorly coated with nHA though alternate soaked and blending techniques. When compared, alternate soaked nanocomposites proved to have better mechanical properties and to stimulate bone-like cells to proliferate more efficiently than blended scaffolds.

Polypropylene, used by Liao *et al.*[99] in their research, displays excellent chemical resistance, good stress crack resistance and flexibility. It has been proved that when implanted in tissue, this polymer retains its tensile strength over a large period of time[118]. However, polypropylene is bioinert. Besides the good properties of the polymer, the nanofillers used by Liao *et al.* are HA nanorods and carbon nanotubes (known for having an exquisite mechanical behavior). This combination provides a great mechanical behavior and superior dimensional stability. On the other hand, the incorporation of HA attributes the necessary bioactive characteristics. The *in vitro* outcomes corroborate these facts entirely.

Chitosan, a natural biodegradable polymer, has a unique characteristic that highlights it from others: it possesses an innate anti-bacterial capacity. Additionally, it is flexible and can be transformed into fibers,[100] membranes,[110] 3D scaffolds or injectable materials.[114] However, reports[119] have been published stating that chitosan's bioactivity and righty are not satisfactory, particularly in the wet state. This same authors state that this disadvantages of chitosan can be resolved by adding reinforcement agents. In the present dissertation, four studies[87,100,110,114] included a report choosing chitosan as a polymer matrix. By adding nHa, silica xerogel and calcium phosphate cement, Liu *et al.,*[100] Lee *et al.*[110] and Moreau *et al.*[114] were able to achieve an excellent outcome regarding chitosan's *in vitro* and *in vivo* performance, fact that corroborates Ignatius *et al.* [119] theory, that states that the addition of bioactive ceramics to chitosan-based polymeric matrixes improves biocompatibility and osteoconductivity of this resorbable nanocomposites. Lu *et al.* [87] used an amphiphilic chitosan derivative, N-carboxyethylchitosan, because it already proved to be more water-soluble and to have higher cell compatibility then chitosan. Unfortunately, for testing the *in vitro* cytocompatibility assey, he used different cells from other authors (rabbit tracheal cartilage cells), reason why it is not possible to clearly compare the results. More research should be conducted in this area.

The natural hydrophilic polysaccharides pullulan and dextran constitute interesting polymers for bone regeneration. They have a biochemical structure that reassembles the extracellular matrix, providing excellent physical support for bone growth and a good biodegradability behavior. Pullulan is a non-immunogenic polysaccharide produced by a fungus, *Aureobasidium pullulans* and has great mechanical properties and biocompatibility[95]. Dextran was first used as a blood replacement constituent and is synthesized from sugar by bacteria. Together they form an easy-to-shape material with good mechanical properties, totally suitable for bone regeneration. In his study, Fricain *et al.*[95] tested a nanocomposite with nHa dispersed in this combination of polysaccharides *in vitro*, by using human bone marrow stromal cells and *in vivo*, in small (rats) and big animals (goats). These results show that nHa/Pullulan-Dextran nanocomposites stimulate bone formation *in vitro* and *in vivo* in a short period of time.

b) The choice of nano-fillers

As referred in Figures 12, 15 and 18, nano-hydroxyapatite is the most used ceramic nano-filler in nanocomposites that were subjected to both *in vivo* and *in vitro* assays. The other relevant nanofillers are nano-apatite,[109] silica xerogel,[110] calcium phosphate cement (CPC),[94,114] bioactive glass (BG) nanoparticule [98] and diopside nanocristals.[96]

Synthetic hydroxyapatite is a calcium-containing biomaterial composed of chemical elements similar to the ones present in natural bone. Due to its excellent biocompatibility, cell affinity, and osteoconductivity, HA stands out as being ideal for bone grafting. Conventional synthetic HA (microparticulated) however, has a large particle size compared with natural bone HA and poor mechanical properties.[120] Moreover, the microparticulated form debonds easily from the matrix, resulting in an inefficient stress-transfer mechanism in the matrix-filler interface. Nano-hydroxyapatite rises than as a solution for bringing the advantages of HA, while granting better mechanical properties in terms of strength, stiffness, toughness and fatigue resistance and being better retained within the polymeric matrix. In addition, nHA resembles more closely to bone

environment and enhances the cellular activity towards bone formation as compared to HA blend scaffolds.

