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Cell-material interaction: Effect of composite systems on cellular behaviour for tissue engineering applications

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AcLDL, acetylated low-density lipoprotein; AII, angiotensin II; ALP, alkaline phosphatase activity; ATP, adenosine triphosphate; ATR-IR, infrared attenuated total reflectance; BAEC, bovine aortic endothelial cells; BMD, bone mineral density; BMP, bone morphogenetic protein; CaP, calcium phosphate; CF, cystic fibrosis; COX, cyclooxygenase; cPLA2, phospholipases 2; CS, chitosan; DIPEA, N,N-diisopropylethylamine; Dll4, delta-like protein 4; DMF, N,N-dimethylformamide; DNA, deoxyribonucleic acid; EC, endothelial cells; ECM, extracellular matrix; EDAX, energy dispersive spectroscopy; EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor; eNOS, epithelial nitric oxide synthase; ERK, extracellular signal–regulated kinases; ES, embryonic stem cells; FBR, foreign body reaction; FBS, foetal bovine serum; FDA, food and drug administration; FGF, fibroblast growth factor; GI, gastrointestinal; GMP, good manufacturing practices; HA, hydroxyapatite; HAEC, human aortic endothelial cells; HDMECs, human dermal microvascular endothelial cells; HLA, human leukocyte antigen; HMECs, primary human microvascular endothelial cells; HPLC, high performance liquid chromatography; HUVECs, primary human umbilical vein endothelial cells; IBS, injectable bone substitute; IFN-γ, interferon gamma; IL-10 , interleukin-10; IL-1β, interleukin 1β; ILs, ionic liquids; iPS, induced pluripotent stem cells; LPS, lipopolisaccaride; MBC, minimum bactericidal concentration; MIC, minimum inhibitory concentration; MIS, minimally invasive surgery; MMPs, matrix metalloproteinases; MSCs, mesenchymal stem cells; NO, nitric oxide; OCN, osteocalcin; OP-1, osteogenic protein-1; PAF platelet-activating factor; PBS, phosphate buffered saline; PEGDA, poly-(Ethylene glycol) diacrylate; PGI2, prostacyclin; PHT, parathyroid hormone; PLA, polylactic acid; PLGA, poly lactic-co-glycolic acid; rhEGF, recombinant human epidermal growth factor; SBF, simulated body fluid; SEM, scanning electron microscopy; TE, tissue engineering; TEM, transmission electron microscopy; TGF-β, transforming growth factor beta; TNF-α, tumour necrosis factor alpha; TOF, time-of-flight; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; α-MEM, eagle's alpha minimum essential medium; β-TCP, beta-tricalcium phosphate.

Preface

Tissue engineering is an interdisciplinary field based on the creation of new tissues and organs. Ideal biomaterials for bone tissue regeneration must possess appropriate chemical, biochemical and biophysical properties. It is well-known that bone regeneration depends on several parameters, such as material properties, chemical and bioactive stimuli, and cells. This thesis provides an overview of innovative strategies in the field of bone tissue regeneration. In particular, this thesis concerns the advantages of using scaffolds and injectable biomaterials in tissue engineering for restorative and regenerative purposes. Recent findings (across the past decade) in stem cell biology and tissue engineering suggest new approaches for bone regeneration. In fact, stem cells have the potential to self-renew and give rise to a variety of cell types to ensure tissue regeneration. In this context, considerable attention has been given to device preparation using different approaches, therein conferring distinctive features on composite materials for bone repair. In fact, it is possible to introduce technologies in the biomedical field, allowing biomaterials with specific features such as specific injectability, architecture, geometry, porosity and interconnectivity to be obtained. These properties are critical for biological and mechanical performance. The principle idea behind this project is to develop new types of bioactive materials in order to repair bone defects associated with diseases such as osteoporosis with a focus on the functional use of "soft" materials for bone regeneration. This thesis explores the study, optimization and evaluation of systems that support bone regeneration by increasing the production of osteoblasts in osteoporotic sites as a new frontier for osteoporosis treatment and more specifically ,the development of porous composite scaffolds based on chitosan for bone regeneration using two different approaches. The study of these scaffolds is based on the fabrication of calcium phosphate injectable materials functionalized with ionic liquids using the sol-gel technique to promote bone regeneration and to prevent microbial infection and inflammation. **Chapter I** focuses on the State of the Art, while **Chapter II** deals with the development of chitosan-based scaffolds bioactivated by osteoinductive signals (*e.g.* hydroxyapatite nanoparticles, BMP-2 peptide) for advanced bone tissue engineering. Scaffolds were prepared through a new foaming method by Dr. Demitri at University of Salento. The method is useful because it permits synthesizing semi-interpenetrating Chitosan-PEGDA scaffolds at different concentrations. Mechanical and morphological characterisations were evaluated, and the *in vitro* effect of bioactivated scaffolds on

cellular behaviour was determined by analysing their osteoinductive properties. Finally, in the last part of Chapter II, the evaluation of the *in vitro* effect of bioactivated chitosan scaffolds on the angiogenesis process is also reported. **Chapter III** describes the fabrication of injectable ionic liquid-hydroxyapatite-based biocomposites obtained via the sol-gel method, which allows work to be conducted at room temperature with thermo-sensitive molecules. Most of the chapter is dedicated to the importance of the use of injectable materials for bone tissue regeneration with particular attention paid to the ability of ionic liquids to prevent and reduce microbial infections. Later, the chapter reports on the evaluation of the effects of these injectable gels on biological performance with a special focus on osteogenic and anti-inflammatory responses. From the beginning, this study involved the development, preparation and characterisation of biomaterials. Over time the work has gained a new focus: evaluating the antimicrobial and osteoinductive properties of the proposed multifunctional injectable systems. Finally, **Chapter IV** includes conclusions and future perspectives.

CHAPTER I

State of the art

1.1 Bone tissue

Bone comprises the majority of the skeleton and plays a key role in the body, both biomechanically and metabolically. There are three main functions associated with skeletal tissue: support, protection and calcium homeostasis. Indeed, the rigidity and hardness of bone maintains the shape of the body and supports it, permitting the transfer of muscular forces from one part of the body to another during normal movement. Moreover, bone exerts a pivotal function by protecting the soft tissue of cranial, thoracic and pelvic cavities. Bone contains minerals, which are a reserve for ions, especially calcium and are involved in the regulation of extracellular fluid composition. By weight, the bone matrix is composed of approximately 28% organic matter, 60% inorganic substance and the remaining 12% water or 38.4% organic matter, 37.7% mineral and 23.9% water by volume [1]. The mineral composition is impure and includes hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$, which contains carbonate, citrate, fluoride and strontium. The organic matrix consists of 90% collagen and about 10% non-collagenous proteins. From a mechanical point of view, the bone matrix is comparable to a composite material: the organic matrix is responsible for giving the toughness to bones, whereas the inorganic matrix has the function to stiffen and strengthen the bone [2]. In normal condition, a balance between resorption and oppositional processes characterises bone. In fact, bone cells are responsible of the processes of bone resorption, formation, and modelling. When an alteration in bone processes appears, osteoporosis and Paget's disease occur [3].

1.2 Osteoporosis

Osteoporosis is a debilitating, progressive, metabolic, systemic, chronic disease characterised by bone mass reduction and micro-architectural structure impairment in tissues. Osteoporosis can be primary (post-menopausal and senile) or secondary. The most common type of osteoporosis is primary osteoporosis, which affects more women

than men. Primary osteoporosis is characterised by a peak in bone mass density at about age 30. After this period, the rate of bone loss slowly increases, while the rate of bone building decreases. The thickness of the bones in early life as well as health, diet, and physical activity at all ages influence the development and progression of osteoporosis. In women, accelerated bone loss usually appears after monthly menstrual periods stop. Post-menopausal osteoporosis is due to a reduction in oestrogen production (usually between the ages of 45 and 55). In men, gradual bone loss typically begins at about 45 to 50 years of age, when a man's production of testosterone slows down. Osteoporosis usually affects women at an earlier age than men, as they generally have lower bone mass. Secondary osteoporosis causes the same symptoms as primary osteoporosis but it occurs because of specific medical conditions, such as hyperparathyroidism, hyperthyroidism, or leukaemia. However, secondary osteoporosis can also occur as the consequence of pharmacological treatments with drugs that induce bone breakdown, such as oral or high-dose inhaled corticosteroids (long-term treatments), too high a dose of thyroid replacement, or aromatase inhibitors. In contrast to primary osteoporosis, secondary osteoporosis can occur at any age.

