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In vitro biological assessment of silicon substituted hydroxyapatite: Angiogenesis and Osteogenesis

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Aos meus Pais, à Teresa e ao João

"Por vezes sentimos que aquilo que fazemos não é senão uma gota de água no mar.
Mas o mar seria menor se lhe faltasse uma gota."
Madre Teresa de Calcutá

Resumo

Um substituto ósseo deve não só promover a regeneração óssea, mas também favorecer o desenvolvimento de uma rede microvascular. A hidroxiapatite modificada com silício é um material bioactivo e osteocondutor, que permite a proliferação e a diferenciação celular.

A primeira parte do trabalho consistiu na caracterização físico-química e estrutural da Hidroxiapatite (HA) e da Hidroxiapatite modificada com Silício (SiHA) como materiais densos e na forma de revestimentos. O titânio é usado como biomaterial em aplicações biomédicas. Apesar das suas boas propriedades mecânicas e de resistência à corrosão, o titânio não tem a capacidade de formar uma ligação ao tecido ósseo, sendo assim este deve ser revestido com um cerâmico bioactivo, já que estes estimulam a ligação biológica entre o implante e o tecido ósseo.

Realizaram-se estudos *in vitro* com culturas de células endoteliais cultivadas em amostras densas de hidroxiapatite (HA) e de hidroxiapatite modificada com silício (SiHA), assim como a avaliação dos parâmetros de crescimento celular e da actividade funcional em determinadas fases do período de cultura, representativas da proliferação/diferenciação celular. Verificou-se que a incorporação de Si^{4+} na malha de HA estimulou a adesão das células endoteliais ao substrato comparativamente com a HA.

O processo de angiogénese pode ser definido como a formação de uma rede vascular a partir de vasos sanguíneos pré-existentes e o processo de osteogénese como a formação de novo tecido ósseo. Estes dois processos estão intimamente relacionados, de modo estrutural, bioquímico e funcional. Sendo assim, após se verificar o efeito positivo do silício na angiogénese em amostras densas, procedeu-se ao estudo da osteogénese da hidroxiapatite modificada com silício, mas na forma de revestimentos da liga de titânio (Ti-6Al-4V). Os resultados obtidos nestes estudos corroboram os resultados descritos na literatura, os quais demonstram o efeito positivo do silício nas células osteoblásticas.

Estes estudos demonstram que a hidroxiapatite modificada com silício quer na forma densa, quer na forma de revestimento pode estimular a regeneração do tecido ósseo.

Abstract

A bone graft should promote bone regeneration and stimulate the development of a vascular net. According to the literatures, the silicon substituted hydroxyapatite is a bioactive and osteoconductive material that stimulates cellular proliferation and differentiation.

In the first part of this thesis the silicon substituted hydroxyapatite (SiHA) and hydroxyapatite (HA) in a dense and coating form were characterized.

Titanium and titanium alloys can be used as biomaterial in several biomedical applications, although its good mechanical properties and corrosion resistance, titanium is not a bioactive material, so it should be coated with a bioactive ceramics, that can form a biological bond between the graft and bone tissue.

In vitro biological studies were performed by seeding endothelial cells in dense samples of HA and SiHA. The colonized materials were evaluated for cellular growth parameters and functional activity at different time points of culture period. The Si^{4+} incorporation in HA lattice induce a positive effect on the development of the endothelial cells. The number of endothelial cells that adhered to SiHA substrate is higher than the number of cells present on the surface of HA.

The angiogenesis process can be defined as the formation of vascular net from preexisting vases and the osteogenic process as the formation of new bone tissue. These processes are structurally, biochemically and functionally related. It has been shown that the presence of silicon stimulates the adhesion of endothelial cells and that in accordance to the literature the osteogenesis in the dense form. So, the second part of this thesis aimed to performed *in vitro* studies with human osteoblastic cells cultured on the surface Titanium alloy coated with HA and SiHA. The results obtained corroborate previous results described in the literature, which showed the positive effect of silicon on bone cells.

Objectives

The aim of this thesis was to study the behaviour of human umbilical vein endothelial cells seeded on the HA and SiHA as dense form, and to study the behaviour of human osteoblastic cells seeded on the HA and SiHA as coating material of titanium alloy, to understand the importance of the silicon incorporated into HA lattice in the angiogenesis and osteogenic process.

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List of Abbreviations

Ang	Angiopoietins
BMPs	Bone morphogenetic proteins
CLSM	confocal laser scanning microscopy
ECs	Endothetial cells
ECM	Extracellular matrix
EPCs	Endothelial progenitor cells
FGF	Fibroblast growth factor
FTIR	Fourier transform infrared
HIFs	Hypoxia-inducible transcription factors
HSCs	Haematopoieitic stem cells
HSPGs	Heparan sulfate proteoglycans
HUVECs	Human Umbilical Vein Endothelial Cells
Id	Inhibitor of differentiation
IMG	Intussusceptive microvascular growth
KBr	Potassium Bromide
MMPs	Matrix metalloproteinases
NO	Nitric oxide
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PECAM-1	Platelet/endothelial cell adhesion molecule-1
PF	Platelet factor
PIGF	Placental growth factor
RTKs	Receptor tyrosine kinases
SEM	Scanning electron microscopy
SMCs	Smooth muscle cells
TGF	Transforming growth factor
TIMPs	Tissue inhibitors of metalloproteinases
TSPs	Thrombospondins
VEGF	Vascular endothelial growth factor
VEGFRs	VEGF signalling receptors
XRD	X-ray powder diffraction
TCP	Tricalcium phosphate

CHAPTER 1
GENERAL INTRODUCTION

CHAPTER 1

1. The bone

Bone is a dynamic connective tissue characterized by its hardness, growth mechanisms and ability to regenerate. It has the functions of support and protection the internal organs and provide attachment for muscles, facilitating the locomotion process. Besides this, bone offers protection for blood-forming marrow and it is the reservoir of mineral ions such as calcium and phosphorous [1].

1.1 Bone composition

Bone is a composite material consisting of an organic matrix that is strengthened by deposits of calcium salts. Type I collagen constitutes approximately 95% of the organic matrix. The remaining 5% is composed of numerous noncollageneous proteins such as: proteoglycans, osteonectin, osteocalcin, osteopontin, sialoprotein, glycoproteins, enzymes and cytokines. Bone apatite is a calcium and hydroxide deficient apatite containing numerous ionic substitutions, such as carbonate, magnesium, potassium, fluoride, sodium, phosphate and others [2-4].

1.2 Bone cells

The cells that are responsible for the structural and functional properties of bone are: osteoprogenitors cells, osteoblasts, osteoclasts and osteocytes.

Osteogenics cells (osteoprogenitors)

This type of cells is mainly in the deepest layer of the periosteum and the endosteum. They have a high mitotic potential and are recruited to repair bone defects [5].

Osteoblasts

Osteoblasts are fully differentiated cells responsible for the bone matrix production. They are typical protein-producing cell, witch secrete type I collagen, noncolageneous proteins of bone matrix and regulate the mineralization of bone matrix. Osteoblasts are derived from mesenchymal stem cells. Factors such as bone morphogenetic proteins

(BMPs) and transcription factors (Cbfa 1) mediate and regulate the induction of mesenchymal stem cells into osteoblastic cells [5,6].

Osteocytes

The osteocyte is a mature osteoblat within the bone matrix and it is responsible for bone maintenance. Each esteocyte occupies a lacunae in the matrix and allows the diffusion of nutrients and metabolites through the mineralised matrix, they develop filipodia connections witch enhance communication between neighbouring osteocytes, bone surface and blood vessels (fig. 1) [5,6].

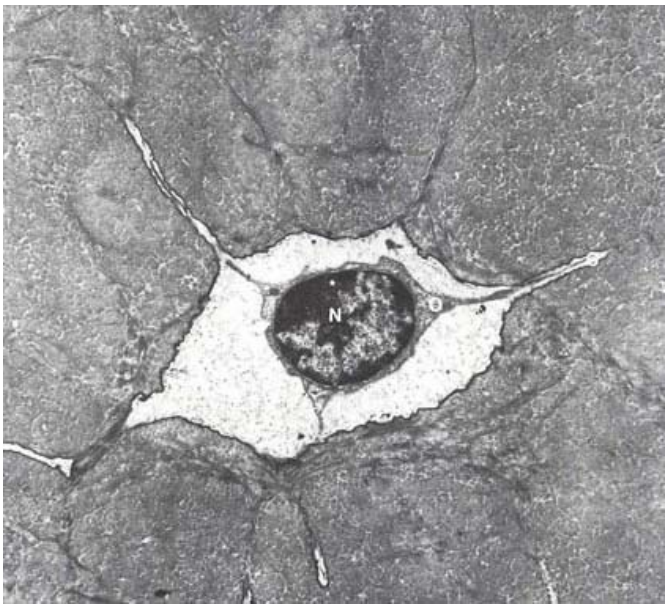

Figure 1 – A microscopic image of an osteocyte [5].

Osteoclasts

Osteoclasts are multinucleated cells responsible for bone resorption. They originated from hematopoetic tissue with common differentiation pathways with macrophages. This type of cell has multiple circumnuclear Golgi stacks, abundant lysosomal vesicles and a high density of mitochondria [5].

1.3 Bone Structure

Histologically there are two histologically defined bone types: dense bone (also known as compact or cortical bone) and cancellous bone (also known as trabecular or spongy bone).

Cortical bone is dense and forms the surface of bones, contributing 80% of the weight of the human skeleton. It is extremely hard, formed of multiple stacked layers with few gaps. In dense bone the tissue is organized in Harvesian systems (osteons) which consist of a channel containing a blood vessel surrounded by concentric and interstitial lamellae (fig. 2) [7-9].

Cancellous bone is spongy, has a low density and strength but has a very high surface area, it fills the inner cavity of long bones. The external layer of cancellous bone contains red bone marrow where the production of blood cellular components (known as hematopoiesis) takes place. Cancellous bone is also where most of the arteries and veins of bone organs are found [7-9].

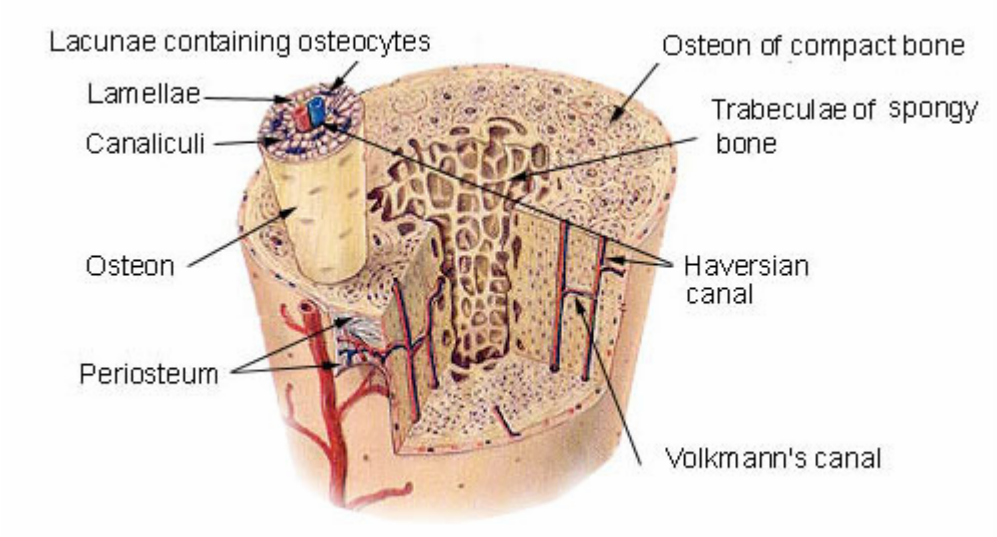

Figure 2 – Compact and cancellous bone [5].

1.4 Bone Growth

The formation of bone during the foetal stage of development occurs by two methods: intramembraneous and endochondral ossification.

Intramembraneous ossification mainly occurs during formation of flat bones of the skull. The steps leading to intramembraneous ossification are: development of ossification centre, calcification, formation of trabeculae and development of periosteum. This type of bone deposition occurs in interaction with vascular tissue [8,9].