There are two major methods for hydroxyapatite synthesis: sintering (high temperature) and precipitation (lower temperature). The structural and chemical vary according to the production method. In fact, hydroxyapatite obtained through the first synthesis method, requires a high temperature sintering (1,250 °C), leading to a higher chemical stability and lower surface activity. This is probably why the majority of authors referred in this dissertation that use nHA, obtain it through the precipitation method.[87,95,104,106,107,111].

By introducing nHA on their surface, polymeric scaffolds become bioactive and osteoconductive. Therefore, it is considered that nHA functionalizes these scaffolds. Among this review, HA functionalized scaffolds, have shown a great capacity to stimulate attachment and proliferation of osteoblasts[94,97,99,115] or osteoblast like cells[96,100,102,103,111-113] and to facilitate the differentiation of mesenchymal stem cells towards osteoblastic lineage[88-90,95,100,108,109,114]. Other interesting fact is that in some studies, the original hydroxyapatite stoichiometry was changed. Liu *et al.*[112] used a partially carbonated HA in his assay, to mimic bone HA and produce a biomimetic nanofibrous-gelatin/nanoapatite as similar as possible to extracellular matrix. Li *et at.*[104] in the other hand, produced a silver ion substituted nano-hydroxyapatite, due to the known antimicrobial activity of silver ions or silver-based compounds. Nano-hydroxyapatite can provide a large reservoir of silver ions, which can be released gradually resulting in long-term antimicrobial activity. Finally, Liao *et al.*[99] have fabricated hydroxyapatite in form of nanorods incorporating carbon nanotubes to construct a nanocomposite with excellent mechanical properties which would be impossible to be reached with the pure HA crystalline form. Zhao *et al.*[109], have grown apatite nanoparticles on the surface of a silk fibroin-polyaspartic acid scaffold though an alternate soaking process. Moreover, these mineralized scaffolds were seeded with bone marrow stromal cells (bMSC) and implanted in dog's mandibles. *In vivo*, they demonstrated bioactivity, showing a bone mineral density very similar to the host bone. When compared to the same seeded scaffolds, without the nanoapatite particles, the first ones show a better *in vivo* outcome. Both the osteoconductive and osteophilic characteristics are then demonstrated in this study.

Silica xerogel was used by Lee *et al.*[110] as the inorganic constituent of the scaffold. It consists in solid nanoparticles formed from a silicate gel by drying with unhindered shrinkage. This material has demonstrated great promise for the biomedical field, since its composition can be turned so they can express, in this specific case, bioactive glasses characteristics. Moreover, low temperature processing enables them to carry biologically active agents useful for drug delivery, for example. Lee *et al.*[110] used this silica xerogel as the nanofiller of a homogeneous chitosan-silica xerogel hybrid nanocomposite. This nanocomposite proved to have excellent mechanical characteristics, great apatite forming ability as well as good cellular responses. Moreover, it promoted *in vivo* bone regeneration on rat calvarium bone defects.

Calcium phosphate cements have been attracting great attention due to their excellent biological behavior (e.g. biocompatibility, bioactivity and osteoconductivity). Nowadays, CPCs can be found commercially such as Eurobone® (Kasios®) or Calcibon® (Biomet®). In addition to their excellent biological behavior, the main advantages of CPCs are that they are presented in a particulated form and can be injected having the ability to harden *in vivo* at body temperature (*in situ*).[121] However, CPCs have major drawbacks: i) without any additives, they have poor injectability properties due to the liquid – solid phase separation, ii) CPC pastes tend to disintegrate upon early contact with blood or biological fluids due to their weak cohesion and finally and most important, iii) CPCs have very poor mechanical properties, not only in terms of strength, but in terms of toughness, brittleness and reliability.[122] Monreau *et al.*[114] created a chitosan/CPC injectable scaffold and tested the biological behavior of mesenchymal stem cell harvested from Wistar Hannover male rats. Firstly, he concluded that the adding of chitosan to the scaffold enhanced the mechanical properties of the CPC/chitosan nanocomposite. In addition, mesenchymal cells responded to the bioactivity of the CPC, adhering, differentiating and proliferating throughout the scaffold. Azami *et al.*[94] performed a similar study using a gel/amorphous calcium phosphate scaffold and assessing the biological behavior of human endometrial stem cells, namely if they differentiate to osteoblast cells. He concluded that human endometrial stem cells were successfully differentiated to osteoblast like-cells by the osteogenic media offered by the produced scaffold.