Osteoporosis is defined as a "*silent disease*" because of the absence of apparent symptoms until a fracture occurs. It has been demonstrated that the bone loss is a silent and progressive process, in which the density and quality of the bone are greatly reduced (Figure 1), [4]. Osteoporosis disorder represents a growing problem in ageing people. It affects a large portion of the population, especially the elderly and postmenopausal women. Younger women, children and pregnant women can be affected as well. It has been noted that 1/3 of women and 1/5 of men are at risk for fractures resulting from osteoporosis. Indeed, the most common fractures associated with osteoporosis are at the hip, spine and wrist. At the time of writing, it's estimated that more than 200,000,000 people in the world suffer from this disease. In the United States and Europe alone, approximately 30% of all postmenopausal women have osteoporosis. At least 40% of women with osteoporosis and 15-30% of men experience one or more fragility fractures in their remaining lifetime [5]. An increasing proportion of the population going through the ageing process is responsible for a major increase in the osteoporosis incidence among postmenopausal women (Figure 2). Osteoporosis has a multifactorial aetiology and its clinical manifestation is connected to the complex relationship between environmental and genetic factors. Indeed, many elements, such as age related (epigenetic) changes, genetic predisposition and intrinsic factors induce an impairment of

bone regeneration and mechanical adaptation. The main risk factors besides age include the following: lifestyle, family history, female gender, hormonal status, long-term use of steroid medications, such as glucocorticoid, and the oestrogen deficiency that occurs with ageing. Oestrogen deficiency is especially problematic in the case of post-menopausal women. Indeed, lower oestrogen levels in the organism are one of the strongest risk factors for bone loss. Decades of research have underlined the effects of oestrogen on bone healing and maintenance through two different types of cells, osteoclasts and osteoblasts. It has been reported that oestrogen has a pivotal role in the modulation of bone metabolism, as a powerful modulator in this crucial process. Indeed, the deficiency in steroid hormone production is responsible for promoting higher resorption as compared to bone formation and therefore delays in fracture healing. Another consequence of the post-menopausal oestrogen level decrease is the loss of bone strength. The deficiency in this sex hormone also induces fatty bone marrow degeneration. Therefore, this imbalance facilitates adipogenic differentiation over osteogenic differentiation and is the basis of bone degeneration. It is well-known that the bone is dynamic tissue that undergoes a continuous physiological process of remodelling. Indeed, bone resorption, under normal conditions, is counterbalanced by bone formation in order to maintain the healthy function of the bone. There is a cross-talk between different kinds of cells in human bone: osteoblasts, osteoclasts, and osteocytes. Osteoblasts and osteocytes possess the role of building new bone and generating healthy tissue, meanwhile osteoclasts remove and break down old bone and reduce defects in the micro-architecture of bone tissue. When an impairment of balance between bone resorption and formation occurs, osteoporotic pathology appears. Alterations in bone remodelling, which promote bone resorption over *de novo* synthesis, lead to diseases such as osteoporosis. The pathological condition of osteoporosis consists of changes in the physical properties of bone matrix and the imbalance causes a decreased bone mineral density (BMD). The osteoporotic bone is characterised by higher porosity and fragility than normal bone, making the bone more sensitive to fractures, among the most common, hip fractures. It has been demonstrated that the disorder can be localised in a specific osteoporotic site or can involve the entire skeleton with high impact trauma and bone fractures. The above leads to serious consequences in the health and life quality of patients and in healthcare costs. Epidemiological studies show that the diagnosis of the pathogenesis of osteoporosis is still difficult, due to the complexity of pathogenesis. This issue encourages the researchers to pay more attention to prevention, and the search for new

therapeutic strategies for the treatment of the disease. In this context, the engineering of complex bones built using several materials (porous scaffolds and injectable gels) combined with differentiation factors, and precursor cells, represents a useful tool for promoting bone tissue regeneration and preventing osteoporotic fractures.

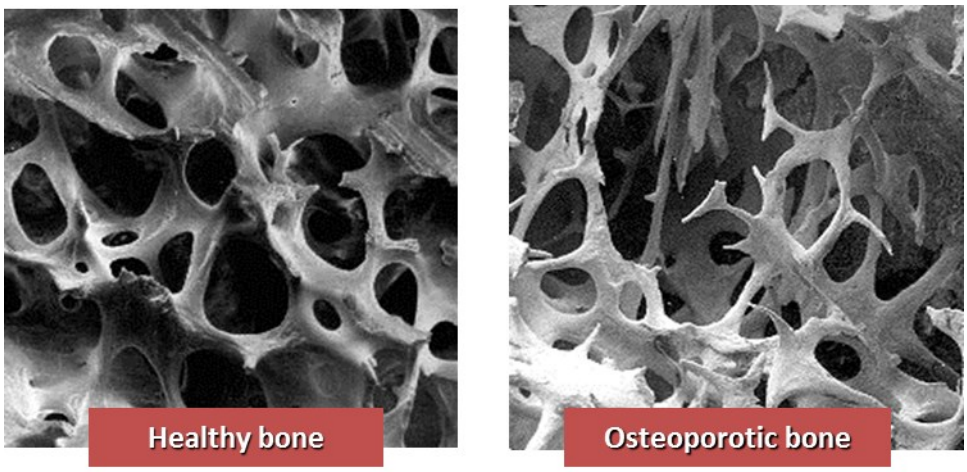

Figure 1. Images of the reduction in osteoporotic bone density compared to healthy bone mass.

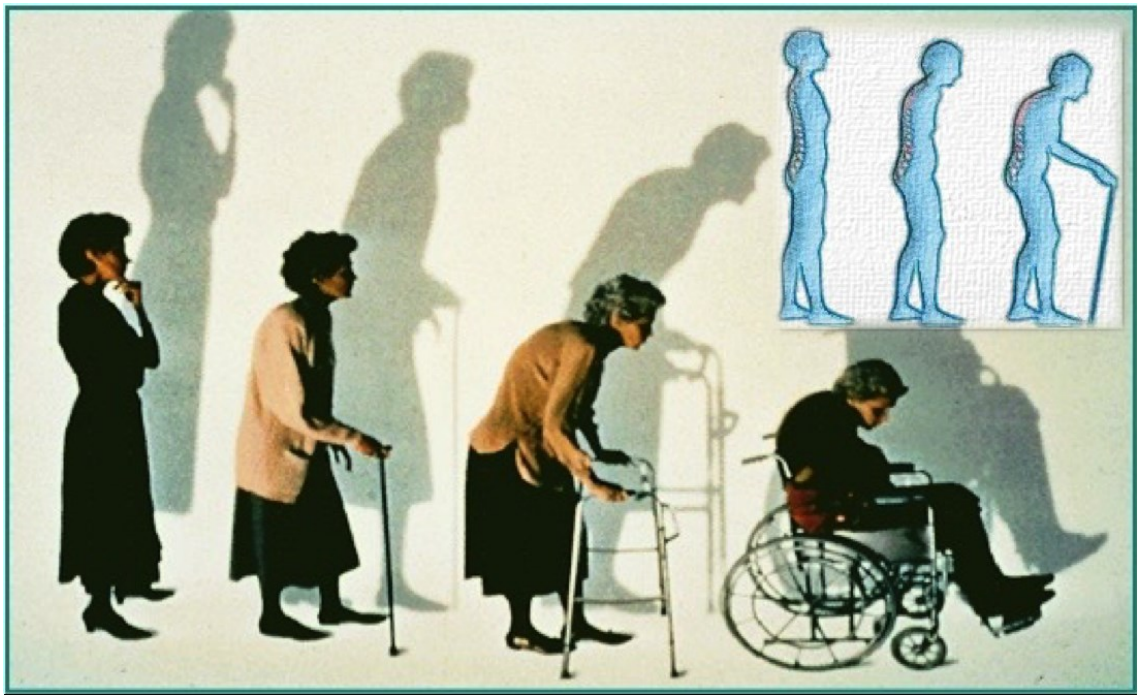

Figure 2. Post-menopausal women affected by osteoporosis and the clinical consequences of spinal column shortening.