Endochondral ossification occurs in long bones, such as limbs. The steps in endochondral ossification are: development of the cartilage model, growth of cartilage model, development of the primary ossification centre, development of medullar cavity,

development of the secondary ossification centre, formation of articular cartilage and epiphyseal plate. Endochondral ossification begins with points in the cartilage called primary ossification centres. They mostly appear during foetal development, though a few short bones begin their primary ossification after birth. They are responsible for the formation of the diaphyses of long bones, short bones and certain parts of irregular bones. Secondary ossification occurs after birth, and forms the epiphyses of long bones and the extremities of irregular and flat bones. The diaphysis and both epiphyses of a long bone are separated by a growing zone of cartilage (the epiphyseal plate). When a child reaches skeletal maturity (18 to 25 years of age), all of the cartilage is replaced by bone, fusing the diaphysis and both epiphyses together (epiphyseal closure). The intrinsic significance of blood vessels in this process is markedly evidenced by the fact that an initial avascular cartilage template becomes replaced by highly vascularized bone and marrow tissue [10].

1.5 Bone remodelling

Remodelling is the process of continuous shaping of the bones, and is the skeletal process that allows mineral ion homeostasis. In healthy bones, the remodelling process involves the coupled actions of bone forming and bone resorbing cells, in an ordered process. The remodelling process involves recruitment of osteoclasts to a point on the bone surface where a resorption pit is created by the removal of bone mineral and matrix. When the osteoclasts moves away, osteoblasts move in and fill the pit with an osteoid which is then mineralised [8,9].

1.6 Bone healing

Bone healing is a proliferative physiological process that requires a timely recruitment of skeletal progenitor cells to the injury site, their proliferation and diferentiation into condrocytes and osteoblasts, the reestablishment of a vascular net to maintein cell viability and bone regeneration through both endochondral and intramembranous ossification mechanism [8].

Bone healing can be arbitrarily divided into overlapping phases: inflammatory, reparative and remodeling phases.

After bone fracture or graft placement, an inflammatory response can occur. Following an injury, the bone is torn and blood vessels are disrupted, leading to the

formation of a haematoma. Close to the fracture line, necrotic marrow and dead bone accumulate and elicit an inflammatory response.

In the reparative phase, mesenchymal progenitors are recruited and differentiate into chondrocytes or osteoblasts. The early reparative phase involves formation of (fibro)-cartilage throughout the callus, particularly in domains distant from ingrowing capillaries (internal callus), and of immature woven (spongy) bone predominantly in subperiosteal areas with profound angiogenesis (external callus). Through endochondral ossification, (fibro)-cartilage is replaced with mineralized bone, forming a hard callus bridging the fracture gap.

In the remodeling phase, osteoclasts and osteoblasts continue to remodel the large callus, ultimately restoring the former shape, strength and functioning of the bone [9-11].

2. Angiogenesis

Angiogenesis is a physiological process that leads to the formation of new blood vessels from pre-existing vessels, by the migration and proliferation of endothelial cells [12-14]. This process occurs during growth and development, wound healing, and in the female reproductive system. Angiogenesis also occurs in different pathological processes such as cancer (Carmeliet and Jain, 2000; Patan, 2004), rheumatoid arthritis, diabetes and cardiovascular diseases. This process is complex and the mechanism behind angiogenesis is not fully understood, although several stimulating factors are known.

2.1 Vessel growth

Small blood vessels are only composed by endothelial cells (ECs), while larger vessels are composed by mural cells, pericytes in medium-sized and smooth muscle cells (SMCs). Endothelial cells are oblong shaped cells that cover the lumen of all blood vessels as a single epithelial cell layer, and they are derived from angioblasts and hemangioblasts [15]. These cells play a major role in vascular biology under normal or pathological conditions (Wiel et al, 2005), including the control of vasoconstriction and vasodilatation (Cosentino and Volpe, 2005), thrombosis and fibrinolysis (Wiel et al, 2005; Chen and Lopez, 2005), angiogenesis (Gerhardt and Betsholtz, 2005; Szekanecz and Koch, 2005), leukocyte adhesion/trafficking and inflammatory processes (Cook-Mills and Deem, 2005;

Aird, 2005; Szekanecz and Koch, 2005). Immature circulating endothelial cells derived from the bone marrow are being referred to as endothelial progenitor cells (Khan et al, 2005).

Vessels can growth by different manners. *Angiogenesis* denotes the formation of new blood vessels from pre-existing ones, while *vasculogenesis* is the term used for the formation of new blood vessels when there are no pre-existing ones by endothelial progenitors, during embryogenesis. Vasculogenesis is related to *in situ* differentiation and growth of blood vessels from mesodermal derived hemangioblasts. Angiogenesis comprise two different mechanisms: endothelial sprouting and intussusceptive microvascular growth (IMG). *Angiogenesis* and *arteriogenesis* refer to the sprouting and subsequent stabilization of these sprouts by mural cells, and collateral growth denotes the expansive growth of pre-existing vessels, forming collateral bridges between arterial networks [14-16]. *Lymphangiogenesis* refers to the growth and formation of new lymphatic vessels, which occurs in a normal development of tissues and also in pathological process (Al-Rawi et al, 2005).

2.2 Endothelial Progenitors

The endothelial progenitor cells (EPCs) contribute to vessel growth in embryo, ischemic conditions, malignant or inflamed tissues in adults [16]. Haematopoietic and other bone-marrow–derived stem cells might be recruited in the context of ischemia to induce neovessel formation [17]. The EPCs have been investigated as therapeutic agents in supply-side angiogenesis under pathological and physiological conditions [18]. ECs can be differentiated from angioblasts in the embryo and from EPCs, mesoangioblasts, multipotent adult progenitor cells, or side population cells in the adult bone marrow [17,19]. EPCs can also contribute to vessel growth by releasing angiogenic growth factors, like the vascular endothelial growth factor (VEGF), placental growth factor (PIGF), angiopoietin (Ang)-1, inhibitor of differentiation (Id) proteins, and different cytokines [20]. EPCs, haematopoietic progenitors and their descendents share common markers and are affected by common signals that will influence each other [21-23]. Identification of the signals that recruit or differentiate these progenitors offers opportunities to manipulate their contributions to vascular growth. The functional contribution of EPCs and haematopoietic stem cells (HSCs) to pathological angiogenesis still undefined [15, 22-25].

2.3 Vascular cell specification

Arteries and veins are distinct vessels anatomically and physiologically. They differ in blood pressure, thickness of their smooth muscle cells (SMC) coat and, ECs and SMCs have a distinct identity and origin [15]. Recent genetic studies show the signals that control the arterial and venous identities of ECs. The Notch pathway, with its ligants and receptors, promotes arterial destiny of ECs by repressing venous differentiation. Sonic Hedgehog and VEGF act upstream, whereas Gridlock probably acts downstream of Notch to determine arterial destiny, even before the onset of flow [26-28]. ECs have a phenotypic plasticity because they can differentiate into either arterial or venous ECs in embryonic development, in neonatal retina and even in adult heart. It means that a selective use of arterial or venous ECs or their precursors may be useful for therapeutic vasculogenesis [29].

Blood vessels in various tissues have specialized functions and ECs have distinct properties, probably as many as the organs in the body. The expression and activity of general angiogenic factors such as VEGF or Ang-1 varies greatly in different tissues. Further more, organ-specific angiogenic factors determine the angiogenic switch, but in a restricted manner in particular organs [30,31].

EPCs differentiate to arterial and venous ECs, which assemble in a primitive capillary plexus. Vessels then sprout and become stabilized by SMCs, differentiating from their progenitors. HSCs contribute to angiogenesis directly and indirectly, by differentiating to leukocytes or platelets. A demarcation of arterial and venous boundaries are required, as well as the establishment of vascular polarity. Ephrin-B2, an Eph family transmembrane ligand, marks arterial ECs and SMCs, whereas EphB4, a receptor for Ephrin-B2, marks only veins. Ephrin-B2-Eph4 participates in the formation of arterio-venous anastomoses by arresting EC migration at the arterial-venous interface [32,33,34].

2.4 Angiogenesis and arteriogenesis

The regulation of angiogenesis by hypoxia is an important component of homeostatic mechanisms that link vascular oxygen supply to metabolic demand. Initially, cells are oxygenated by simple diffusion of oxygen, but when tissues grow beyond the limit of oxygen diffusion, hypoxia triggers vessel growth by signalling through hypoxia-inducible transcription factors (HIFs) [35].

ECs built resistant channels and efficiently distribute blood to the various parts of the body. They have long half-lives, several years, but when triggered are capable of rapidly sending out sprouts in a coordinated and directional manner. Cells within the vessel wall communicate with each other and with cells inside and outside of vessel lumen, they sense changes in blood flow and pressure, and dynamically interact with the internal cytoskeleton and surrounding ECM. When ECs migrate during vessel sprouting, these contacts are transiently dissolved but later re-established, once ECs assemble a new sprout [15].

Cellular interactions with the extracelular matrix (ECM) contribute to the biochemical processes that regulate angiogenesis. The ECM provides necessary contacts between ECs and the surrounding tissue, prevents vessels from collapsing, and regulates the formation of new vessel sprouts. In quiescent vessels, the vascular cells are encased by a basement membrane of collagen IV, laminin and other components, and pericytes and ECs are also embedded in the same basement membrane. An interstitial matrix of collagen I and elastin between vascular cells provides visco-elasticity and strength to the vessel wall. When vascular cells migrate to form new sprouts, the matrix network is proteolytically broken down and its composition is altered. The EC and SMC are induced to migrate because proteinases expose new cryptic epitomes in ECM proteins (such as in collagen IV) or change their structure (fibrillar versus monomer collagen). Further more, a provisional matrix of fibronectin, fibrin and other components provides a support scaffold, guiding ECs to their targets [36].

The cell-surface receptors of specific ECM molecules known as integrins are crucial for vascular cells to build new vessels. Integrins are involved on the regulation of proteolytic enzymes activity which degrades the basement membrane (the initial barrier to surrounding tissue). Integrins are essential for cell migration and invasion, not only because they directly mediate adhesion to the extracelular matrix, but also because they regulate intracellular signalling pathways that control cytoskeleton organization, respond to intracellular cues and modify the way they interact with the extracelular environment. The binding to ligands in the extracelular matrix initiates several pro-survival mechanisms to prevent apoptosis [37]. The $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins have been considered to positively regulate the angiogenic switch. However, genetic deletion studies suggest that vascular integrins inhibit angiogenesis by suppressing VEGF and Flk-1 mediated EC survival, by trans-dominantly blocking other integrins or by mediating the antiangiogenic activity of

thrombospondins (TSPs) and other angiogenesis inhibitors (such as tumstatin, endostatin, angiostatin and PEX) [38].

Remodelling of the ECM during vessel sprouting requires breakdown by proteinases, including plasminogen activators, matrix metalloproteinases (MMPs and tissue inhibitors of metalloproteinases (TIMPs)), heparinases, chymases, tryptases and cathepsins [39-41]. Proteinases also facilitate EC sprouting by liberating matrix-bound angiogenic activators (basic fibroblast growth factor (FGF), VEGF and transforming growth factor (TGF) -β) and proteolytically activating angiogenic chemokines. In proteolytic remodelling of the ECM, insufficient breakdown prevents vascular cells from leaving their original position, but excessive breakdown removes critical support and guidance cues for migrating ECs and, in fact, inhibits angiogenesis. Proteinases can also have a role in the resolution of angiogenesis, as they liberate matrix-bound inhibitors (TSP-1, canstatin, tumstatin, endostatin and platelet factor (PF)-4) and inactive angiogenic cytokines [41,42].

Establishment of a functional vascular network further requires that nascent vessels mature into durable vessels. Nascent vessels initially only consist of ECs and vessel maturation requires a mix of angio- and arteriogenic factors for a sufficient duration, so that ECs can tighten up and become covered by mural cells and ECM. Flow is a critical determinant of vessel maintenance and durability. When insufficient angio- and arteriogenic factors are present and angiogenesis inhibitors are present, EC channels remain naked, leaky and fragile, are easily ruptured and bleed, what reduce flow and result in vessel regression. The association of pericytes and SMCs with newly formed vessels regulates EC proliferation, survival, migration, differentiation, vascular branching, blood flow and vascular permeability. Platelet-derived growth factor (PDGF)-BB and its receptor, PDGF-β, have essential roles in the stabilization of nascent blood vessels by recruiting PDGF-β-positive mesenchymal progenitors [43,44].

Tie-2 receptor is involved in vessel maintenance, growth and stabilization, which binds to angiopoietins (Ang-1 and Ang-2) [45,46]. Members of the TGF-β superfamily contribute to the resolution and maturation phases of angiogenesis, but in a pleiotropic manner [47].