Bioactive glasses are composed by calcium and phosphate which are present in a proportion similar to the bone hydroxyapatite. These glasses have an excellent biocompatibility and a great ability to form surface apatite, bonding to bone. Kouhi *et al.*[98] used these materials as a nanofiller for a poly(ε-caprolactone) matrix. Though electrospinning, increased crystallinity nanofibers were produced and its biological behavior was accessed as well as the drug delivery of Simvastatin. The nanocomposite nanofibrous web demonstrated excellent bioactivity, inducing the precipitation of bone like apatite mineral on its surface under a simulated physiological medium. The nanocomposite nanofibrous web demonstrated excellent bioactivity, inducing the precipitation of bone like apatite mineral on its surface and the bioactive particles allow the controlled drug release of simvastatin throughout time.

Diopside ($CaMgSi_2O_6$) is a mineral that has been reported as a feasible biomaterial as a bone substitute since it demonstrated to possess more potential of apatite formation ability and higher mechanical strength than hydroxyapatite itself.[123] Ghorbanian *et al.*[96] produced a 3D porous scaffold by combining diopside nanocrystals with silk fibroin through a freeze-drying process. This combination turned out to provide better mechanical characteristics than the SF scaffolds alone. In addition, diopside increased the wettability of the scaffold, enhancing the cell migration towards the scaffold. The fact of having such good mechanical properties, makes this type of scaffolds an excellent resource for maxillofacial bone regeneration.

In vivo Assays

Biocompatibility is often used incorrectly with *in vitro* tests. In fact, biocompatibility can only be used in the case of animal or humans (*in vivo* tests), with the correct term being cytocompatibility for *in vitro* tests.[124]

Animal models are considered to be an essential step in the testing of orthopedic strategies, prior to clinical use in humans. When selecting the animal model, some factors should be taken under consideration: ability to observe numerous subject over a relatively short time frame, availability, cost to acquire

and care for animals, acceptability to society, lifespan suitable for the duration of the study, tolerance to captivity and ease of housing, low maintenance care, ease of handling, resistance to infection and disease, inter-animal uniformity, biological characteristics analogous to humans, tolerance to surgery, adequate facilities and support staff and an existing database of biological information for the species.[124] Specifically for study of bone regeneration materials, an understanding of the species specific bone characteristics such as bone microstructure and composition, as well as their bone modeling and remodeling properties is needed and these features should be as similar to human bone as possible. According to Pearce *et al.*[124], the most important characteristics that should be compared between human's bone and the animal model's bone are: Macrostructure, microstructure, bone composition and bone remodeling. The similarity between human and the most used animal model bone is compared in table 7.

Table 7- Summary of the four attributes in terms of similarity between animal and human bone [124]

	Canine	Goat	Pig	Rabbit	Rat
Macrostructure	++	+++	++	+	+
Microstructure	++	+	++	+	+
Bone composition	+++	++	+++	++	+
Bone remodeling	++	++	+++	+	+

Legenda: + least similar, ++ moderately similar, +++ most similar

No species fulfills all the requirements of an ideal model, since each animal model has its unique advantages and disadvantages, regarding mechanical and physiological approximation of human bone. International standards established that the species suitable for testing implementation of materials in bone are dogs, sheeps, goats, pigs or rabbits.