1.2.1 Current therapies for osteoporosis treatment

There are several therapeutic targets for osteoporosis. The main goals of osteoporosis treatments are to reduce the loss of bone mass, to prevent bone fractures, and to treat clinical consequences and symptoms associated with the disease. It is well known that bone loss can be reduced also by building new bone or inhibiting osteoclasts, which leads to a breakdown of the old bone. There are many different approaches, including pharmacological therapy, physiotherapy interventions, and surgical treatment. However, many osteoporotic patients do not tolerate the currently available options, due to several side effects, as among them renal and gastrointestinal diseases associated with the administration of some conventional drugs. Clinically approved therapies to treat osteoporosis include oestrogen replacement therapy, the use of bisphosphonates (*e.g.,* alendronate and zoledronic acid), the systemic use of parathyroid hormone (PHT), calcitonin and strontium ranelate, (not approved in the United States). Strontium ranelate is a first-line treatment for post-menopausal osteoporotic patients. The effectiveness of treatment with anti-osteoporotic drugs has been documented in a wide range of patient profiles varying by age, number of prevalent vertebral fractures, body mass index and family history of osteoporosis. Indeed, strontium ranelate, due to its double and simultaneous action mechanism, has the ability to increase bone formation by inhibiting or slowing the bone resorption process. It affects BMD, in addition to restoring bone turnover and osteoporotic fractures. The rebalancing in bone replacement is based on the improvement of bone geometry, cortical thickness, trabecular bone morphology and intrinsic tissue quality. Several controlled trials emphasize the importance and the efficiency of strontium ranelate in osteoporosis to reduce the risk of vertebral and hip fractures. However, its systemic administration has many limitations. Even though the drug possesses a wide spectrum of efficacy, high tolerance and safety, sometimes it can cause undesirable side effects (*e.g.* gastrointestinal disorders, venous thromboembolism, myocardial infarction) and low *compliance* at the site of interest. In this context, tissue engineering is a good approach to reducing problems associated with systemic administration. New controlled-release systems in osteoporotic centres can be developed. A modern approach is based on the combination and the synergistic use of suitable polymeric and/or ceramic structures (in terms of biocompatibility, biodegradability, chemical-physical, mechanical and biological properties), biomolecules and cells. A recent study suggested that 8.5% of patients reported low adherence to osteoporosis medications and 21.6% of them stopped their medication completely over the following

two years [6]. Therefore, there is a compelling need to find alternatives for the treatment of osteoporosis. Several materials with different properties (in terms of stability, functionality, degradability, biocompatibility and antigenicity) have been clinically developed. These "intelligent" materials are useful in the field of tissue engineering because they have the ability to mimic the natural environments of the cells [7].

1.3 Bone tissue engineering

Indeed, tissue engineering (TE) represents a valid alternative for treating and restoring bone defects, considering that every year in the United States there are approximately 900,000 hospitalizations due to bone fractures which require intervention [8]. TE was only defined in the mid-1980s. Skalak and Fox firstly defined tissue engineering as "*the application of principles and methods of engineering and life sciences towards the fundamental understanding of structure-function relationships in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain or improve tissue function"*[9]. The discipline applies the principles of engineering and sciences, such as biology and medicine to biological substitutes (Figure 3), which can be used to restore, maintain or improve bone tissue function [10]. TE seeks to maintain and restore the function of human bone tissue by combining cell biology, materials science and principles of engineering principles.

The general principle is based on the development of matrices, which consequently can be incorporated into the body. Matrices are generally composed of natural or synthetic polymers. Indeed, TE is a promising alternative approach to treat the loss of tissues or organs without the limitations of current therapies. Specifically, the current research emphasizes biodegradable polymers, combining specific structures with osteogenic precursor cells. Several biodegradable materials have been considered for the preparation of three-dimensional porous scaffolds for bone tissue engineering [11]. The use of these materials is explained by their similarity to extracellular matrix, chemical versatility, biological performance and cellular interactions. The scaffolds or three-dimensional (3D) constructs provide support to cells to proliferate and maintain their differentiated function [12]. Figure 4 shows that TE is composed of different steps: 1) cells need to be extracted from the patient or a donor; 2) cells then can grow and expand in *in vitro* conditions; 3) it then becomes possible to implant the cells in a matrix, defined as a *scaffold*. 4) The

scaffold should offer a suitable environment to promote cell growth and differentiation; 5) scaffold-containing cells can be implanted into the patient by the surgeon [13].

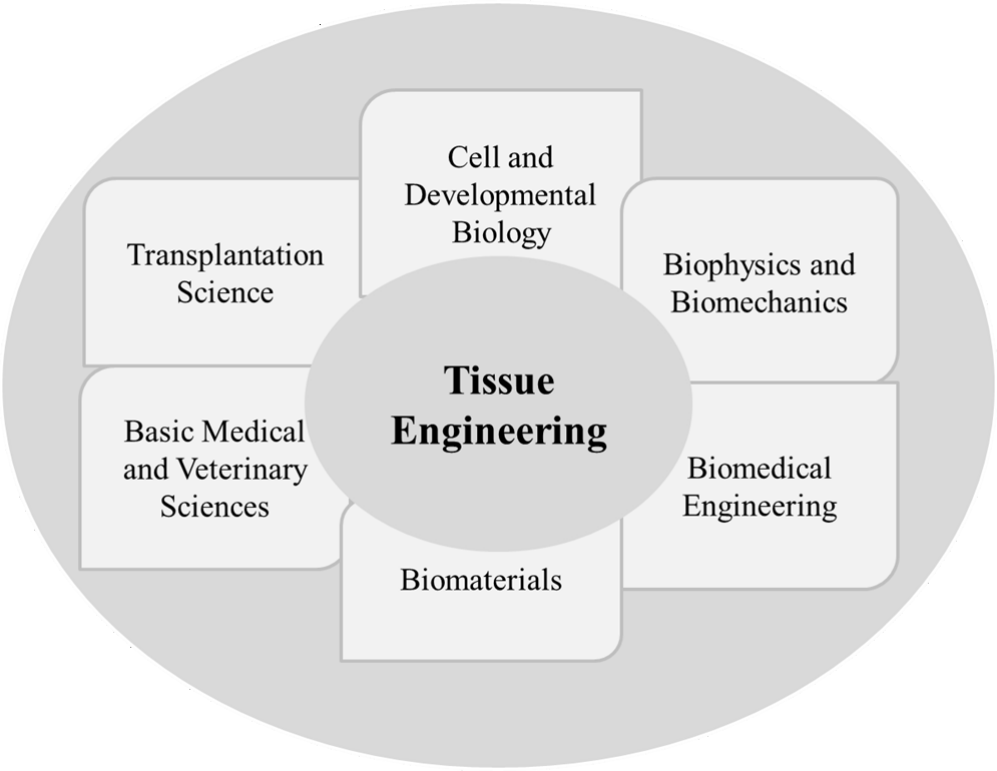

Figure 3. Multidisciplinary tissue-engineering.