2.5 Collateral growth

When an artery is occluded its vascular territory becomes ischemic. Arterial systems are often interconnected by pre-existing collateral vessels that can enlarge and save the ischemic region [48]. The mechanisms of angiogenesis and collateral growth differ significantly, due to the large pressure difference between the perfusion territories. In collateral growth, the increased shear stress activates ECs, which then recruit monocytes. These cells produce growth factors and proteinases, which enables SMCs to migrate and divide [49, 50]. Cytokines that attract monocytes or prolong their life span, enhance collateral growth, whereas anti-inflammatory cytokines are inhibitory [51]. PIGF also enhances collateral growth, not only because it recruits monocytes, but also because it stimulates EC and SMC growth [52]. Delivery of acidic FGF, FGF-4 or basic FGF (together with PDGF-BB) stimulates collateral growth, in part by upregulating PDGFR expression [53].

2.6 Leukocytes and angiogenesis

Inflammation and immune driven angiogenesis affect numerous disorders (cancer, vascular malformations, obesity, psoriasis, diabetic retinopathy, arthritis, synovitis, osteomyelitis, osteophyte formation, endometriosis, osteoporosis, Alzheimer disease, atherosclerosis, and others) in part because most leukocyte subtypes produce a myriad of angiogenic factors such as VEGF, PIGF, PDGF, basic FGF, Ang-2, epidermal growth factor, TGF-β1, MCP-1 and various interleukins and proteinases (tryptase, chymase, MMPs, heparanase and uPA) [54,55]. Leukocytes affect many angiogenic processes. Monocytes are a source of EPCs and can differentiate into endothelial-like cells [56]. Leucocytes and vascular cells influence each other in other ways. Angiogenic factors amplify the inflammatory process by recruiting leukocytes and affecting their function.

2.7 Coagulation and angiogenesis

Homeostasis and angiogenesis are closely linked. Fibrin-rich clot formation and platelet aggregation precede infiltration of blood vessels into a wound. Platelets release large stores of angiogenic factors such as VEGF, PDGF, TGF-β, IL-6, thrombin and sphingosine-1-1phosphate, which stimulates the growth and stability of nascent vessels by

tightening their junctions and recruit mural cells. Platelets also contain antiangiogenic factors (TSP-1, PF-4 and others) that may have a role in the resolution of angiogenesis once the wound has healed [57,58].

2.8 Vessel regression

Vessel regression occurs when the nascent vasculature consists of too many vessels, and also constitutes the basis of many antiangiogenic therapeutic strategies. Abnormal vessel regression also contributes to the pathogenesis of numerous disorders. Removal of angiogenic stimulus causes vessels to regress, as in tumours and in heart, especially when vessels have only been recently assembled and are still immature. When angiogenic stimulus are provided for a sufficient length of time, new vessels mature and persist for months, even after the angiogenic stimulus are withdrawn. Flow may have an important role in determining whether neovessels regress or persist, by affecting several factors (including MMPs, PDGF, basic FGF, integrins and nitric oxide (NO)). Flow stimulates hyperplasia of ECs and SMCs, and induces the reorganization of endothelial junctions and deposition of ECM, all of which contribute to vessel maturation. Thus, insufficient perfusion may lead to regression, whereas sufficient perfusion promotes vessel persistence. Pericytes also determine the susceptibility of vessels to regression, because once vessels are surrounded by pericytes, they become resistant oxygen-induced regression. In contrast, disruption of endothelial-pericyte associations results in regression of vessels [43,44,47].

Angiogenesis inhibitors also contribute to vessel regression. TSP-1 inhibits angiogenesis through direct effects on ECs and indirect effects on growth factor mobilization or activation. Upregulation of endogenous TSP-1 and TSP-2 contributes to the resolution of angiogenesis and vessel stabilization after ischemia, and overexpression of TSP-1 or TSP-2 in cancer cells results in reduced tumour vascularisation and tumour growth. Macrophages contribute to vessel regression by releasing TGF-β1. Inhibitory PAS dominate protein functions as a dominant-negative regulator of hypoxia-induced angiogenesis to maintain an avascular phenotype in certain tissues. Additional inhibitors include chemokines binding CXCR3, soluble receptors (Flt-1 and Tie-2), clotting antagonists and others. It is being discovers inhibitors including cleavage products of matrix components, proteinases, enzymes or plasma proteins [43,44].

2.9 Types of angiogenesis

There are tow types of angiogenesis: sprouting angiogenesis and intussusceptive angiogenesis [41,42].

2.9.1 Sprouting angiogenesis

In the sprouting angiogenesis the angiogenic growth factors activate receptors present on endothelial cells in pre-existing venous blood vessels. The activated endothelial cells begin to release enzymes called proteases that degrade the basement membrane in order to allow endothelial cells to escape from the original vessel walls. After that the endothelial cells proliferate into the surrounding matrix and form solid sprouts connecting neighbouring vessels. As sprouts extend toward the source of the angiogenic stimulus, endothelial cells migrate in tandem, using integrins. These sprouts then form loops to become a full-fledged vessel lumen as cell migrate to the angiogenesis site. Sprouting occurs at a rate of several millimetres per day, and enables new vessels to grow across gaps in the vasculature [59].

2.9.2 Intussusceptive angiogenesis

In the intussusceptive angiogenesis the capillary wall extends into the lumen to split a single vessel in two. The two opposing capillary walls establish a zone of contact, the endothelial cell junctions are reorganized and the vessel bilayer is perforated to allow growth factors and cells to penetrate into the lumen. A core is formed between the two new vessels at the zone of contact that is filled with pericytes and myofibroblasts. These cells begin laying collagen fibres into the core to provide an extracelular matrix for vessel lumen growth. Finally, the core is fleshed out with no alterations in basic structure. Intussusceptive angiogenesis allows a vast increase in capillaries number of without a corresponding increase in endothelial cells number [59].

2.10 Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF) is an important signalling protein involved in both vasculogenesis and angiogenesis. It is a key regulator of physiological

angiogenesis during embryogenesis, skeletal growth and reproductive functions. VEGF activity is restricted mainly to cells of the vascular endothelium, although it has effects on a limited number of other cell types (e.g. stimulation monocyte/macrophage migration). *In vitro* VEGF has been shown to stimulate endothelial cell mitogenesis and cell migration. VEGF has also been implicated in pathological angiogenesis associated with tumours, intraocular neovascular disorders and other conditions. Upregulation of VEGF is a major component of the physiological response to exercise and its role in angiogenesis is suspected to be a possible treatment in vascular injuries. *In vitro* studies demonstrate that VEGF is a potent stimulator of angiogenesis because in the presence of this growth factor, plated endothelial cells will proliferate and migrate, eventually forming tube structures resemble capillaries [60-64].

VEGF covers a number of proteins that result from alternative splicing of mRNA from a single, 8 exon, VEGF gene. The different VEGF splice variants are referred to the number of amino acids they contain (in human: $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$). These proteins differ by the presence or absence of short C-terminal domains encoded by exons 6A, 6B and 7 of the VEGF gene. These domains have important functional consequences for the VEGF splice variants as they mediate interactions with heparan sulfate proteoglycans (HSPGs) and neuropilin co-receptors on the cell surface, enhancing their ability to bind and activate the VEGF signalling receptors (VEGFRs) [60-64].

The VEGF splice variants are released from cells as glycosylated disulfide-bonded homodimers. Structurally VEGF belongs to the PDGF family of cystine-knot growth factors. Subsequently, several closely-related proteins were discovered (Placenta growth factor (PIGF), VEGF-B, VEGF-C and VEGF-D) which together comprises the VEGF sub-family of growth factors. VEGF is sometimes referred to as VEGF-A to differentiate it from these related growth factors [65].

All members of the VEGF family stimulate cellular responses by binding to tyrosine kinase receptors (the VEGFRs) on the cell surface, causing them to dimerize and become activated through transphosphorylation. The VEGF receptors have an extracelular portion consisting of 7 immunoglobulin-like domains, a single transmembrane spanning region and an intracellular portion containing a slit tyrosine-kinase domain. VEGF-A binds to VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1). VEGFR-2 appears to mediate almost all of the known cellular responses to VEGF [66-69]. VEGF causes a massive signalling cascade in endothelial cells. Binding to VEGFR-2 starts a tyrosine kinase signalling

cascade that stimulates the production of factors that variously stimulate vessel permeability (eNOS, production NO), proliferation/survival (bFGF), migration (ICAMs/VCAMs/MMPs) and finally differentiation into mature blood vessels. The increased flow also causes a large increase in the mRNA production of VEGF receptors 1 and 2 [60]. The function of VEGFR-1 is less well defined, although to modulate VEGFR-2 signalling. Another function of VEGFR-1 is to act as a dummy/decoy receptor, sequestering VEGF from VEGFR-2 binding. A third receptor has been discovered (VEGFR-3), however, VEGF-A is not a ligand for this receptor. VEGFR-3 mediates lymphangiogenesis in response to VEGF-C and VEGF-D [66-69].

VEGF production can be induced in cells that are not receiving enough oxygen. When a cell is deficient in oxygen, it produces HIF, Hypoxia Inducible Factor, a transcription factor. HIF stimulates the release of VEGF, among other functions. Circulating VEGF then binds to VEGF receptors on endothelial cells, triggering a Tyrosine Kinase Pathway leading to angiogenesis [70,71].

VEGF has also effects on bone marrow-derived cells. It promotes monocyte chemotaxis and induces colony formation by mature subsets of granulocyte-macrophage progenitor cells. [72,73]. VEGF delivery to adult mice inhibits dendritic cell development and increases production of B cells and generation of immature myeloid cells [74,75]. VEGF is known also as vascular permeability factor, based on its ability to induce vascular leakage. It induces an increase in hydraulic conductivity of isolated microvessels, and this effect is mediated by increased calcium influx [76-78].

2.11 Matrix Metalloproteinase

The matrix metalloproteinase (MMP) help to degrade the proteins that keep the vessel walls solid. This proteolysis allows the endothelial cells to escape into the interstitial matrix as seen in sprouting angiogenesis. Inhibition of MMPs prevents the formation of new capillaries, and these enzymes are highly regulated during the vessel formation process because wanton destruction of the extracellular matrix would destroy the integrity of the microvasculature [60, 79].

2.12 Angiogenesis and bone

The VEGF have the ability to induce neovascularisation. Studies had show that neutralizing VEGF receptor decreased angiogenesis, bone formation, and callus mineralization in femoral fractures. Exogenous VEGF enhanced blood vessel formation, ossification, new bone (callus) maturation in mouse femur factures, and promoted bone bridging of a rabbit radius segmental gap defect [80].

Fracture of bone disrupts its circulation and leads to necrosis and hypoxia of adjacent bone. Under normal circumstances, fractured bone undergoes the orderly regeneration of its component tissues with complete restoration of mechanical properties. Reestablishment of the circulation is an early event in fracture healing. Bone repair is a multistep process involving migration, proliferation, and activation of several cell types. Expression of particular growth factors, such as FGFs, PDGFs, TGF-βs, VEGF, and bone morphogenetic proteins (BMPs), during the course of healing suggests a possible role for these secreted factors in bone repair [81,82]. VEGF is expressed in the fracture callus in animal models in much the same temporal and spatial pattern as during long bone development. Although VEGF can control hypertrophic cartilage structure and vascularity within the development growth plate, the role of VEGF in bone repair has not yet been determined [80, 83-85]. The fracture callus contains many factors that could promote bone healing by coordinating angiogenesis with bone homeostasis [86].

2.13 Osteogenesis and angiogenesis

Bone formation is dependent on the signalling of BMPs, molecules initiating vascularisation (e.g. VEGF) and osteogenic precursor cells capable of responding to these cues and forming bone tissue [87]. Tissue development and regeneration are regulated by interplay among various tissue inductive growth factors, formation of an appropriate vascular bed to support the metabolic needs of the forming tissue mass, and a cell population capable of responding to the chemical cues and creating the new tissue. BMPs are responsible for initiating cartilage and bone progenitor cell differentiation and sequencing new bone formation through endochondral ossification [88,89]. Angiogenesis is also involved in the initiation of fracture healing and promotion of endochondral and intramembranous ossification in bone growth. These factors must act on a population of cells capable of responding to local factors and forming bone tissue. Multipotent stem cells

originating from the bone marrow stroma, or bone marrow stromal cells (BMSCs), are a particularly attractive source for osteogenic precursors for bone tissue engineering, because they can be easily harvested and expanded *in vitro* and induced to differentiate into bone-forming cells [87,90].

3. Bone Tissue Engineering

Biomaterials can be defined as a natural modified or synthetic material that interacts with the biological system to improve, cure or substitute any tissue, organ or function of the body (Williams, 1999) [2]. Biomaterials should be biocompatible, absence of harmful or toxic effect for the organism and have biomechanics properties to support the dynamic and static requests that will be subject during its useful life [91].