Among the *in vivo* referenced studies, rats stand out as being the most used animal (Figure 13), in both biocompatibility evaluation through non-osseous

implantation and osseous implantation. The use of rodents (rats and mice) is popular among research groups due to the fact that they are inexpensive, easy to house, have high availability and have a satisfactory immune system.[125] Therefore, these animals qualify for a starting point of the *in vivo* performance analysis of a bone regeneration material, which means their biocompatibility assessment through non-osseous implantation. Some authors[92,95, 106] reported in this review used this strategy. However, rodent's bone has a very significant dissimilarity to the humans bone at many levels. As a result, the regeneration outcomes of implanting biomaterials in rats bone can hardly be used to predict the materials behavior in humans.[124]

Rabbits are reported to be one of the most used animal models in medical research.[124] As rats, they have a major drawback related to its size that makes it not suitable for the assessment of multiple implant materials in the same model. In fact, international standards state that the maximum amount of implanted materials in rabbits is 6, which is half the maximum number of implants recommended for bigger sized animal models such as dogs or goats. Moreover, rabbits bone structure continues to be very different from human bone. Despite all this disadvantages, rabbits have a convenient bone maturity rate of 6 months after birth and a faster skeletal change and bone turnover. This allows faster results in the *in vivo* assays, but at the same time, moves further from the similarity to humans bone remodeling mechanisms. Zhang *et al.*[93], in their study, used rabbit mandibles to understand if the biomimetic nHA/polyamide scaffold that they produced was suitable for maxillofacial bone regeneration. The outcome of the assay is a complete regeneration of the created bone defects and based on it, the authors refer that this material has an interesting potential for maxillofacial reconstructive procedures in load-free areas, but further studies are warranted. Other referred authors[105,106] that recurred this animal models have a similar position, stating that they should be followed by larger animal models testing.

Goats arise as animal models that have a body size suitable for the implantation of multiple implants *per* goat. Moreover, and even though the composition of goat's bone does not match entirely human bone, goats have a metabolic and bone remodeling rate very similar to that of humans. However, the

rate at which a bone graft is vascularized and converted into vital bone structure is faster in goats, occurring at approximately 3 months, in comparison to 8 months in humans. Only one of the reported articles, Fricain *et al.*,[95] used goats in his study. In fact, by using two different animals (mice and goat), the authors ensured the great potential of the created pullulan/dextran/nHA nanocomposite, since the change of environment did not compromise the successful regeneration final outcome.

In terms of bone composition and density, dogs and pigs are the animal models that most closely represent the human anatomy and phisiology. However, and even though pigs demonstrate a great likeness with human bone, difficulties may arise in relation to their increasing size with time, which makes it an inconvenient animal model.[95] In terms of composition, water fraction, organic fraction, volatile inorganic fraction and ash fraction of dog and human's bone are reported to be extremely similar.[126] Moreover, the load bearing capacities of human's bone is comparable with dog's bone, especially in the maxillofacial area, even though bone loading in dogs is bigger. Zhao *et al.*[109] choose to evaluate the maxillofacial regenerative capacity of an apatite-coated silk fibroin scaffold in dog mandibles, combining the scaffold with mesenchymal stem cells extracted from the same animal. As a result, he obtained excellent mineralization of the canine inferior mandibular border defects, the scaffold was able to bear the high bone loading of that specific site and the author successfully proved that this nanocomposite strategy is suitable for maxillofacial applications.

a) Characterization and properties of the nanocomposites

Nanocomposite scaffolds, in order to be considered as part of a valid bone regeneration strategy, have to be cytocompatible. Another essential property of cytocompatible scaffolds is its surface properties and surface to bulk ratio, which increases with increasing porosity[80] or decreasing size in particulate systems.[65] Depending on the specific application it is possible to find nanocomposites in different forms. For instances, Zhang *et al.*[93] developed a porous hydroxyapatite/polyamide nanocomposite to implant in mandibular critical size defects, while Fu *et al.* [106] created an injectable nanocomposite with a poly(ethylene glycol)-poly(ε-caprolactone)-poly(ethylene glycol) (PEG-PCL-PEG) copolymer and collagen matrix filled with nano-hydroxyapatite particles for bone injection in a minimally invasive manner. When considering applications where a considerable extension of bone needs to be regenerated porous scaffolds are preferred to particulate systems due to its superior mechanical stability. A porous scaffold can provide an ideal physical structure for bone cells to infiltrate the scaffold and to produce new bone. Additionally, it contributes for implant stability by biological fixation.[93] A porous structure can be induced as a consequence of the processing method. Processing methods that induce porosity can use temperature as in the phase inversion process reported by Li *et al.*[34] and Liu *et al.*[112] or can evaporate a component, as reported by Li *et al.*[104,107] Freeze-drying (also called lyophilization or solvent sublimation), is another technique used to induce porosity that results from freezing a solution and then allowing the frozen water in the material to sublimate by reducing the pressure as reported in this review.[87,94-96,103,106,108,110,111] Inducing porosity by a chemical way is also possible, by using a chemically induced phase inversion, as Raucci *et al.*[88] described or by using organic solvents, as presented by Nemati *et al.*[115]