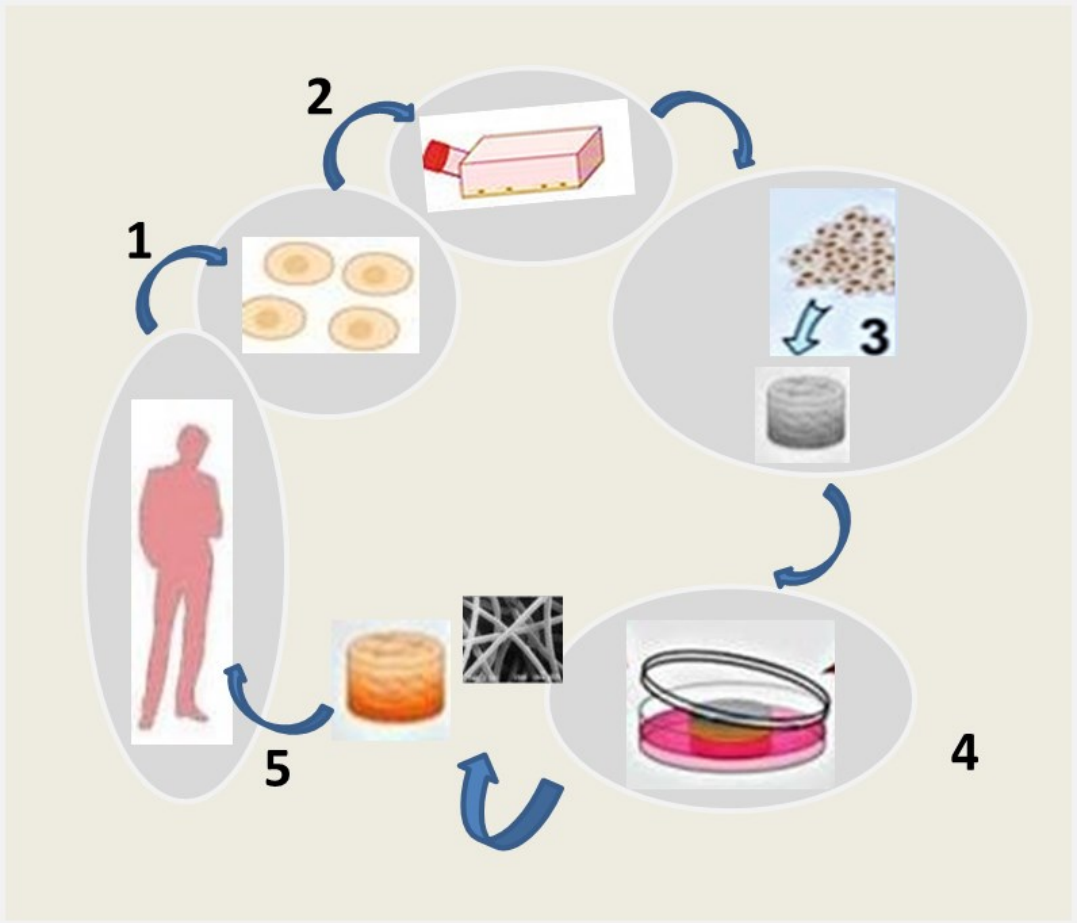

Figure 4. Steps in the tissue engineering approach.

1.3.1 Tissue engineering approach

In the last decade, as life expectancy continues to increase, the need for new bone substitutes in order to promote bone replacement is growing very rapidly. As a result, there is a great demand for biomaterials with specific anti-inflammatory, antibacterial and regenerative properties [14]. In recent years, considerable attention has been given to regenerative medicine and tissue engineering to replace bone tissue. In this context, the main challenge in tissue engineering is to develop biomaterial-bases which induce stem cell response in terms of osteogenic differentiation. Since the 1990s, numerous materials for supporting cell attachment, growth, and differentiation, as well as new stem cell sources and bioactive molecules have been identified in order to improve bone tissue regeneration following lesions derived from osteoporosis. Research has developed new experimental methods for tissue regeneration using the combination of 3 key elements, namely, stem cells, bioactive molecules (*e.g.,* growth factors), and scaffolds [15]. Scaffolds mimicking extracellular-matrix endow mechanical support, promote biological response and regulate bioactive molecule effects [16].

1.3.2 Cell-material interaction

The success of a biomaterial in tissue regeneration processes depends on its ability to interact with the physiological environment and specifically with cells [17]. The cell-material interaction process is composed of different stages. The first stage consists of the contact between material surface and water molecules forming a water coating layer. This step is highly dependent on the material surface properties. The second step is characterised by the interaction of all the existing macromolecules in the physiological medium, namely sugars, lipids and proteins with the material surface which influences material biocompatibility. Surface features, surface energy and the chemical composition of the material strongly influence the cell-material interaction process determining the nature of the proteins adsorbed to the surface and their orientation and conformation, which are crucial parameters. Finally, in a third stage, cells directly interact with material. Cell-material interaction takes place through cell adhesion proteins known as integrins that interact with particular peptide motifs from the protein adsorbed to the material surface. Integrins are cell transmembrane proteins that possess two units of glycoproteins (α and β) and three domains (cytoplasmic, transmembrane and extracellular). The

extracellular domains of the α and β units possess receptors for the specific recognition of cell adhesive peptide motifs that are contained in some adhesive proteins present in the ECM [18-20].

1.4 Mesenchymal stem cells: tools for bone tissue regeneration

Several research studies have been performed on mesenchymal stem cells (MSCs) capable of generating several tissue types including bone tissue. It was widely reported that MSCs isolated from bone marrow in combination with scaffolds and growth factors are able to regenerate bone tissue both in *vivo* and in *vitro* experimental models [21]. At present, the main challenge in regenerative medicine is to identify the ideal scaffold to enhance MSCs' residing response in terms of cell growth, spreading, adhesion and differentiation. Phenotypically, MSCs express the CD13, CD29, CD44, CD59, CD73, CD90, CD105, CD146 and STRO-1 surface antigens, and they do not express CD45 (a leukocyte marker), CD34 (the marker for primitive hematopoietic progenitor and endothelial cells), CD14 and CD11 (markers for monocytes and macrophages), CD79 and CD19 (B cell markers), or HLA class II. Most current tissue engineering approaches suggested the use of MSCs taken from sites that are even more accessible and rich in stem cells because the ability of stem cells to renew themselves indefinitely and differentiate into multiple types of more specialized cell phenotypes. However, these regenerative mechanisms decrease with age and cells lose the ability to repair damaged tissues [22]. Tissue engineering introduced the combination of biomaterials, growth factors and stem cells to overcome a lack of "self-renewal" in damaged tissue [23]. In this context, as previously reported, scaffold properties are crucial to enhancing MSCs' biological response. To satisfy the aims of regenerative medicine MSCs should demonstrate the following features: they should be in abundant in number, able to differentiate in multiple cell lineages, and able to be isolated using minimally invasive procedures, produced according to *GMP* (Good Manufacture Practice) and transplanted safely [24-26]. Commonly three main types of stem cells are used for tissue repair: *i)* embryonic stem cells derived from embryos (ES); *ii)* adult stem cells that are derived from adult tissue; and *iii) induced* Pluripotent Stem (iPS) cells that have been produced artificially via genetic manipulation of the somatic cells [27]. ES and iPS cells are pluripotent stem cells because of their ability to differentiate into all types of cells from

all three germinal layers. On the other hand, adult stem cells are multipotent because they are only able to differentiate into a restricted number of cell types. Initially, it was widely reported that each type of tissue possesses a specific area called the "stem cell niche" containing adult stem cells. MSCs were isolated from bone marrow for the first time by Friedenstein *et al.* in 1974 [28]. To date, MSCs can be isolated from different tissues including peripheral blood, umbilical cord blood, and amniotic membrane, adult connective, adipose and dental tissues [29]. Mesenchymal stem cells (MSCs) represent a great source of specific biomaterials that can manipulate the fate of stem cells and lead to high quality tissue regeneration for bone defect therapy [30]. In fact, besides controlling the fate of stem cells, the biomaterials play a pivotal role in regulating MSCs' many physiological functions including survival and host immune system control [31]. The immune response plays a key role in several bone tissue degenerative and regenerative processes. In fact, it was widely reported that pro-inflammatory mediators such as TNF-α (tumour necrosis factor alpha) and IFN-γ (Interferon gamma) induced down-regulation of osteogenesis thus inhibiting MSC-mediated bone regeneration [32]. Thus, by using biomaterials functionalised with specific signals it is possible to protect MSCs from the host immune cell/cytokine insult and regulate the crosstalk between immune cells and MSCs. These new tissue-engineering approaches make stem cell-based therapies a very promising long-term alternative in the treatment of bone defects due to the possibility of regenerating bone tissue and keeping the structural integrity and physiological functions of bone. Furthermore, by using new biomaterials it is possible to control innervation and vascularisation in stem cell niche homeostasis, thus promoting stem cell response in terms of angiogenesis, another important stage in new bone tissue formation [33]. In order to generate the formation of new bone tissue MSCs are isolated, expanded in culture and finally seeded within or onto a natural or synthetic scaffold that can reproduce the shape of the newly forming tissue and then the newly formed "organoid" can be transplanted into the patient. In this context another approach consists of the direct implantation of a cellular scaffold into the bone defect so the body cells can populate the scaffold to form the new tissue *in situ*.