Medical problems can emerge from bone trauma, diseases and ageing. In order to solve these problems, autografts (autogenous grafts), allografts (tissue harvested from a different human patient) and xenografts (tissue harvested from an animal) have been used in bone surgery [91]. The main advantage of an autograft is related to its osteogenic, osteoinductive and osteoconductive properties. This graft contains cartilage matrix minerals, osteogenic proteins and precursor cells [91]. Allografts can lead to the transmission of viral diseases as HIV, hepatitis B and hepatitis C, and can induce immunological reactions. Although, these risks can be diminished through sterilization with gamma radiation, its structure can be affected and the bone graft may lose its osteoinductive and osteogenic properties because most of the cells can be damaged during the sterilization process. Xenografts can give rise to unfavourable immune response and viral contamination. Due to the limitations described above related to autografts, allografts and xenografts, researchers have been focus on the development of synthetic alternatives, as metals, polymers, ceramic, and composites [91].

3.1 Biomaterials

Several materials can be used as biomaterials, namely metal, polymer, ceramic, and composites.

Metal and metal alloys such as titanium, titanium alloys, aluminium, chromium and cobalt alloys, are able to support mechanical loads. However, these materials do not have

bioactive properties required for a proper osteointegration into the host tissue; they are hard and have toxic effect, due to the release of several ionic species, which can induce metallises, inflammatory reactions and fibrosis encapsulation [91].

Polymer bone grafts are easily tolerated by the host tissue. However they can induce necrosis of the surrounding tissue and can be toxic for the organism [2]. Polyethylene, polypropylene, polyurethane, acid polyactinic (PLA) and the poli(methyl methacrylate) (PMMA) are examples of polymers used in implantology. Some of these polymeric grafts can cause a pathological response through the release of toxic monomers or allergic sensitization. The use of polymeric materials is better then the metallic ones, because the difference in the elasticity modulus between graft and bone is reduced, and polymers can be degraded *in vivo* [7].

Composites are synthesized to improve the properties and the biological behaviour of a biomaterial in clinical applications [92]. The bone tissue is composed of collagen fibres and minerals, being a good example of a natural composite with excellent biomechanics properties.

Ceramics are defined as inorganic, non-metallic materials which consist of metallic and non-metallic elements bonded together primarily by ionic and/or covalent bonds. The ceramic materials are fragile, hard, highly resistant to acid attack, stable at high temperatures and are electric insulators [92].

Bioceramics play an extensive role in biomedical materials. The development of these materials and diversity of manufacturing techniques has broadened the applications that can be used in the human body, such as dental, maxillofacial and orthopaedic applications. They can be in the form of thin layers on metallic implants, composites with a polymer component, or even just porous networks. Common materials used for bioceramics include alumina, zirconium, calcium phosphate, glass ceramics, and pyrolytic carbons.

3.2 Interaction between bone tissue and bioceramics

The bioceramics when are implanted in the body elicit a response of the host tissue, and both can suffer physical and chemical modifications. This response between bioceramic and host tissue is related to implant site, material properties and surface treatment, implant design, surgical procedures and patient conditions. Bioceramics can be

divided in tree different types based on different attachments and interactions between the implant and tissue [91].

Bioinert ceramics (or nearly inert), for example Al_2O_3 or ZrO_2, are nontoxic and biologically inactive, causing the formation of fibrous tissue. The interface between ceramic and host tissue is not chemically or biologically bonded, therefore there is a relative movement which cause the development of a nonadherent fibrous capsule. These materials attach by bone growth onto surface irregularities, by cementing or by morphological fixation [91].

Biodegradable ceramics can be chemically dissolved or digested by macrophages being gradually replaced by the natural host tissue. The main purpose of these materials, for example, β-TCP and some bioactive glasses, is the regeneration of tissues instead of their replacement. However, there are two main problems with the use of resorbable materials: the maintenance of strength and stability of the interface during degradation period and the difficulties of materials resorption rate with body tissues repairing rate. The degradation can be due to: the solubility of the material and local pH, fragmentation into small particles and biological factors (biological dissolution) [91].

Bioactive ceramics are nontoxic and biologically active forming an interfacial bond which is called bioactive fixation. The interfacial bond seems to be a hydroxycarbonate apatite layer, which is chemically and structurally similar to the mineral phase of bone. Bioactive ceramics include HA, some composites such as polyethylene-HA, some glasses and glass-ceramics.

3.3 Hydroxyapatite

The synthetic HA can be prepared by several methods, one of them is the chemical precipitation, through the reaction of calcium hydroxide and orthophosphoric acid with a molar ratio calcium/phosphorus of 1.67, corresponding to a phase pure HA. The HA has as the following chemical formula $[Ca_{10}(PO_4)_6(OH)_2]$, a molecular weight of 1004.8 g/mol and its crystalline system is hexagonal having a space group of $P6_3/m$. The axles lengths of the HA lattice are a=b=9.423Å and c=6,875Å, and finally its density is 3,16g/cm3 [10,91].

The HA lattice contains two kinds of calcium positions, assigned for Ca(1) and Ca(2) (fig. 3). Ca(2) is surrounded by six atoms of O that belongs to the PO_4^{3-} group and one OH^- group, while the atom of Ca(1) is almost octahedral and surrounded for six O

atoms. The Ca(2) atoms form triangles that are piled up on axle c, encircled mutually by 60º in relation to each one. The OH^- group is next to the centre of the Ca(2) triangle and atom P is encircled by four O atoms and forms a tetrahedron. The PO_4^{3-} tetrahedron is practically regular, with only one small distortion [10].

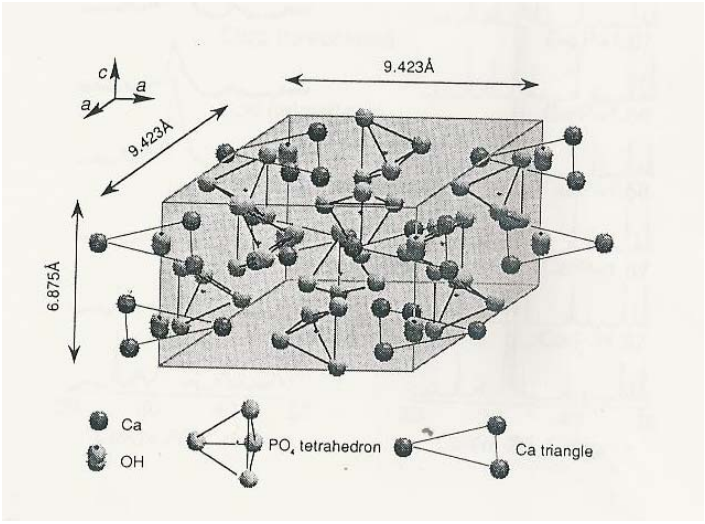

Figure 3 – HA structure [10].

The HA allows the graft osteointegration and is osteoconductive *in vivo* allowing its use in medical applications of orthopaedic and verbal surgery, for example [91]. However, its dissolution rate is very slow when is compared with the bone regeneration rate.

3.4 Silicon substituted hydroxyapatite

The silicon (Si^{4+}) is an agent of bone mineralization, increasing the biocompatibility and bioactivity of the HA adding Si^{4+} [94-96].

Si has atomic number 14, the atomic weight is 28.09, and the parameters of its unitary cells are a = b = c = 543,09 pm, $\alpha = \beta = \gamma = 90{,}00º$ [92] (fig. 4).

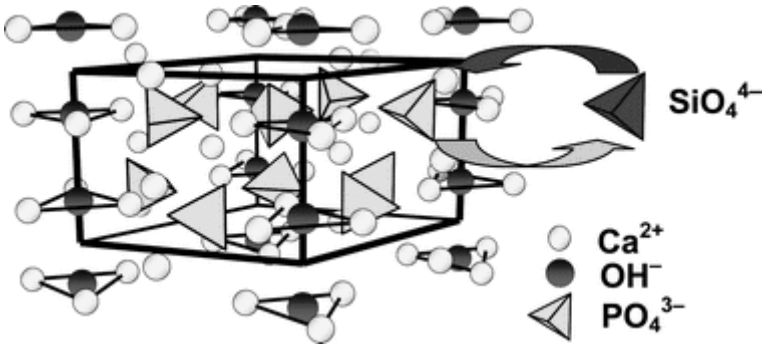

Figure 4 - SiHA structure [10].

Combining the properties of HA and Si a new material was developed and called silicon substituted hydroxyapatite (SiHA), that can be prepared by a chemical precipitation, with similar procedure of the HA preparation, but in this case there is the addition of silicon tetra-acetate [$Si(CH_3CO_2)_4$] as source of silicate ions. The substitution of silicon in the HA, $Ca_{10}(PO4)_{6-x}(SiO4)_x(OH)_{2-x}$ (x is the moles number of silicon), reduces the number of the hydroxyl groups to compensate the extra-negative load of the silicate group [97].

PO_4^{3-} group is located preferentially on the surface of HA, and the substitution of these ions for SiO_4^{4-} results in a reduction of the surface charge, indicated by its zeta potential [98]. The more electronegative surface of SiHA can promote a preferential place for the amorphous calcium phosphate apatite nucleation to occur, through the absorption of Ca^{2+} ions into the electronegative surface, resulting in a surface charge increase and in the attraction of the phosphate groups [98].

The incorporation of Si^{4+} in HA lattice increases the hydrophobicity of the material and increases its surface tension. Si^{4+} is more easily polarized than the P^{5+} due its low atomic number. The phosphate group in SiHA has a smaller symmetry what can increase its polarity [99]. The silicon incorporation did not affect only the HA chemical properties but also its physical properties, as the size of grain, number of grain boundaries and triple junctions.

Another study demonstrated that silicon has a preferential dissolution from the SiHA material, promoting the carbonate apatite nucleation surrounding the graft [100]. The bioactivity increase in the SiHA can be due to changes in protein adhesion [101]. In a dense form SiHA increases the proliferation of osteoblastics cells when compared with HA [102]. Therefore, the human osteoblastics cells are affected by the presence of silicon in

HA lattice and the duration of these effects can depend on the degree of substitution by silicon [103]. SiHA also has a stimulator effect in the osteoclasts [104].

The increased structural defects in SiHA can be very important in the increase of the material solubility and in bond between bone and ceramic. The *in vivo* dissolution decreases in the following order 1.5 wt% SiHA > 0.8 wt% SiHA pure > HA and it is particularly observed on the grain boundaries and triple junctions [105-107].

An *in vivo* study [108] demonstrated that the morphology of the apatite deposition and the sequence of events in the interface between bone/HA and between bone/SiHA are different. The organized staple fibres of collagen appear earlier in the interface bone/SiHA then in the synthetic HA grafts. It was observed a trabecular zone in bone in contact with the SiHA graft and the collagen staple fibres form a strong bond with SiHA ceramic graft [108]. The beneficial effects of silicon have been reported for many years, namely by Schwarz [109] and Carlisle [100], whom demonstrated that a deficient diet in silicon in rats and chicks retarded the growth and disturbed the development of bone structure. Reffit *et al* (2003) [111] showed that at a physiologic concentration, silicon stimulates collagen I synthesis, differentiation and alkaline phosphatase activity in human osteoblastic like cells.

Botelho *et al* [103] demonstrated that dense SiHA stimulated the adhesion, proliferation and differentiation of human osteoblasts. It was also possible to observe calcium phosphate mineral deposits on cell layer. Additionally, Botelho *et al* [112] demonstrated that this material allows the differentiation of osteoclasts precursors into mature osteoclasts. These cells grown on the surface of the material expressed typical phenotype characteristics, such as: actin rings, several nuclei, TRAP expression and expression of vitronectin receptors.

It is know that titanium or titanium implants have a low bioactivity, so one way to improve its bioactivity is by coating its surface with a ceramic biomaterials. Therefore, in order to take advantage of the positive effect of the SiHA, a titanium alloy was coated with SiHA and its biological behaviour assessed.

3.5 Coating techniques

Many techniques are available for the deposition of ceramic coatings, including physical vapour deposition techniques, conversion of superficial metallic fractions to ceramic-like oxides, immersion in a ceramic melt, direct melting or chemical reaction of components placed directly onto the surface, electroforming, gas-pressure bonding,

welding by diffusion, ultrasound or field-assisted diffusion bonding, reaction with gas induced by laser, and covering with refractory salts or metallic oxides by plasma spraying [91].

Plasma spraying technique is used to coat a metal substrate surface with ceramic through a plasma flame. In the coating process an electric arc is struck between two electrodes and stream of gases is passed through the arc. The coating powder is injected into the plasma with a carrier gas (usually argon or nitrogen), and the heated powders melt and reach the substrate with high velocity. There are many variables in the process including the gases used, the electrical settings, the nozzle/substrate separation and the morphology, particle size, and particle size distribution of the powder [91,109].