Particle leaching is another technique that has been used to induce porosity. It consists in using a porogen (which can be constituted by different materials that typically must have a high dissolution capacity) dispersed in the

material's bulk and removing it by a leaching technique. Once the porogen is dissolved, a porous structure is formed. Raucci *el al.*[88] and Wang *et al.*[89] have used this technique with sodium chloride particles, which are leached out after immersion in water (the so-called salt-leaching technique) . Liu *et al.*[112] used paraffin in a matrix of gelatin that was further soaked in hexane to leach out paraffin spheres. One of the advantages of this technique is that the porosity can be easily controlled by the size and shape of the sacrifice porogen.

Processing methods such as electrospinning, create a material constituted by randomly disposed fibers, generating a subsequent porous structure. Several authors[90,97,98,100,102] have used electrospinning to produce porous scaffolds. However, significant challenges still exist in using this technique to fabricate complex 3D scaffolds shapes or to generate designed internal pore structure, limiting its potential for many tissue engineering applications.[80]

The size, number of pores and its interconnectivity are as important as their existence. It was reported[127,128] that a satisfactory porosity level is achieved for percentages of more than 80% and that the ideal pore size is between 200 and 400 µm.[129,130] Moreover, the interconnectivity of pores together with the pore size can modulate cellular ingrowth in the scaffold.[130,131] Pores of less than 10 µm inhibit cellular in-growth, while pores between 15 and 50 µm help fibrovascular colonization. Pores between 50 and 150 µm determine osteoid growth and pores higher than 150 µm facilitate internal mineralized bone formation.[131] From the 30 selected articles, only 8 specified the percentage of porosity existing in the nanocomposite and 13 specified the pore size. Among these, Liu *et al.*[112] and Tan *et al.*[108] were able to create the highest pore percentages, with 97,5 % and 97,9%, for a gelatin/apatite nanocomposite and a calcium alginate hydrogel as matrix to carry nHA and collagen particles, respectively. In fact, Tan *et al.*[108] was able to establish a direct relationship between the concentration of calcium alginate in the nanocomposite and the pore percentage – as the alginate concentration increases, porosity decreases – achieving high porosity level with a 2% alginate concentration. Other authors achieved lower amounts of porosity such as 65 - 70%[93], 70%[96], 72% ± 4%[34], 82%[94] and 88,5%[107]. Regarding pore size, almost all articles that mentioned this value are included among the considered ideal gap from 200 µm to 400 µm. However, if in one hand porosity

enhances the biological capacities, in the other, it decreases the nanocomposite's mechanical properties, making the structure too brittle and fragile.[103] The work of Tan *et al.*[108] constitutes a good example of this, reporting that the nanocomposite that he created and evaluated has excellent biological response *in vivo*, but does not fulfill the mechanical requirements for being implanted in a load bearing site.

Other very important property of a nanocomposite surface is hydrophilicity. In fact, a certain affinity to water can help to immobilize growth factors and diffuse nutrients in bone native tissues and scaffolds, enhancing the adhesion of host cells. To support this theory, Fuard *et al.*[132] proved that the addition of a hydrophilic substrate (Poly-Di-Methyl-Siloxane) to a silicone polymer structure highly favors the cell adhesion. However, this authors were not able to establish the ideal value of hydrophilicity. In order to measure the hydrophilicity of a nanocomposite, water angles should be measured and analysed - the smaller water contact angles are, the best hydrophilicity is presented by the nanocomposite. Ghorbanian *et al.,*[96] by measuring the water contact angles in a diopside/silk fibroin scaffold with and without the diopside nanoparticles, was able to conclude that, as the diopside content increased, the water contact angle decreased. This means that diopside nanoparticles conferred better hydrophilicity, which is this case has provided a better environment for cell attachment.