1.5 Human umbilical vein endothelial cells (HUVECs)

Endothelial cells (EC) are of particular interest in regenerative medicine [34]. Endothelial

cell structure and function alterations may significantly contribute to diseases of blood vessel such as thrombosis, atherosclerosis, and vasculitis. The construction of stable blood vessels is an important challenge in the field of tissue engineering. It is possible to introduce genes for bone tissue regeneration in devices that can enhance the survival and/or proliferation of vascular cells and endothelial cells (EC) in order to extend the lifespan of the engineered vessels [35]. EC have an important role in physiological hemostasis, blood vessel permeability [36] and blood vessel response to physiological and pathological stimuli [37]. Therefore, EC are currently used as *in vitro* model systems to investigate various physiological and pathological processes, especially in angiogenesis research. In recent years, many researchers have established and characterised EC lines. It is well known that endothelial cells are involved in many biological events. Several studies have suggested that these cells are very sensitive to growth factors and biological agents and they can repeatedly be extended. In fact, Fibroblast Growth Factor (FGF) or thrombin stimulates these cells, inducing a proliferative response [38]. Human vascular endothelial cells also respond to Epidermal Growth Factor (EGF), but their rate of proliferation is slower than when maintained with FGF and thrombin [39]. Thus, the set of factors controlling vascular endothelial cells proliferation may depend on the vascular endothelial cells' origins. Generally, EC include cell types of various origin; primary and immortalised bovine aortic endothelial cells (BAEC), primary human umbilical vein endothelial cells (HUVECs), primary human microvascular endothelial cells (HMECs), primary human dermal microvascular endothelial cells (HDMECs) and immortalised microvascular endothelial cells (HMEC-1). The availability of these cells has played a crucial role in the development of the field of vascular biology [40]. Particularly, human umbilical vein endothelial cells (HUVECs) have played a major role as a model system in order to study endothelial cell function regulation. It is no simple matter to decide which cell line is better to use for the final aim of research. In this context, HUVECs have a crucial role in the response of the blood vessel wall to stretch, shear forces, and the development of atherosclerotic plaques and angiogenesis. In fact, HUVECs are popular and easy to isolate in laboratories [41]. The human umbilical cord is one of the most important sources of vascular endothelial progenitor cells. HUVECs are isolated from freshly obtained human umbilical cords through the collagenase digestion of the interior of the umbilical vein. A sterile technique is used in all manipulations. After birth, the umbilical cord is severed from the placenta and placed in a sterile container filled with cord buffer

(0.14 M NaCl, 0.004 M KCI, 0.001 M phosphate buffer, pH 7.4, and 0.011 M glucose). Then, perfusion of the human umbilical cord vein with collagenase permits the attainment of a pure preparation of the single layer of endothelial cells that line this vessel [42]. The cells at initial passages, which grow in the presence of heparin and pituitary extract, maintain nearly the same features as native vascular endothelial cells. Characteristics include the expression of endothelial cell specific markers such as von Willebrand factor and an endothelial cell specific adhesion molecule, CD31, expression of receptors for growth factors, cytokines, vasoactive ligands, and specific signalling pathways for vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), transforming growth factor-β, tumour necrosis factor (TNF-α) and angiotensin II [43]. Hence, under *in vitro* culture conditions, HUVECs show a typical "cobblestone" morphology and spontaneously form tubules. Furthermore, several studies demonstrate that these cells can be induced to form tubules in cell culture if deprived of endothelial cell growth factor (ECGF) for 4 to 6 weeks [44]. Additionally, scanning electron microscopy analyses show that cultured human endothelial cells grow as monolayers of closely opposed, polygonal large cells. Under transmission electron microscopy, the cells seem to contain cytoplasmic inclusions (Weibel-Palade bodies) characteristic of *in situ* endothelial cells. These inclusions were also found in endothelial cells lining umbilical veins but were not seen in smooth muscle cells or fibroblasts in culture or *in situ*. [45]. Indeed, HUVECs provide a critical *in vitro* model for major breakthroughs in molecular medicine including seminal insights into cellular and molecular events in the pathophysiology of atherosclerosis and plaque formation, and mechanisms for angiogenesis control or neovascularisation in response to hypoxia and inflammation in tumours, ischemic tissue, and in embryogenesis [46]. Moreover, monolayers of HUVECs have been studied for endothelial cell layer/leukocyte and macrophage interaction in vascular tissues. This was useful in the discovery of adhesion molecules, chemokines and kinases, which mediate the interaction of inflammatory cells with the endothelial surface and their migration into the media. HUVEC monolayers are generated on deformable surfaces or in chambers, which allow for the study of the effects of shear stress and pulsatile flow on cell signalling in order to reproduce the effects of blood flow on endothelial cell function *in vivo*. These monolayers have been used to identify transcription factors such as KLF2, which regulate the expression of adhesion molecules such as vascular cell adhesion molecule-1 and endothelial adhesion molecule E-selectin in response to stress and proinflammatory cytokines such as TNF-α which mediate changes in cell adhesion and

migration. This plays a role in early changes in atherosclerosis. Cultured endothelial cells contain a large amount of smooth muscle actomyosin and ABH antigens appropriate to the tissue donor's blood type; these antigens are not detectable on cultured smooth muscle cells or fibroblasts. This evidence demonstrates that it is possible to culture morphologically and immunologically identifiable human endothelial cells for a period of up to 5 months. HUVECs are endothelial cells of human origin with characteristics similar to the parent endothelial cell. They exhibit positive acetylated low-density lipoprotein (AcLDL) uptake, express eNOS, CD31 and V-cadherin, and spontaneously form capillary-like structures when grown on Matrigel. HUVECs also maintain endothelial cell characteristics at the mitogenesis, kinase activation and vasodilator production level. Moreover, they express key proteins necessary for vasodilator production, including epithelial nitric oxide synthase (eNOS), HSP 90, cav-1 and -2, cPLA2, and COX-1 and -2. In HUVECs, receptors for angiotensin II (AII), bradykinin, ATP and growth factors have been detected. Thus HUVECs appear to be functionally very similar to primary cell line and they will prove a valuable tool for future studies in therapeutic sciences. Moreover, the data strongly suggests that HUVECs are a pure population of endothelial cells and the production of vasodilators such as nitric oxide (NO) and prostacyclin (PGI2) by endothelial cells is an essential factor when it comes to maintaining the balance between vasodilation and vasoconstriction [46]. More substantially, HUVECs represent a valid tool for studying the angiogenesis process in order to assess the potential angiogenic effect of biocomposites in tissue engineering applications. Consequently, the research pays great attention to HUVECs use in the study of angiogenesis and vasculogenesis processes.

1.6 Porous scaffold for tissue engineering

A wide variety of polymer scaffolds, both synthetic (*e.g.*, poly [lactic] acid) and natural (*e.g.*, collagen), ranging from macroporous structures obtained through salt leaching/solvent casting and gas foaming, to nanofibrous scaffolds processed via electrospinning, self-assembly, and phase-separation have been realized for regeneration of bone tissue [47]. In order to mimic bone mass structure, approaches in scaffold design must be able to create 3D hierarchical porous structures for the desired mechanical function and mass transport (that is, permeability and diffusion) properties. Porous

hierarchical structures present pores of different size at scales from the nanometre to millimetre level that influence mechanical functions, nutrients transport and cell migration. Biodegradable scaffolds with these features provide temporary mechanical support to bone tissue regeneration. In fact, they must degrade into biocompatible products, ideally on a time scale comparable to that of new tissue development. Many scaffolds are typically fabricated with biocompatible polymers, proteins, peptides, and inorganic materials. Today, four types of material have been studied experimentally and clinically as scaffold material: (A) synthetic organic materials; (B) synthetic inorganic materials: hydroxyapatite, tricalcium-phosphate; (C) organic materials of natural origin: collagen, hyaluronic acid; (D) inorganic material of natural origin: coralline hydroxyapatite [48]. The ideal scaffold should be highly biocompatible so as not to elicit immunological or clinically detectable primary or secondary foreign body reactions. However, biocompatibility or tissue tolerance is not enough. Henche and Ethridge expressed a general theory of biomaterials in 1982 [49].