Plasma spray process parameters affect the structure of the coatings, specially the crystallinity, porosity, density, adhesion, cohesion, and as a consequence the bone bonding mechanism and the rate of bone formation [91, 92, 113]. The structure of the coatings will depend on the time of performance of the particles in the flame, and on the solidification and cooling conditions. The high temperatures of the plasma flame and the high cooling rates promote the formation of amorphous phases. The way by which a coating adheres to a substrate is very complex and it is not fully understood. However, many factors seem to influence the establishment of coating-to-substrate adhesion: mechanical anchorage, Van der Waals physical interaction forces, chemical interaction and metallurgical process [114].

3.6 *In vitro* biological studies

The most important characteristics that will allow the selection a material for medical use are its biocompatibility, absence of harmful or toxic effect for the organism and its biomechanics properties capable to answer to the dynamic and static requests that will be subject during its useful life. Cell death, reduced cell adhesion, altered cell morphology, reduced cell proliferation and reduced biosynthetic activity are examples of toxicity *in vitro* [115].

In vitro research is used to assess the good performance of a biomaterial. With fewer variables and perceptually amplified reactions to subtle causes, results are more discernible. Evaluation under *in vitro* conditions may provide rapid and not expensive data on biological interaction. However, these results may allow us to partially predict its performance *in vivo*, because there are many variables that are not controlled. The biomaterials must include biocompatibility studies, cell cultures, prior to any *in vivo* testing

[116] due to the legal and ethical rules that restrict animal experimentation and because the correlations between *in vitro* and *in vivo* tests provide a quite good results of the expected biological performance of a biomaterial.

The studies *in vitro* are important because it can have an important influence for the studies *in vivo* and also because the *in vitro* approach is the unique possibility to test human cells reaction on the material.

CHAPTER 2
EXPERIMENTAL PROCEDURES

Chapter 2

Introduction

The objective of this chapter is to describe the laboratorial procedures:

1. The preparation and characterization of HA and SiHA as dense samples and as plasma-sprayed coated disc of Ti;

2. *In vitro* biological studies with Human Umbilical Vein Endothelial Cells;

3. *In vitro* biological studies with Human Osteoblastic Cells;

The HA and SiHA powders were prepared through a precipitation method.

The physico-chemical and structural characterization of the bioceramics prepared were performed using the X-ray diffraction (XRD), Fourier transform infrared (FTIR) and Scanning Electron Microscopy (SEM) techniques.

In vitro biological studies with human umbilical vein endothelial cells cultured in dense samples of HA and SiHA were evaluated through MTT assay and Confocal laser scanning microscopy (CLSM).

In vitro biological studies of human osteoblastic cells cultured on the surface of Ti alloy coated with HA and SiHA, were evaluated through MTT assay, CLSM and SEM.

1. Preparation of HA and SiHA

1.1 HA preparation

HA was prepared through a precipitation method consisting on the reaction between calcium hydroxide ($Ca(OH)_2$) and orthophosphoric acid (H_3PO_4), following the reaction:

$$10Ca(OH)_2 + 6H_3PO_4 \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 18H_20$$

During the synthesis process 0.500 moles of calcium hydroxide ($Ca(OH)_2$) were dissolved in 1 litre of deionised water, and 0.299 moles of orthophosphoric acid (H_3PO_4) in 1 litre of deionised water. The solution of orthophosphoric acid was added drop to drop to the calcium hydroxide solution, at room temperature, for a period of 3 hours, and the pH of the suspension was kept at 10.5 by the addition of ammonia (NH_4OH). The suspension was stirred for one hour and left aging for 6 hours.

1.2 SiHA preparation

The method used in the preparation of SiHA was similar to that used in preparation of HA, but in this case silicon tetra-acetate [$Si(CH_3COO_4$] was added to the mixture, as a source of silicate ions.

In the preparation of SiHA the amount of reagents used to prepare was calculated on the basis of the following equation:

$$10Ca^{2+} + (6\text{-}x)PO_4^{3-} + xSiO_4^{4-} + (2\text{-}x)OH^- \rightarrow Ca_{10}(PO4)_{6\text{-}x}(SiO4)_x(OH)_{2\text{-}x}$$

(x is the moles number of silicon tetra-acetate)

To prepare 0.8 wt (%) of SiHA each reagent was dissolved in 1 litre of deionised water: 0.500 moles of ($Ca(OH)_2$), 0.285 moles of (H_3PO_4) and 0.014 moles of [$Si(CH_3COO_4$]. The solutions were mixed for 30 minutes and the orthophosphoric acid solution was added drop to drop to the $Ca(OH)_2$ solution, for a period of 3 hours. Such as in the preparation of HA, during the precipitation reaction, the pH of the suspension was kept above 10.5 by the addition of ammonia. The suspension was stirred during one hour and left aging for 6 hours.

1.3 Dense Samples

HA and SiHA dense samples of approximately 15 mm of diameter of were prepared by filling a cylindrical die with 1g of powder of each material, and then they were pressed using a uniaxial press until 180 Bar. The HA and SiHA dense samples were then sintered in a furnace at 1300ºC using a heating rate of 2.5ºC/min with 120 min dwelling time, followed by natural cooling inside the furnace.

1.4 Milling and sieving

After uniaxial pressing and sinterization, the samples were, milled and sieved until obtain the following distribution (optimize to for plasma spray):

30% - 90 μm and 125μm

40% - 75μm-90μm

20% - 63μm-75μm

10% - 45μm-63μm.

1.5 Plasma-spray

A commercial Ti rod (Ti-6Al-4V) of 14 mm of diameter was coated with HA and SiHA. The thickness of the coating was 120 μm.

2. Physical-chemical and structural characterization of HA and SiHA

X-ray diffraction (XRD), Fourier transform infrared (FTIR), Scanning Electron Microscopy (SEM) and Confocal Laser Scanning Microscopy (CLSM) were used to characterize the HA and Si-HA samples.

X-ray powder diffraction (XRD)

To determine the phase purity of the HA and SiHA, the samples were grounded to a fine powder and analysed using a Rigaku Dmax-III-VC X-ray diffractometer, with CU-Kα radiation (Kα = 1.54056 Aº). Data was collected from 4º to 80º (2θ), with step size of 0.02º/s.

Fourier transform infrared (FTIR)

The infrared spectra analysis was performed with a *System 2000 FT-IR, Perkin Elmer*, with a 4cm^{-1} resolution and 100 scans.

Scanning electron microscopy (SEM)

For morphological evaluation, the HA and SiHA samples were coated with gold and were observed in a JOEL JSM-63 10F scanning electron microscope, with detection of secondary electrons and Backscattered electrons.

3. *In vitro* biological studies of Human Umbilical Vein Endothelial Cells

3.1 Human Umbilical Vein Endothelial Cells

Human Umbilical Vein Endothelial Cells (HUVECs) (5º passage) were cultured in M199 medium, supplemented with 10% of Simulated body fluid (SBF), 1% of Penicillin/Streptomycin, 1% of heparin (10mg/ml) and 2 µl/ml of ECGS. After cells reached confluence, they were enzymatically released by trypsin-EDTA solution and cultured on the HA and SiHA dense surface at a density of 3x10^4 cells/cm^2. The samples were previously coated with 0.2% gelatine (1hour immersion at 37ºC).

3.2 Cell proliferation

For cell proliferation evaluation, the colonized samples were incubated with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrasodium bromide (MTT) (5mg/mL) at 37º C for 4 hours. After treatment with DMSO, the absorbance of the solution was evaluated at 610 nm, at day 1 and 3.

3.3 Morphologic Evaluation

Cells were fixed with 4% formaldehyde (methanol free), permeabilized with 0.1% triton and incubated in 10 mg/ml bovine serum albumin (BSA) with 100µg/ml RNAse. F-actin filaments were stained with Alexafluor®-conjugated phalloidin and nuclei were counterstained with 10µg/ml propidium iodide. Samples were washed with phosphate

buffered saline (PBS) and covered with Vectashield. Images were acquired on a Leica TCP SP2 AOBS with a LCS software.

To prove that the cells in the material surface were in fact endothelial cells, they were staining with a platelet/endothelail cell adhesion molecule-1 (PECAM-1). After cells fixation with 4% formaldehyde, the cells were permeabilized with triton 0.1% during 5 minutes, after which they were incubated with the primary antibody, Pecam-1 anti-rabbit, in 1:50 dilution on 1% BSA/PBS solution during 45 minutes. The samples were then washed with PBS and incubated with secondary antibody AlexaFlour 488 anti-rabbit in 1% BSA/PBS solution, in a 1:1000 dilution for 45 minutes. The nucleus was stained with 1.5μg/ml propidium iodide for 7 minutes.

One of the characteristics of endothelial cells is the capacity to form tubular structures, so 1.5% of collagen gel was added to the samples. The gel was added during 24 hours. As control positive, endothelial cells were cultured in specific cell culture material.

4. *In vitro* biological studies of Human Osteoblastic Cells

4.1 Human osteoblastic cells

The human osteoblastic cells were isolated from bone marrow of the kneecap of a 28 years old male patient. Trabecular bone samples were mechanically disaggregated and washed with standard culture medium, as result a single-cell suspension was obtained. The cells were cultured in minimum essential medium Eagle, alpha modification (α-MEM) containing 10% foetal bovine serum (FBS), 100μg/ml penicillin, 10IU/ml streptomycin and 2.5 μg/ml fungizone. Incubation was carried out in a humidified atmosphere of 95% air and 5% CO_2 at 37ºC. The passage was made when primary culture was near confluence. Adherent cells were enzymatically released by trypsin-EDTA solution and counted using a hemocytometer. The human bone marrow cells (first subculture) were seeded on surface biomaterials at a density of $2x10^4/cm^2$ for 28 days, at conditions favouring osteogenic differentiation. The cells were cultured at 37ºC in a humidified atmosphere of a 95% air and 5% CO_2 in plates containing 10% of foetal bovine serum (protein source), 1% of fungizone and streptomycin/penicillin (antibacterial), 50μg/ml ascorbic acid (vitamin C source for collagen formation), 10nM dexamathasone (increases

the cellular proliferation and it induces osteoblasts differentiation) and 10mM β-glycerolphosphate. The culture medium was changed every three days.

4.2 Cell proliferation

For cell proliferation evaluation, the colonized samples were incubated with MTT (5mg/mL) at 37º C for 4 hours. After treatment with DMSO, the absorbance of the solution was read at 610 nm, at days 7, 14, 21 and 28.

4.3 Morphologic Evaluation

For the morphologic evaluation, the cells were analyzed by Confocal Laser Scanning Microscopy (CLSM) and Scanning Electron Microscopy (SEM).

Confocal laser scanning microscopy (CLSM)

In this case a similar method to the HUVECs was used. Cells were fixed with 4% formaldehyde (methanol free), permeabilized with 0.1% triton and incubated in 10 mg/ml bovine serum albumin (BSA) with 100µg/ml RNAse. F-actin filaments were stained with Alexafluor®-conjugated phalloidin and nuclei were counterstained with 10µg/ml propidium iodide. Samples were washed with PBS and covered with Vectashield. Images were acquired on a Leica TCP SP2 AOBS with a LCS software.

Scanning electron microscopy (SEM)

For SEM observation the cells were fixed with 1.5% glutaraldehyde in 0.14 M sodium cacodylate, and then dehydrated in graded series of alcohols and critical-point dried. Specimens were mounted onto aluminium supports using araldite and then sputter-coated with gold and observed in a Joel JSM 35C scanning electron microscope equipped with an X-ray energy dispersive spectroscopy voyager XRMA System, Noran Instruments, at days 3, 7, 14, 21 and 28.

RESULTS

RESULTS

1. Hydroxyapatite and Silicon substituted apatite characterization

XRD analysis

XRD analysis showed that HA and SiHA were both phase pure. Phase identification was carried out by comparing the peak positions of the diffraction patterns with ICDD (JCDS) standards. The incorporation of silicon into the HA lattice did not affect phase composition, as no secondary phases, such as tricalcium phosphate (TCP) or calcium oxide (CaO), were formed (figure 5).