b) Biodegradation and mechanical strength

Since bone regeneration strategies are commonly intended to serve as temporary replacement for the extracellular matrix, they should present, besides excellent biocompatibility, suitable biodegradability and sufficient mechanical strength to ensure tissue functionality.[106] In other words, the mechanical load should be supported initially by the scaffold and gradually transferred to the newly forming bone, according to the biodegradation profile of the composite.[55] Within a nanocomposite, it can be ideally considered that the bioceramic filler will induce a bioactive behavior towards bone regeneration by self-degradation, so the space formed by that degradation can be replaced by new bone. On the other hand, the

polymer matrix would have to degrade slowly, to compensate the quick degradation of the nanofiller, so both materials end up giving space newly formed bone.

Among the retrieved published papers in the last 5 years several studies propose nanocomposites based on synthetic polymers and others on natural polymers. Polycaprolactone, is a very interesting polymer, because it offers two excellent degradation features: it degrades slowly in an *in vivo* environment, showing a good mechanical stability while the degradation process and its degradation products do not generate an acidic environment in the surroundings. Therefore, this polymer does not cause a major inflammatory reaction.[97] These reasons can help to explain the fact that polycaprolactone is the most used polymer in the articles that constitute this review. [88,89,97,98,101]

Silk fibroin, due to its structural conformation, degrades considerably slowly as well and has relatively good mechanical properties. However, Kweon *et al.*[105] reports that during an *in vivo* study using a scaffold made of nano-hydroxyapatite implanted in rabbit's parietal bony defects, an inflammatory reaction appeared which was attributed to degradation of the silk graft materials. In contrast, Zhao *et al.*[109], used a silk-fibroin matrix in a *in vivo* assay in dog's mandibles and did not report any inflammation episode.

Gels, hydrogels and other gelatin-based matrixes were the selected polymers for some of the analyzed nanocomposites[94,100,103,106,108]. This class of nanocomposites are considered very attractive because they have good biocompatibility, low immunogenicity, low cost, biodegradability, increased cell adhesion, migration, differentiation and proliferation. However, their very poor mechanical properties limit its application, especially for load bearing tissues or very big defects. The addition of nanomaterials as structural reinforcement is an interesting solution for this problem. Nanoparticles, because of the physical properties associated to their size, immediately work as reinforcement materials. Chiu *et al.*[103] found that the addition of nHA was not enough for significantly improving the low mechanical properties of the gelatin matrix and proposed to reinforce it with TiO_2 nanoparticles and a siloxane cross linker. Interestingly, the presence of these particles reduced the gelatin's degradation rate.

In order to access the biodegradation properties of the polymer-ceramic evaluated nanocomposites, some authors performed *in vitro* degradation assays. Thermal degradation tests[87,103] and weight loss measurements after immersing the scaffolds in phosphate buffered saline[89,98,102,110] or in fetal bovine serum[90] are the most common methods to investigate the degradation kinetics of a scaffold.

In order to evaluate the mechanical properties, the main performed tests were compressive tests to measure the compressive strength and respective modulus. The elastic modulus was measured in some studies as well. It is important to refer that the aim of any biomaterial used for bone regeneration is to match the mechanical properties of native bone. Zhang *et al.* [93], for example, was able to produce a porous hydroxyapatite/polyamide nanocomposite that possessed a compressive strength of 3-7 MPa, which is similar to living cancellous bone (2-15 MPa), [93] being therefore considered to be a good candidate for *in vivo* applications. Additionally, Azami *et al.*[94] was able to determine that the elastic modulus of a biomimetic nanocomposite scaffold based on a GEL/calcium phosphate constitution is 80 ± 5 MPa. This value is comprised in the known elastic modulus of spongy bone (20-500 MPa).

c) *In vitro* bioactivity evaluation

Bioactivity is defined as the capacity of a certain material to bond to natural body structures, like bone or soft tissues. In polymer-ceramic nanocomposites, the bioactivity is related with the nano-bioceramic used in its composition. Therefore, the nanocomposite's bioactivity can be defined as the ability of the ceramic component to establish a chemical bond with the host bone tissue. This includes enhancing the ability of apatite formation, osteoblast differentiation and bone matrix formation.[133] Currently, two common methods have been used for testing the *in vitro* bioactivity. One method is to evaluate the apatite-formation ability of bioceramics in the simulated body fluid solutions (SBF). The other complementary method is to investigate the *in vitro* bone-related cell response to assayed materials. In this review, some of the *in vitro* studies have performed the SBF assay,[98,101,110] while others have used biological assays using bone-related cells.[87,94,96,97,99,102-104,110-113,115]