An ideal implant material should perform as if it were equivalent to the host tissue

- The tissue at the interface should be equivalent to the normal host tissue

- The response of the material to physical stimuli should be like that of the tissue replaced

Moreover, the 3D scaffold surface can be functionalised to promote cell adhesion through specific cell–matrix interactions. In fact, the formation of complex tissues from single cells and tissue maintenance needs large amounts of information, which must be transported from cell to cell, and from cells to ECM. Scaffold activity is also conferred by chemical composition, which determines the identity of ligands; the specific surface of the porous network; the orientation of pore channels, which determines the spatial configuration of ligands; and degradation rate of the scaffolds which depends on the chemical composition as well as the cross-link density of the macromolecular network and determines the duration of the active surface. In bone tissue engineering, an understanding of the bone-forming behaviour of cells must be combined with progress in material science, to achieve guided bone regeneration. Starting from the 1950s, in bone regenerative applications there was a predominant use of metal implants and associated devices with a good effectiveness on local tissues. In the 1970s and 1980s, polymers and synthetic materials able to enhance cell biological responses began replacing metal

implants. Recently, many efforts have been made to design both natural and degradable scaffolds in three dimensions, that are structurally more acceptable and more able to completely regenerate tissue [50]. Recent findings have shown that alginate and/or chitosan (natural polymers) are also useful in achieving injectable biomaterial-based scaffolds for clinical applications aimed at regenerating teeth including dentinal-wall-thickening, root maturation, and, in some cases, the formation of reparative cementum-like tissue [51]. In the context of porous scaffolds, a natural polymer, chitosan has received much attention in the regenerative field because of its positive properties. Many studies on chitosan-based scaffold preparation and their *in vitro* biological response were performed in order to promote bone regeneration [52]. It is well-known that chitosan is biocompatible, non-toxic and mechanically stable. It is also suitable for interactions with soft tissues due to its porous structure and possesses chemical groups, which can be easily functionalized or bio-activated chemically. Furthermore, chitosan has a high affinity for *in vivo* macromolecules and seems to be a good potential material for other tissues such as skin, adipose, cornea, liver, nerve and blood vessel.

1.6.1 Biomimetic treatment of scaffold for bone regeneration

Many bioactivation strategies may be applied in order to obtain scaffold with osteoinductive properties for bone regeneration applications. In this thesis, the scaffold surface was bioactivated with dual osteogenic signals: organic and inorganic. Organic approaches include the osteogenic peptides and proteins immobilization on the scaffold surface. In fact, short sequences mimicking proteins of Bone Morphogenic Protein (BMP) such as BMP-2 can be synthetized and immobilised on scaffold in order to improve the osteoinductive properties of biomaterials. On the other hand, inorganic approaches consist of coating with apatite using a supersaturated SBF solution (5xSBF). The solution induces hydroxyapatite nuclei formation, according to the method described by Kokubo and co-workers [53]. Specifically, we used an accelerated treatment, which combines the preliminary use of a supersaturated SBF solution ($5xSBF_1$) to stimulate the formation of nuclei, while a fresh chemically modified solution ($5xSBF_2$) is used, in order to promote the growing of apatite nuclei, once nuclei are formed. Meanwhile, $5xSBF_1$ was prepared by sequentially dissolving $CaCl_2$, $MgCl_2 \cdot 6H_2O$, $NaHCO_3$ and $K_2HPO_4 \cdot 3H_2O$ in distilled water. Solution pH was lowered to 6 by adding hydrochloric

acid HCl (1M) to increase the solubility. Na_2SO_4, KCl were added until the previous solution become clear. The final pH was adjusted with 1M NaOH to reach a final pH equal to 6.5 ($5xSBF_1$). Therefore, Mg^{2+} and HCO_3^- free 5xSBF was prepared by subsequently adding $CaCl_2$, $K_2HPO_4 \cdot 3H_2O$ to distilled water up to obtain a fresh solution ($5xSBF_2$). In this case, the solution pH was lowered to 6.0 with hydrochloric acid to increase the solubility. Both solutions were buffered at pH 6.5 and 6 respectively, by using Tris hydroxymethyl aminomethane-chloric acid (Trizma-HCl). Table 1 reported the ionic concentration of blood plasma, SBF, $5xSBF_1$ and $5xSBF_2$. All solutions were prepared freshly before use.The biomimetic treatment consists of two steps in a pH-controlled environment: during the first step, samples of the pre-ordered size were soaked into $5xSBF_1$ at pH=6.5 where the 5xSBF solution volumes have been calculated in respect to the total scaffold material surface by using an exposed surface to SBF volume ratio equal to 10 mm^2/ml, as reported in the literature [54].

Solution temperature was fixed at 37 °C during the treatment. After the sequential immersion in $5xSBF_1$ (4 days) and in $5xSBF_2$ (3 days), all scaffolds were gently rinsed in distilled water to remove excess ions and, then dried overnight under a laminar flow hood.

Table1: The ionic concentration of human blood plasma, SBF, $5xSBF_1$ and 5xSBF

Ion Concentration (mM)	Na^+	K^+	Ca^{2+}	Mg^{2+}	HCO_3^-	Cl^-	HPO_4^-	SO_4^{2-}
Blood plasma	142	5	2.5	1.5	27	103	1	0.5
SBF	142	5	2.5	1.5	4.2	148	1	0.5
$5xSBF_1$	710	25	12.5	7.5	21	740	5	2.5
$5xSBF_2$	710	10	12.5	-	-	735	5	-

1.7 Injectable calcium phosphate materials

Minimally invasive surgery (MIS) in the treatment of routine and complex orthopaedic conditions presents a useful approach for the patient [55]. The traditional surgical approach typically involves an incision of five inches or larger. These larger incisions can cause many risks, which may include significant blood loss, tissue or nerve damage, and a greater risk for infection. Due to these large incisions, surgery may be delayed for weeks. Such delays may have undesirable consequences for patients such as prolonged

pain, increased leave from work, and even further complications. The MIS approach, which typically involves pin size incisions resulting in less blood loss and tissue damage, reduces risk for post-operative infection. When compared to the traditional approach, MIS shows certain benefits, among them a much faster healing and recovery period. As a result of the increasing popularity of MIS, a interest in the use and development of injectable biodegradable materials for regeneration of bone has grown, too. Tissue engineering strategies are based on the use of very promising injectable materials for use in bone regeneration. These injectable biomaterials permit the incorporation and the release of therapeutic agents, such as antimicrobial and anti-inflammatory drugs as well as bioactive molecules that can trigger stem cell differentiation to aid in the regeneration of bone structure. To this end, after calcium phosphate-based ceramics such as hydroxyapatite (HA), beta-tricalcium phosphate (β-TCP) and the HA/β-TCP association that replaced bone autografts thanks to a chemical composition closely related to that of bone mineral, a ready-to-use injectable bone substitute (IBS) has been developed. The effectiveness of these IBS depends on the fact that the newly formed bone contains the same Ca and P values as in basal bone and makes it possible for IBS to satisfy immediate implantation requirements. For this reason, injectable composite biomaterials are of primary importance for clinical applications such as socket filling and pre-implant reconstruction. The sol-gel method has recently attracted much interest as a new technique for IBS synthesis because it permits improvement in the chemical homogeneity of the resulting HA compared to conventional methods such as solid-state reactions, wet precipitation, and hydrothermal synthesis. In particular, the Sol-gel method is useful for the synthesis of HA-based injectable materials to possibly obtain nanoparticles that are able to rapidly improve stability at the artificial/natural bone interface [56]. Hydroxyapatite is the most studied biomaterial for medical applications due to both its high biocompatibility and for being the main constituent of the mineral part of bone [57]. Hydroxyapatite, HA [$Ca_{10}(PO_4)_6(OH)_2$] possesses a chemical structure similar to the mineral components of bones. In fact, its structure looks like crystalline bioapatite mineral composed of 45% by volume, and 65% by weight of the mineral fraction of human bone. However, the extracellular matrix of bone has been described as a composite material composed of collagen type I fibrils mineralized with hydroxyapatite nanocrystals. Approximately 70% of bone by weight is composed of calcium salts, with hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$ as the primary mineral constituent. In other words, bone mineral is not purely hydroxyapatite, and the presence of ion impurities has actually