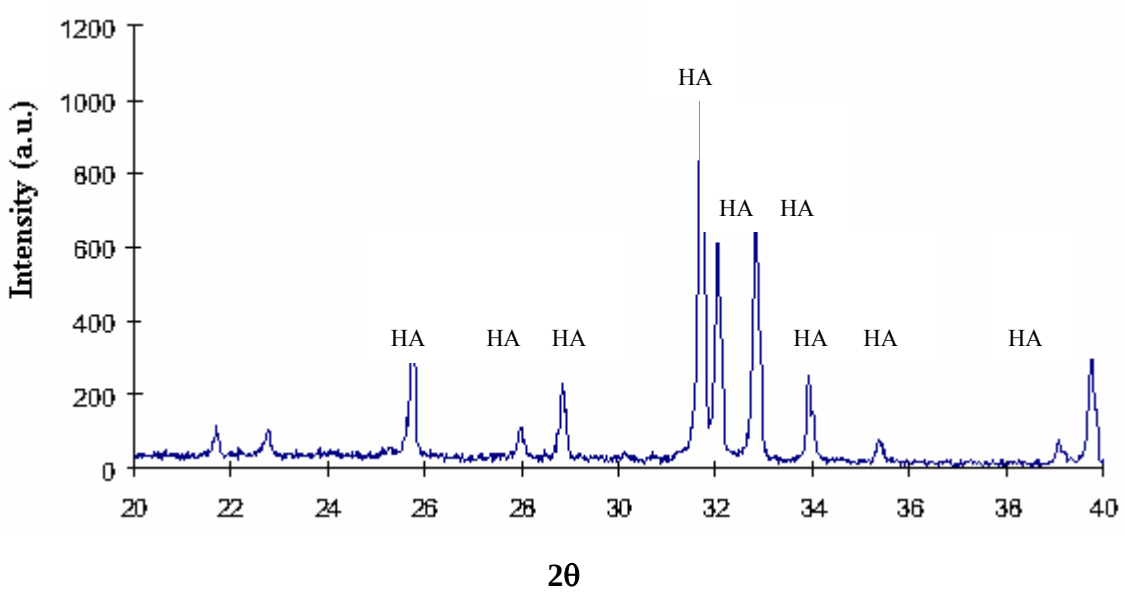

Figure 5 – XRD spectrum of phase pure SiHA.

FTIR analysis

In the spectrum of HA (fig. 6) is possible to observe the peaks that correspond to PO^{3-}_4 groups at 1089, 1046, 958, 600 and 569 cm^{-1} and to the OH^- group 630cm^{-1}, whose wavelengths are in accordance with literature values [117].

For the 0.8wt % SiHA two additional peaks were detected at 888 and 504 cm^{-1} that can be assigned to the SiO_4^{4-} group. It is also worth noting that the peak that corresponds to the OH^- group at 630cm^{-1} underwent a significant decrease in intensity (fig. 7).

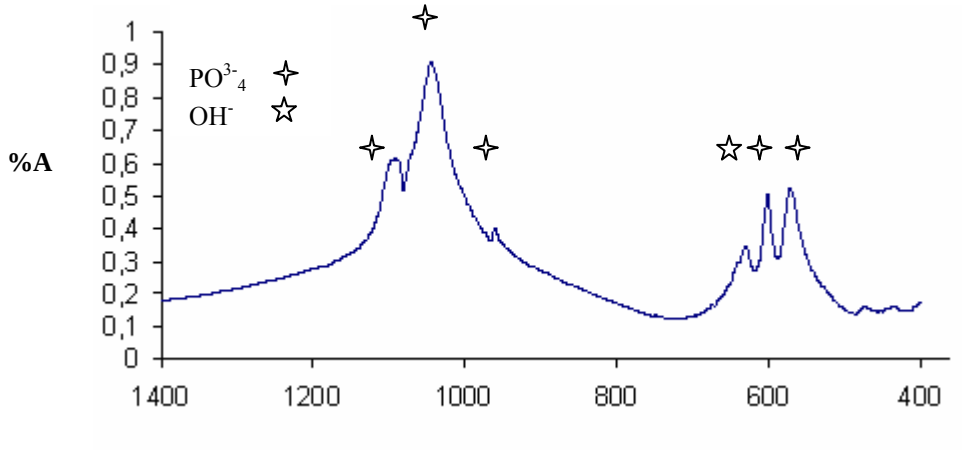

Figure 6 – FTIR spectrum of phase pure HA.

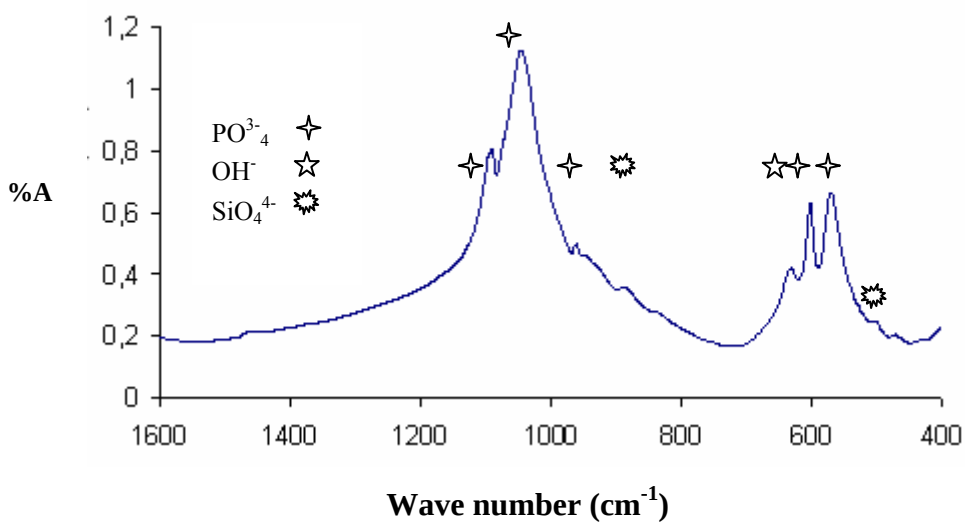

Figure 7 – FTIR spectrum of phase pure 0.8 wt% SiHA.

Through the analysis of the FTIR pattern and XRD it was possible to see that the HA and SiHA compositions prepared were phase pure. Additionally, due to the decrease on the intensity of the band corresponding to the OH^- group and the presence of a band at 881cm^{-1} and 496cm^{-1} on the FTIR spectra it was possible to identify the proposed mechanism of silicon substitution by phosphorous, in accordance with the results previously reported [97].

SEM analysis

The results from SEM analysis showed difference between HA and SiHA morphology. It was possible to see a significant difference on the grain size present on the

HA and SiHA. The grains on the SiHA sample are considerably smaller (fig. 8). Thian *et al* [123] showed that the incorporation of Si into the HA lattice inhibited grain growth.

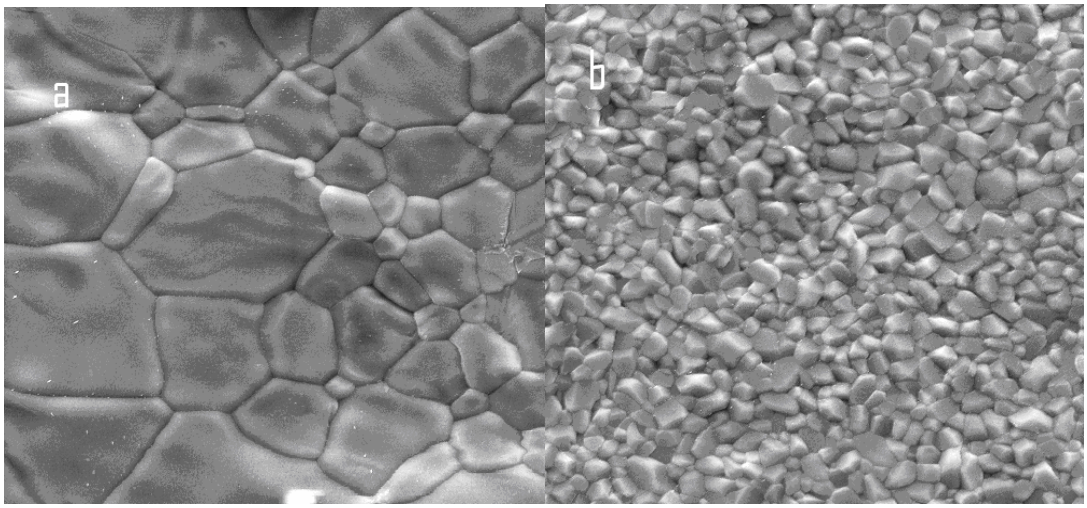

Figure 8 – SEM image of HA dense (a) and SiHA dense (b).

SEM analysis showed that the surface of the HA and SiHA plasma-sprayed coated samples are very rough and have small spherical particles (fig. 9).

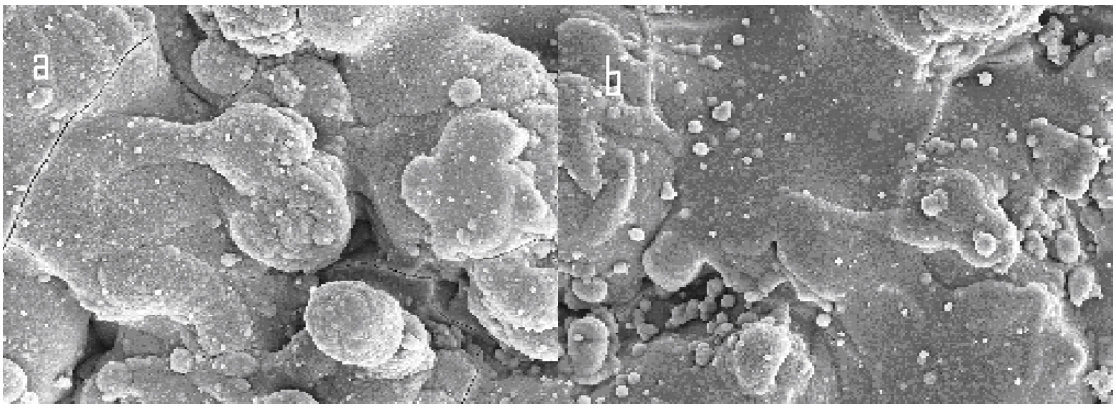

Figure 9 - SEM appearance of the (a) HA plasma-sprayed coated disc and (b) SiHA plasma-sprayed coated disc (original magnification, x 2000).

2. *In vitro* biological studies of Human Umbilical Vein Endothelial Cells

MTT assay

The number of HUVECs that adhered to SiHA substrate was higher in comparison to the number of cells present on the HA substrate, being this difference statically significant for day 1 and day 3 ($p<0.05$), (fig.10).

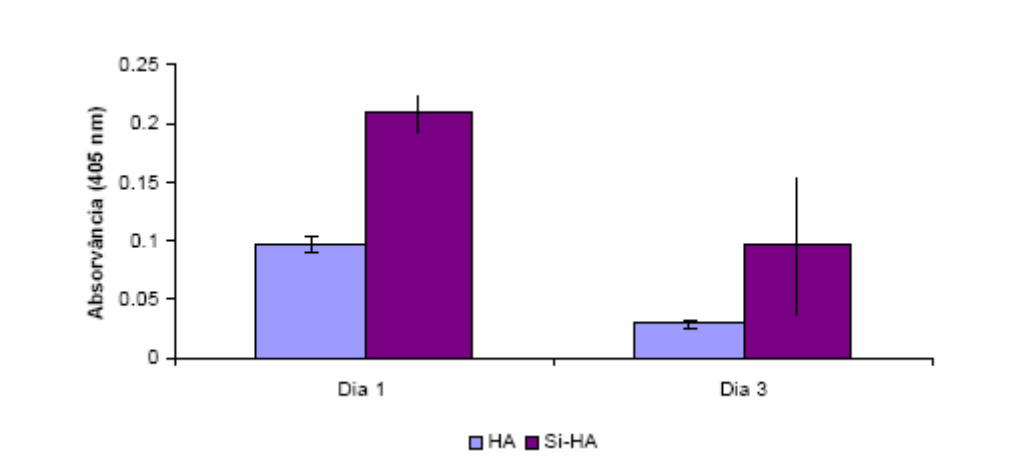

Figure 10 – Cell viability/proliferation (MTT assay) of endothelial cells cultured on the surface of dense samples of HA or SiHA.

CLSM analysis

After 1 day of culture, the samples were covered with endothelial cells (fig. 11 a) and showed a positive staining for Pecam-1 (fig. 11 b). It was also seen that after 3 days of culture, cells had formed tubular structures on the surface of the control samples (tissue culture slides) (fig. 11 c) and showed a positive staining for Pecam-1 (fig. 11 d).