Bioactivity assessment using a SBF solutions was reported as being an effective way of reproducing the formation of bonelike apatite on the surface of the tested material.[134] However, when this procedure is used, it is important to understand that the biochemistry of *in vivo* bone formation is different for each bioceramic. Silicate-based bioceramics, as silicate bioglasses or diopsides, bond to natural bone through the formation of bone like apatite layers due to the dissolution of Ca^{2+} or other metal ions, followed by the deposition of CaP in the surrounding tissue.[133] In contrast, phosphate-based bioceramics such as HA or β-TCP, bond to natural bone directly.[133] Therefore, nanocomposites that have in their constitution a silicate-based bioceramic, are more likely to produce apatite crystals on its surface when exposed to SBF then nanocomposites having phosphate-based bioceramics.[133] It can be then considered that the bioactivity test using SBF alone would be better complemented by an *in vitro* cell study to confirm the results. In his article, Lee *et al.*[110] uses both assays to evaluate the bioactive behavior of the hybrid chitosan-silica xerogel membrane, but Rezaei *et al.*[101] on the other hand, finds the SBF assay sufficient to evaluate the bioactivity of a hydroxyapatite/polycaprolactone (HA/PCL) nanocomposite even though it is constituted by a silicate-based bioceramic.

At a cellular level, the nanocomposites were tested using two major cell types: (a) mesenchymal stem cells, which are multipotent stromal cells that have the capacity to differentiate in different kind of cells according to the external stimuli and (b) osteoblastic cell lines which are already differentiated bone-like cells with the ability to produce mineralized bone. The bioactivity of nanocomposites was then tested by evaluating if they had the capacity of stimulating mesenchymal cells to differentiate into an osteoblastic cell line[88,114] or if they were able to promote osteoblasts adherence, proliferation and ECM production.[99,102,113] In both cases, the consequence is native bone formation.

Grow-factors Incorporation

Bone healing is a complex integrated process where there is temporal-spatial interaction between various factors resulting in regeneration. During bone healing, the body itself generates a natural scaffold on which cells differentiate and under influence of various factors ultimately leads to timely regeneration of the lost tissues. Thus, an interesting approach to successful bone tissue regeneration is to provide the repair site with sufficient osteogenic progenitor cells in a suitable deliver vehicle to ensure osteoblastic differentiation and optimal secretory activity.[135] Li *et al*.[34] studied the effect of MSCs transfected with BMP-7 implanted in an n-HA/PA scaffold. After an *in vivo* evaluation of the bone regeneration capacity of this assembly in rabbit mandibles, the authors concluded that MSCs accelerated the bone regeneration after implantation during the early stages.

However, BMPs are unstable and high-doses therapies are costly. In order to find a solution for this disadvantage, Li *et al.*[107] created P24, a novel short peptide obtained from peptides 73 to 92 of the knuckle epitope of BMP-2 and incorporated it in a nHA/collagen/poly(L-lactic acid) scaffold, using the biodegradable properties of the poly(L-lactic acid) to promote a controlled release of this novel peptide. After assessing the improved osteoinductivity of P24 *in vitro*, the authors implanted the same nanocomposite with P24 and with rhBMP2 in rats to assess if their bone regeneration capability. They were able to conclude that the incorporation of 3 mg of P24 in the nanocomposite resulted in a similar effect that identical nanocomposites filled with 1 mg of rhBMP-2.