led to the consensus that a more accurate term for the inorganic component is carbonate hydroxyapatite with the formula $(Ca,Mg,Na)_{10}(PO_4HPO_4CO_3)_6(OH)_2$. Devoid of an organic component, calcium salts such as hydroxyapatite are biocompatible, non-immunogenic components of bone and are considered osteoconductive. Consequently, there has been much interest in designing synthetic osteoconductive grafting materials based on these naturally occurring calcium salts. HA is widely used to repair, fill, extend and reconstruct damaged bone tissue. This crystalline molecule was used as a coating for metallic implants in order to improve the osteoconductive and osteointegrative properties of prostheses. It can be synthesized via reactions in solid state, coprecipitation, hydrothermal methods, or sol-gel process, among others. Precipitation in aqueous solutions is the experimental procedure most employed because HA is insoluble in water. The molar ratio of calcium to phosphorus Ca/P varies from 1.2 to almost 2 in HA. The stoichiometric molar ratio of HA is 1.67; however, this is not the value observed in the organism because small amounts of other materials such as carbon, nitrogen, iron and other elements are incorporated. Thus, the general chemical formula is $Ca_{10}x(HPO_4)x(PO_4)_6x(OH)_2$. Young and Posner determined the crystal structure of HA. Hydroxyapatite can be produced with either a dense or a macroporous morphology, and is typically sintered at temperatures above 1000 °C in granular or block forms. The high heat of sintering produces a material that cannot be reshaped to fit into a bone defect (i.e. a block form) and is non-resorbable. Beta-tri-calcium phosphate (β-TCP) has the formula $Ca_3(PO_4)_2$ and like hydroxyapatite is a brittle material with low fracture resistance. A variety of CaP compounds have been used for the solid phase such as dicalcium phosphate, dicalcium phosphate dihydrate, calcium-deficient hydroxyapatite, and amorphous calcium phosphate. These cements are injectable and mouldable for variable periods before hardening. They are also described as resorbable, though clinical experience has demonstrated the retention of the material over extended periods. At the beginning of the 1970s, it was shown that HA displayed two crystal systems, hexagonal and monoclinic. HA contained in teeth and bones and mineral HA present a hexagonal structure; unlike dental enamel HA has a monoclinical structure. HA obtained in the lab presents a structure which depends on the of method synthesis. For example, IBS biomaterials based on HA precursors and obtained by using the sol-gel method can be enriched with therapeutic molecules and compounds. In the last decade, greater attention has been paid to the study of devices for bone regeneration, which possess a dual function: to stimulate the newly forming bone tissue and prevent bacterial infections.

Microbial infection prevention is important because the bone implant failure often occurs due to implant-associated bacterial infections and inflammation, which inhibit adequate osseointegration [50]. As result, these infections can cause systemic pathologies, thus increasing the costs of hospitalization for patients. Several approaches were attempted in order to obtain antibacterial effects by using biomaterials functionalized with antimicrobial agents. Over the last few years, among these new approaches a wide class of ionic liquids (ILs) have been counted as antimicrobial agents. Ionic liquids are large organic cations, such as imidazolium or pyridinium, with alkyl chain substituents that alter the hydrophobicity of the molecule. Recently, several studies showed the possibility to combine ILs with such biomaterials or bone precursors in order to obtain osteoinductive and antibacterial effects.

2. Thesis aim

Osteoporosis is a musculoskeletal disorder with a high social impact. In fact, fractures derived from the inflammatory bone loss associated with osteoporosis cause long-term disability in the elderly and significant economic burdens. Current conventional therapy for osteoporosis consists of systemic administration of bisphosphonates and anti-inflammatory drugs. However, these drugs in long-term treatment induce complications and serious side effects such as gastrointestinal (GI) diseases, fever and articular pain. The main aim of this thesis is to propose new alternative therapies for osteoporosis in the field of tissue engineering, in order to overcome the drawbacks related to systemic therapy for osteoporosis care. In particular, the present study suggests the possibility of preventing and treating locally osteoporotic bone fractures by using biomimetic 3D scaffolds (chitosan-based scaffolds) with osteoinductive and angiogenic properties. Moreover, in the current study we have analysed the effect of injectable CaP based biomaterials functionalised with ionic liquids (ILs), useful for the regeneration of some osteoporotic lesions. The choice of ILs is due to their well-known antibacterial and antifungal properties in order to combine the bone regenerative effects of CaP with the opportunity to prevent and treat the bone infections associated with osteoporotic fractures with ILs.

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CHAPTER II

Organic and inorganic bioactive signals for the preparation of biomimetic chitosan-based scaffolds for bone tissue regeneration

1. Introduction

Living tissue and organ functions are often compromised or removed as a result of tumours, trauma or accidents. Therefore, there is a need for the development of devices able to support and replace the function of living tissues. The use of biomaterials dates back to ancient civilizations. Indeed, some Egyptian mummies have artificial eyes, ears, teeth and noses. The tissue engineering approach is based on the development of three-dimensional scaffolds, which act as temporary substitutes for the removed tissue and promote cell proliferation and differentiation [1]. Tissue engineering is an emerging field of science that was first defined in 1987 by the National Science Foundation as *"an interdisciplinary field that applies the principles of engineering and the life sciences towards the development of biological substitutes that restore, maintain or improve tissue function"*. At present, the loss or failure of a tissue or an organ is one of the most frequent and expensive problems in the human health care system. Therefore, the gold standard in regenerative medicine is the development of new strategies to regenerate human tissue or to reconstruct entire organs using tissue-engineering approaches. Bone tissue engineering seeks to maintain and restore human bone tissue function by using cell biology combined with materials sciences and engineering principles. Therefore, the three main elements for TE are harvested cells, recombinant signalling molecules, and 3D matrices. Cells and signalling molecules such as growth factors can be incorporated into highly porous biodegradable scaffolds, cultured *in vitro*, and then the scaffold can be implanted into bone defects to induce and stimulate new bone growth. The substratum obtained allows cells to attach, proliferate, and differentiate to osteoblast phenotypes in order to favour bone health according to the scaffold degradation rate (Figure 1) [2].

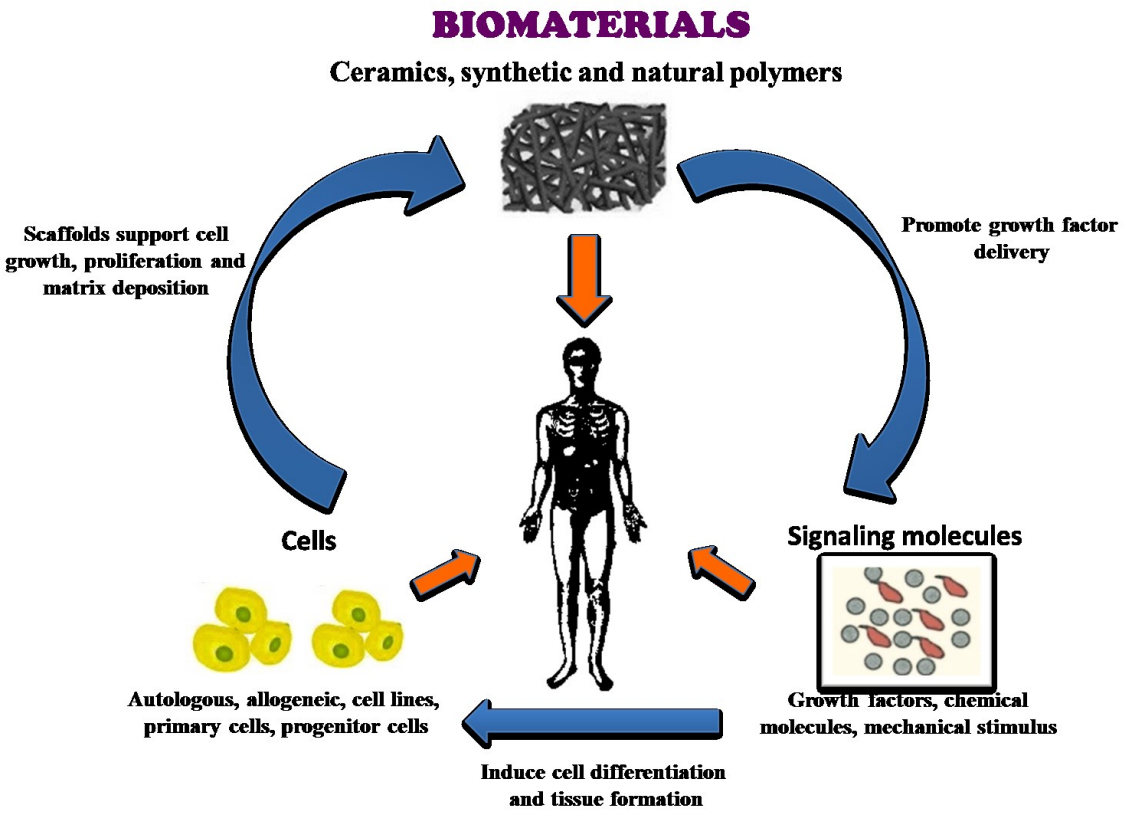


Figure 1. Bioactive biomaterials functions in terms of biological response.