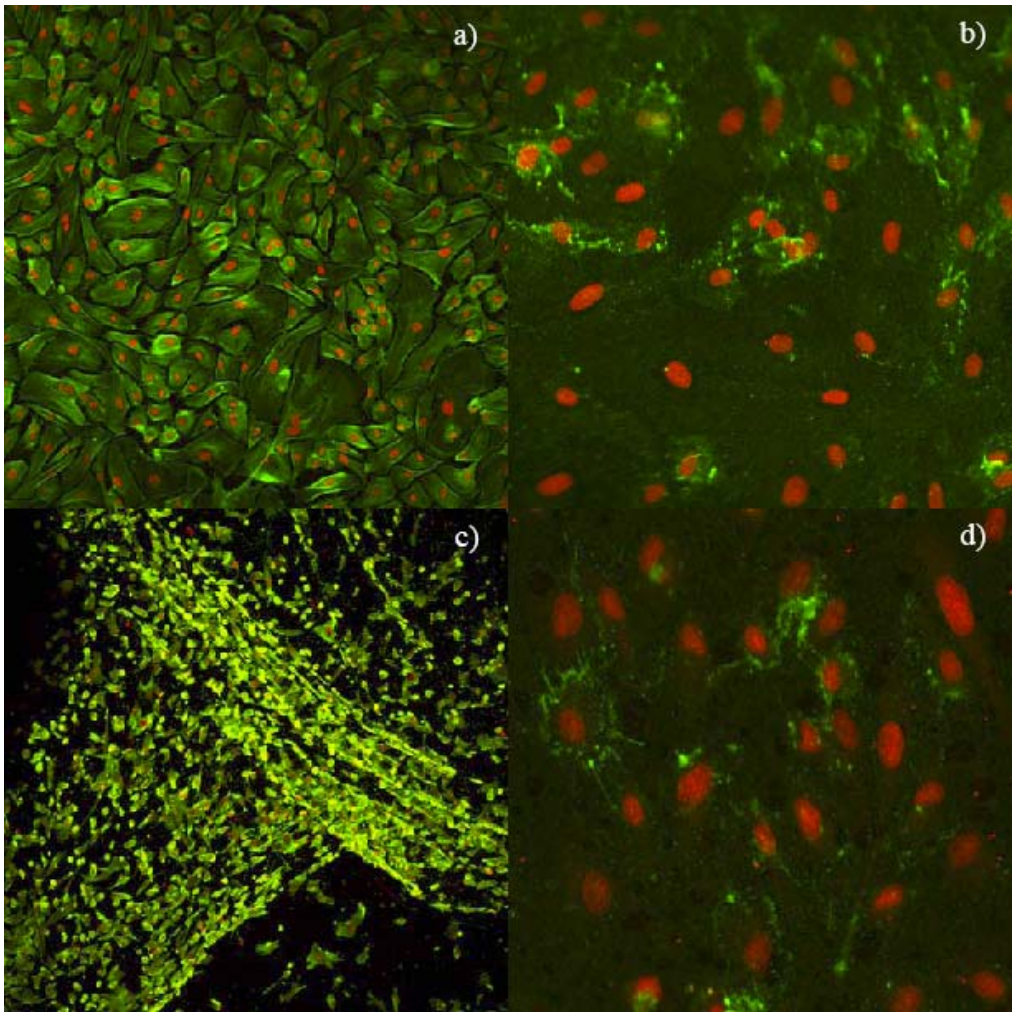

Figure 11 - Morphology of endothelial cells after 1 day and 3 days of culture; (a and c) actin staining and (b and d) Pecam-1 staining.

The results obtained by confocal microscopy analyses, confirm the results obtained on the cellular proliferation assay. After 1 day of culture, it was possible to observe the presence of endothelial cells in HA and SiHA samples (fig. 12). In both cases cells showed a positive stain for Pecam-1.

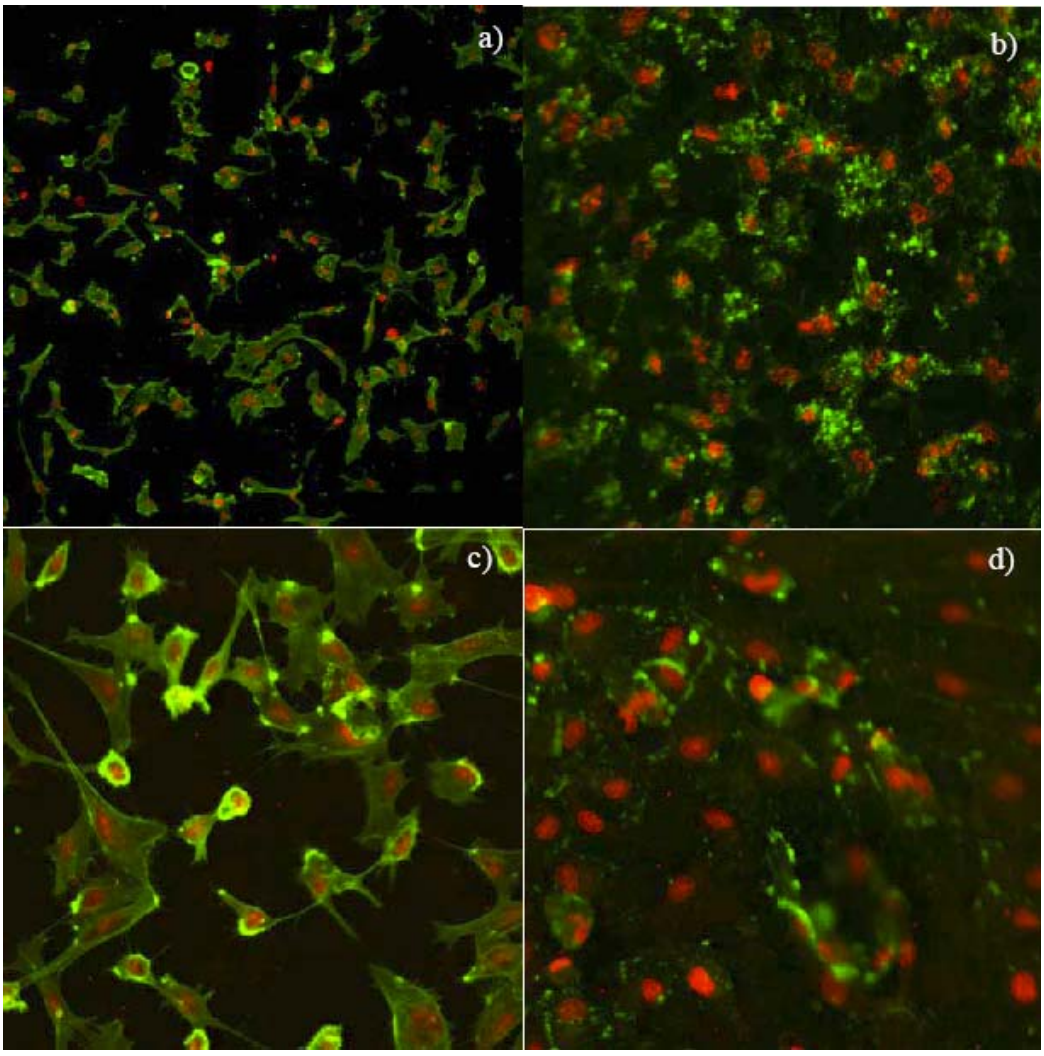

Figure 12 – Morphology of endothelial cells after 1 day of culture; (a and c) actin staining of endothelial cells in HA and SiHA respectively and (b and d) Pecam-1 staining of endothelial cells in HA and SiHA respectively.

These results showed again the positive effect of the incorporation of silicon into the HA lattice.

3. *In vitro* biological studies of human osteoblastic cells

MTT assay

Cultures grown on the surface of SiHA and HA coated titanium samples showed an increase in the cell proliferation untill day 21, and a decrease afterwards (fig.13). At day 7,

MTT reduction values were similar in both materials, although the rate of cell proliferation was higher on the SiHA samples. At day 21, MTT values in SiHA samples were significantly higher than those observed for the HA samples (fig. 13).

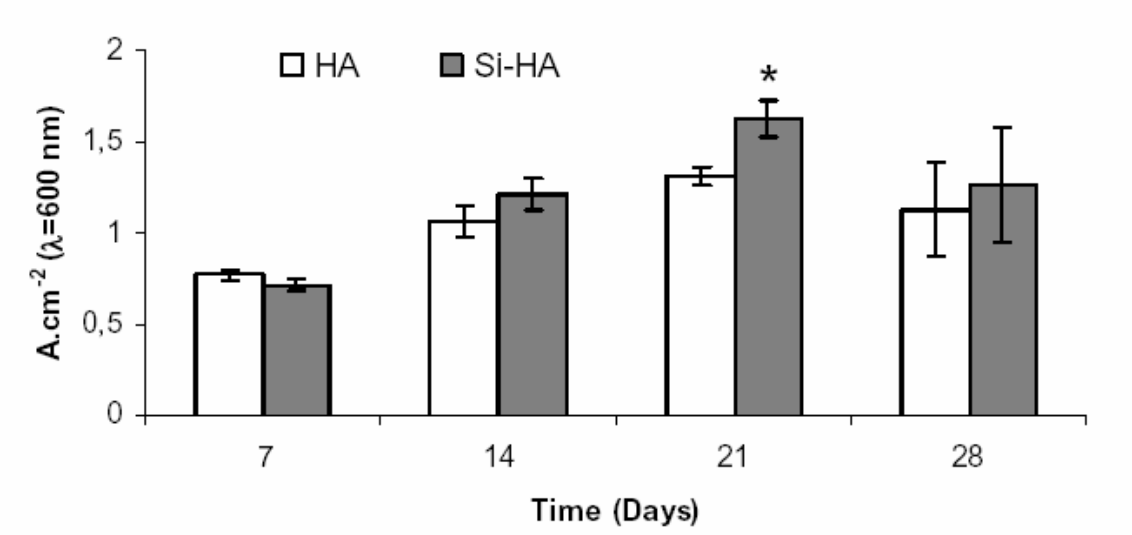

Figure 13 – Cell viability/proliferation (MTT assay) of human osteoblastic bone marrow cells cultured on the surface of Ti alloy coated with HA or SiHA.

SEM analysis

Through SEM analysis it was possible to see that at 3 days of culture cells adhered to the substrate and established contact points between them. At 7 days, the osteoblasts appear to be flattened on the substrate surface presenting a fibroblastic morphology with several cytoplasmic processes contacting directly with the coating material (fig. 14 (a)).

The cell culture reached confluence around day 14 (fig. 14 (b)) and formed cell multilayers (fig. 14 (c,d)). Mineral deposition, closely associated with the established cell layer, was verified from day 21 onwards (fig. 14 (d)). At a high magnification it is possible to mineral deposits that can be related to the formation of an apatite layer on the surface of Si-HA coated samples (data not shown).

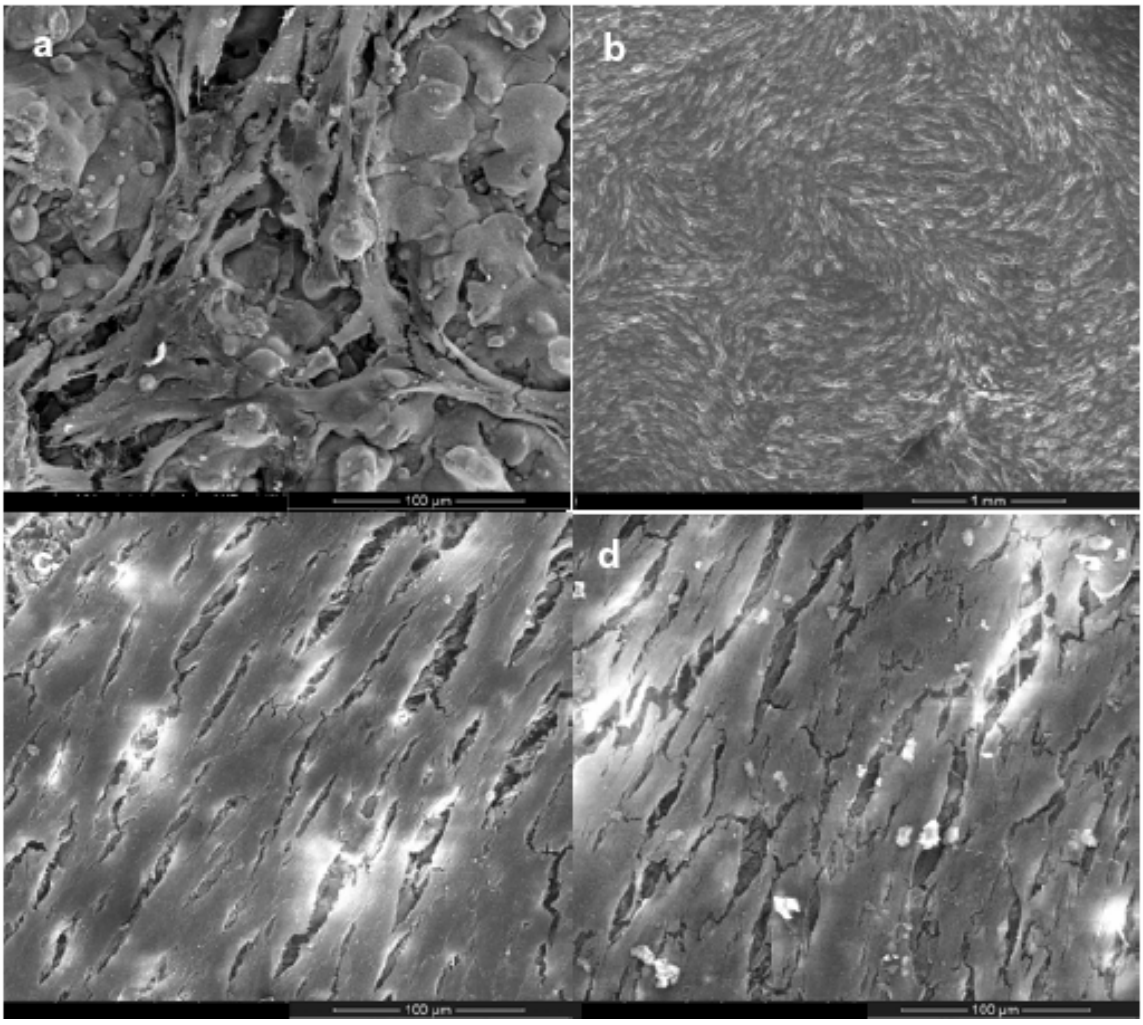

Figure 14 – SEM images of human osteoblastic bone marrow cells grown on the surface of Ti alloy coated with SiHA, at different periods of the culture: a - 7 days, b – 14 days, c – 21 days and d –28 days.