Antibacterial Agents Incorporation

Nanocomposites implanted *in vivo* are often affected by bacterial colonization. If the aimed regenerating site is the alveolar bone, bacteria like *Porphyromonas gingivalis*, *Streptococcus mutans* and *Fusobacterium nucleatum*, which are the major pathogens of periodontitis and periimplantitis are likely to infect the nanocomposite and result in an inflammatory reaction and

subsequent failure of the bone regeneration procedure. Therefore, the incorporation of an antibacterial component can be an effective way to improve the nanocomposite functionality. Silver ions (Ag+) in the composite membrane can inhibit the adhesion and proliferation of bacteria. It has been reported[136] that Ag+ can cause bacterial inactivation by binding both to microbial DNA and to the sulfhydryl groups of the metabolic enzymes of the bacterial electron transport chain as well as by preventing bacterial replication. Two authors[92,104] considered in this dissertation used Ag to accomplish an antibacterial effect of the nanocomposite. The first,[95] used a composite slurry from silver ion-substituted nano-hydroxyapatite, titania nano-particles and polyamide 66 to create a novel antimicrobial membrane. The kinetics of the antibacterial release was positively assessed, since the selected biodegradable polymer provided the ideal conditions to a controlled release of the silver ions. Additionally, this nanocomposites provided a complete closure of 5-mm bone defects created in the skull of rats, without demonstrating any adverse reaction. The second, [97] used the exact same membrane, produced by the previous author,[95] and studied its biosafety, biocompatibility and regeneration capacity, using an e-PTFE membrane as a positive control. By subcutaneous implantation, the nanocomposite in study proved to possess a great biocompatibility, showing no adverse reactions. By osseous implementation, it proved to have osteogenic activity comparable with conventional e-PTFE membranes, a material that has excellent osteogenic properties, but is non-resorbable, having the huge disadvantage of needing a second surgical procedure, as already referred through this study.

V. Conclusions and Future Perspectives

V. Conclusions and Future Perspectives

Polymer-ceramic nanocomposites are innovative materials that due to their constitution can allow for mimicking the complex architecture of native bone tissue. The introduction of theise materials in the field of bone regeneration field is sustained by a good mechanical performance allied with great biocompatibility *in vivo*, leading to a successful outcome.

In order to accomplish great performance, different types of polymers and ceramic nanofillers are joined together. Polymers can be natural or synthetic and biodegradable or non-biodegradable, and these characteristics determine whether they constitute a good candidate as a nanocomposite matrix or not. In fact, it was concluded that biodegradable polymers such as polycaprolactone are suitable for bone regeneration, since they are degraded *in vivo* though a specific degradation rate, providing space for new bone to grow and provide the regeneration of the implanted defect successfully. Moreover, synthetic polymers are proven to be better for integrating nanocomposites, since the can be modeled to have suitable properties, conferring in the end better mechanical and biological behavior. Natural-based polymers have been gaining more and more interest in the latest years mostly since they can better mimic the extracellular matrix of bone, can provide an adequate environment for establishing a chemical bond with the inorganic nanofiller and also because they are able to generate non-toxic products upon their degradation process.

As far as nanofillers are concerned, nano-hydroxyapatite demonstrated to be the most studied and reliable option. Because it matches with the natural ceramic component of bone, its dispersion in the polymeric matrix ensures bonding to bone structure and stimulates native regeneration.

A good dispersion of the nanoparticles in the polymeric matrix, provided by an effective incorporation technique is essential to ensure mechanical properties, since it avoids the creation of voids that provide conditions for cracking as well as of nanoceramic agglomerates that leads to the nanocomposite failure. Incorporation protocols such as melt blending, alternate soaking and high pressure solidification proved to be effective.

Porosity of the final regeneration scaffold is essential for providing physical structure for bone ingrowth. It can come as a result of the nanocomposite construction or it can be stimulated by specific procedures. Size and number of pores has to be taken in consideration as well.

The addition of other components to nanocomposites, can enhance their features. Adding silver nanoparticles confers antibacterial properties to the scaffold and titanium particles apparently increase bone formation *in vivo*. Moreover, seeding stem cells from the host to the nanocomposite, increases its bioactivity. When implemented in the nanocomposite, stem cells receive specific stimuli and differentiate into bone-cells that have the ability to produce new bone.

In the future, much more research is needed to understand the mechanism of nanocomposite–tissue interactions and to optimize the composition, structure and properties of different polymer-ceramic nanocomposites, in order to finally extract the full potential of nanocomposites for bone tissue regeneration.

VI. Bibliography

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VI. Annexes

Annex 1 – Research Diagram