CLSM analysis

CLSM showed similar results regarding cell morphology and growth. The immunostaining of the actin cytoskeleton revealed a characteristic morphology with distinct microfilaments being visualized at day 3 (fig. 15). Active proliferation was verified with the coating being completely covered by an exuberant cell layer after two weeks of incubation (fig. 15 (b,c)).

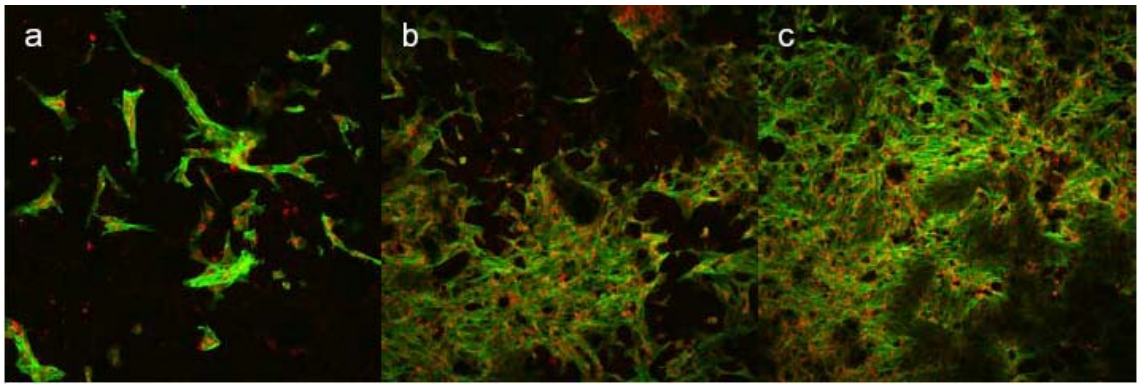

Figure 15 – Confocal laser scanning microscopy of the human osteoblastic bone marrow cells grown on the surface of Ti alloy coated with SiHA, at different periods of the culture: a – 3 days, b – 7 days, c – 21 days. Cytoskeleton and nuclei staining magnification: a – 250x, b and c - 100x.

These studies show the positive effect of silicon in the adhesion of endothelial and osteoblastic cells. So, this material, as in dense form as a coating material, probably will stimulate *in vivo* angiogenesis and osteogenesis.

DISCUSSION AND CONCLUSIONS

DISCUSSION

In this thesis it was proposed to characterise Hydroxyapatite and Silicon Substituted Hydroxyapatite, in a dense form, regarding its ability to stimulate angiogenesis and HA and SiHA as a coating material regarding its ability to stimulate osteogenese.

The angiogenesis was evaluated by *in vitro* cultures with human umbilical vein endothelial cells, which were seeded on the surface of HA and SiHA. Several parameters were assessed, namely cellular growth and functional activity at time points of the culture. The osteogenesis evaluation was performed by culturing human osteoblastic cells on the surface of Ti alloy coated samples with HA and SiHA.

If a coating material is too soluble, there is high probability that it will dissolve completely into the physiological environment, even before it is able to accelerate the promotion of direct attachment at the bone/implant interface [118]. Variables like temperature, pH, dissolution time, reagent type, must be controlled because small changes in these variables can be responsible for disarrangement of chemical reaction. According to the literature, [119, 120] the XRD pattern of the Si-HA powder before plasma spraying has a similar pattern to crystalline phase pure HA and after plasma spraying the SiHA coating becomes more amorphous and suffers some conversion to secondary phases, having the following chemical composition: HA (34.1%), calcium oxide (CaO: 12.6%) and tri-calcium phosphate phases (α-TCP: 27.4% and β-TCP: 25.9%).

On the FTIR spectra it was possible to see the characteristic wavelengths for the phosphate groups present on the HA lattice. When comparing the FTIR spectra of HA and SiHA it was possible to observe a decrease on the intensity of the band corresponding to the OH^- group. It is known that PO^{3-}_4 groups are preferentially located at HA surface and therefore the substitution of these ions for SiO_4^{4-} groups results in a decrease in surface charge [121]. The decrease in intensity of the peak on the FTIR spectra that corresponds to the OH^- group on the SiHA was expected since according to the mechanism described by the following equation: $10Ca^{2+} + (6\text{-}x)PO_4^{3-} + xSiO_4^{4-} + (2\text{-}x)OH^- \rightarrow Ca_{10}(PO4)_{6\text{-}x}(SiO4)_x(OH)_{2\text{-}x}$, the substitution of the phosphate group for the silicate group leads to the loss of some OH^- to maintain the charge balance [122]. Si^{4-} ions are structurally incorporated into the HA lattice in solid solution, and are not segregated as a secondary phase.

Thian *et al* [123] showed that with increasing Si addition, the crystalline size of SiHA decreased and the incorporation of Si inhibited grain growth, being the effect more significant as the level of Si increased. These results are in agreement with the results obtained through SEM analysis, that showed a significant difference between the grain size of HA and SiHA dense sample, being smaller in the SiHA. Additionally, it would be expected that an increased of Si would cause HA crystals to be more soluble, releasing more Ca^{2+} and P^{5+} ions into the culture medium. A rapid re-precipitating and formation of a newly-formed CaP-rich layer on the coating surface, with features very similar to the mineral phase of bone, would be an ideal site for osteoblast cells to attach, grow and form new bone [124]. Thian *et al* [123] showed that although the addition of Si increased the unit cell parameters and reduced the crystalline size, the XRD showed no changes in the relative intensities. In this work, XRD also showed no changes in the relative intensities what can be explained because Si^{4+} and P^{5+} are adjacent in the Periodic Table (differ by only 1 in atomic number) and the Si level is relatively low (< 5 wt.%), although such changes are normally expected when there is an atomic substitution [123].

Implantation involves tissue trauma, which evokes an inflammatory response, coupled to a wound healing reaction, involving angiogenesis, fibroblast activation and matrix remodelling. Three principal fields of research can yield useful data to better understand these phenomena: physical-chemical studies of biomaterials, *in vitro* studies and animal models. Until now the type and extent of such reactions to give optimal integration of various biomaterials are practically unknown [125]. *In vitro* biological studies with endothelial cells allows a better understanding of mechanisms involved in the interactions between cells and a material surface, important for the rational development of medical devices with optimal biocompatibility [126,127]. The success of biomaterial applications is not only dependent on the growth and function of the tissue-specific cells on the biomaterials but is also dependent in most cases on successful vascularization after implantation [128]. So, there is a significant importance to characterise the biological response of HA and SiHA in the presence of HUVECs.

The MTT assay measures the metabolic activity of the cells, which can be correlated with the number of viable cells. The number of endothelial cells that adhere to SiHA substrate was superior to the number of endothelial cells that adhere to HA substrate, being this difference statistically significant at day 1 and day 3 ($p<0.05$). Similar result was obtained at day 3 of culture. On the surface of the control used in this study (tissue culture

plastic), the HUVECs formed tubular structures and also showed a positive stain for Pecam-1, which proved that the cells culture on the surface of these materials expressed the endothelial phenotype. Therefore, the results obtained by confocal microscopy analyses confirm the results obtained in cellular proliferation assay. These results showed again the positive effect of the incorporation of silicon into the HA lattice.

Bone formation is dependent on several molecules such as BMPs, molecules that will initiate vascularization and osteogenic precursor cells. BMPs are also involved on the molecular cascade that will lead to the formation of cartilage and bone progenitor cell differentiation. Multipotent stem cells originating from bone marrow stromal cells are a particularly attractive source for osteogenic precursors for bone tissue engineering, because they can be easily harvested and expanded *in vitro* and can differentiate into bone-forming cells. Human osteoblastic cells were used to evaluate osteogenesis on coated HA and SiHA. *In vitro* studies with human osteoblastic cells showed that cultures grown on the surface of SiHA coated titanium had an increase in cell proliferation untill day 21, and a decrease afterwards. This behaviour is in accordance with the literature, where it is described the *in vitro* development of osteoblastic phenotype. At day 14 and 21, cell growth on the SiHA was higher (statistically significant) compared with the HA as expected according to the literature [94,95,102,108].

Ferraz *et al* [129] showed that the resulting surface modifications improved osteoblasts growth, probably due to the formation of a relatively stable calcium-phosphorous rich layer on the coatings surface that is a strong indicator of bioactivity. Furthermore, the adsorption of biological molecules from the culture medium by the material, have an important role in the adhesion process and functional behaviour of the osteoblasts cells [123,130]. The differences in the proliferation behaviour of human osteoblasts seeded into the surface of HA and of SiHA, are probably related to differences in surface characteristics, as their chemical composition and physical properties differ [94,95,102,108, 98-101, 130].

Although, in this study, no osteoblastic differentiation markers were assessed, the cell morphology and the formation of a mineralized matrix indicate that the cell culture reached a differentiated stage on the SiHA coated substrate. Compared to the HA coated titanium, the enhanced behaviour of the SiHA coating might be explained by the presence of silicon into the culture medium. It is known that the formation of an apatite layer is

preceded by a dissolution stage, which indicates that the silicon could be released into the culture medium and incorporated by the osteoblasts. Similar results were reported by Botelho *et al* [103] who showed that the SiHA, in a dense form, stimulates osteoblasts proliferation, differentiation and formation of mineral deposits. Thian *et al* [123] also reported that a SiHA coating prepared by magnetron co-sputtering can also stimulate the proliferation of osteoblasts. This is in line with that reported by several authors suggesting that silicon is a key element for bone regeneration [102,107].

These results clearly demonstrate the beneficial effect of silicon on the adhesion of endothelial cells and osteoblastic cells.

CONCLUSIONS

The main conclusions of this work are:

Analysis of the SiHA compositions prepared for this study using XRD and FTIR confirmed the substitution mechanism of silicon by phosphorous, in accordance with the results reported previously.

In vitro biological studies of human umbilical vein endothelial cells cultured in dense samples of HA and SiHA showed the positive effect the incorporation of Si into the HA lattice.

The SiHA coating allowed the adhesion, proliferation and differentiation of human bone marrow osteoblastic cells, which suggest that this coating is suitable to be used in several biomedical applications. Although, this study revealed an enhanced activity of the osteoblasts cultured on the SiHA coating, the mechanism behind this behaviour is still unknown and further evaluation is required.

The results achieved in this work are in agreement with previous *in vitro* studies suggesting that SiHA has better characteristics for bone cell growth and function than HA, using human osteoblasts to evaluate the biological response of the materials.

SiHA is suitable material to be used as bone graft, as in dense form or as a coating material. This material elicits a positive response in different cell types, such as: human umbilical vein endothelial cells and human osteoblastic cells.

As future work it is proposed to optimize the ideal amount of Si to incorporate into the HA lattice in order to improve the response of human umbilical vein endothelial cells to the material in a dense form and as a coating.

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