The scaffold must possess certain requirements to be used successfully in bone tissue regeneration. Indeed, it must be biocompatible, must have a suitable interconnected microstructure and an optimal porosity to promote cell proliferation and migration. Moreover, it needs to support the vascularisation of the newly forming tissue. Indeed, the scaffold must guarantee a porosity of 90% and pore diameter of at least 100 μm. It also must possess good mechanical properties to stimulate new tissue growth in accordance with its degradation behaviour. In addition to the above, the scaffold must be able to promote cell adhesion and retain the metabolic functions of the attached cells. Mechanical properties depend on the nature of the biomaterials but also on the preparation and fabrication processes, which influence the biological response. The ideal *scaffold* for bone TE should be characterised by a good porosity to organise tissue regeneration and guide new tissue in-growth. Also of utmost importance is, creating a suitable microarray environment to promote osteoblast proliferation and the osteogenesis process. It is well known that the natural bone is a complex inorganic-organic nanocomposite material, in which hydroxyapatite (Ca_{10} $(PO_4)_6$ (OH) $_2$) nanocrystal and collagen fibrils are well organized in a hierarchical architecture [3]. Polymers can serve as a matrix to support cell growth and they can be easily modified and altered by changing the constituents of monomers in different ratio, controlling polymerisation conditions or introducing various bioactive functional groups. Polymers including poly (glycolic acid) (PGA), poly (lactic acid) (PLA), poly [lactic-co-(glycolic acid)] (PLGA), polyethylene glycol, chitosan, collagen, hyaluronic acid and alginate, have been widely used in the tissue engineering of cartilage, bone, skin, and ligament [4]. Collagen, drugs or constructs can be contained in therapeutic or diagnostic systems and are comprised of biologically derived components (amino acids or peptides). The research pays focuses on natural rather than synthetic polymers for bone tissue applications due to its higher biocompatibility and biodegradability [5]. Furthermore, these polymers contain an extracellular substance "*ligand*" which allows specific cell receptor interactions. It has been demonstrated that polymer structure can guide the growth cell at different stages of development and at the same time, stimulate an immune response. Among the polymers, chitosan and its derivatives represent very attractive candidates in scaffold composites, thanks to their degradation behaviour. In fact, chitosan degrades as soon as new tissue is formed without inflammatory reactions or toxic degradation [6, 7]. Moreover, chitosan (CS) is more widely used because of its ability to create porous natural scaffolds, its biodegradability and adsorption properties. Because its structure is similar to the

glycosaminoglycan in the extracellular matrix of bone and cartilage, CS has been used in bone tissue engineering [8]. From a chemical point of view, it is an amino-cationic polysaccharide derived from the partial deacetylation of chitin, a component in the exoskeleton of crustaceans [9] (Figure 2). Its chemical properties allow cell adhesion and proliferation, including osteoblasts [10-11], fibroblast, keratinocyte and endothelium cells [12].

According to several studies, it has also been reported that CS induces a great response in macrophages and neutrophils both *in vitro* and *in vivo* systems [13]. Additionally, the presence of the N-acetylglucosamine moiety on CS also suggests related bioactivities. It has been shown that CS oligosaccharides have a stimulatory effect on macrophages and both chitosan and chitin are chemo-attractants for neutrophils in both *vitro* and *in vivo* [14]. Moreover, the literature demonstrates host tissue response to CS-based implants. Generally, CS biomaterials exhibit a minimal foreign body reaction, with little or no fibrous encapsulation [15]. Furthermore, CS *in vivo* implantation in vertebrates has demonstrated that it may act as a substrate, which enhances specific types of progenitor cells *(i.e.* osteoblast differentiation), migration and differentiation [16]. This property is probably due to the ability of CS to be moulded into various shapes as well as its porous structure [17]. Nowadays, considerable research effort is being dedicated to improving the bioactivity and biological properties of CS scaffolds through the incorporation of a bioactive solid signal, such as hydroxyapatite (HA). Biomineralized materials can be produced in different ways; one way concerns the use of an inorganic biomimetic treatment, which comprises the precipitation and creation of apatite nucleation sites using a simulated body fluid (SBF) [18]. The natural hydroxyapatite and chitosan composites have good tissue biocompatibility and also excellent osteoconductivity. However, these materials are lacking when it comes to mechanical properties. To overcome this drawback, in this study the development of semi and grafted interpenetrating polymer networks based on poly(ethylene glycol) diacrylate (PEGDA) and chitosan was considered. The quickness of the reaction results from the presence of the double acrylate groups, which are able to react in the presence of free radicals. Furthermore, PEGDA polymer exhibits higher solubility in water and water solutions. Finally, it possesses specific chemical, mechanical and biological properties, which allow the use of PEGDA-based systems in a wide range of biomedical applications (*e.g.,* drug delivery vehicles, tissue engineered scaffolds and *in vitro* applications). The most widespread foaming techniques are salt leaching, gas foaming, phase separation, freeze-drying and

electrospinning [19]. CS-PEGDA based porous scaffolds were developed through a foaming method [20]. The approach is based on the combination of two consecutive steps: 1) physical foaming is induced using Pluronic as blowing agent; 2) the foaming continues in a microwave reactor until it is arrested by a chemical reaction, which stabilizes the porous structure.

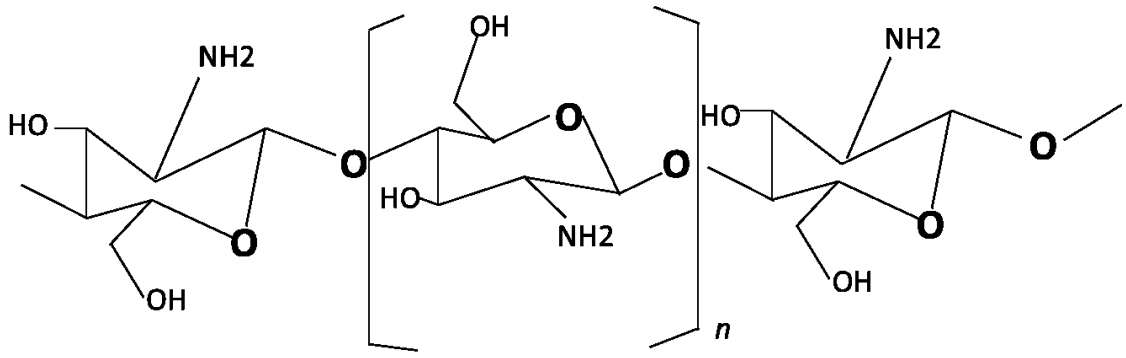


Figure 2. Chitosan chemical structure.

The microwave-induced reaction allows a rapid and homogeneous heating of the sample, avoiding the typical drawbacks of a conventional oven-heating process. Moreover, this is beneficial for the creation of a uniform interconnected porous structure without the presence of gradients between the core and scaffold surfaces. At the end of the process, an interpenetrated network is obtained because CS does not participate in the crosslinking reaction. The influence of different process parameters, such as time, power of microwave curing, and the effect of washing in acetone have been qualitatively evaluated through infrared attenuated total reflectance (ATR-IR) analysis. *In vitro* degradation of CS-PEGDA-based scaffolds was investigated to evaluate thermal stability, pore morphology, structure and weight loss, as a function of degradation time [20].

The **aim** of the present study was to develop bioactivated CS-PEGDA scaffolds using two different approaches (organic and inorganic cues), prepared in collaboration with Dr. Christian Demitri of University of Salento, in order to evaluate their effect on cellular behaviour in terms of proliferation and their potential to promote osteogenic differentiation in human mesenchymal stem cells (hMSC). It is well known that bone is a highly vascularised tissue relying on the spatial and temporal proximity between blood vessels and bone cells, in order to maintain skeletal integrity. There is a close relationship between osteogenesis and angiogenesis processes [21]. In fact, bone defect repair remains a major clinical orthopaedic challenge. In this context, angiogenesis plays a pivotal role in skeletal development and bone fracture repair. For this reason, by combining therapies using mesenchymal stem cells, and endothelial cells with polymeric bioactive biomaterials, it is possible to promote osteogenesis and angiogenesis simultaneously. Biomaterials with angiogenic and osteoinductive properties are widely under evaluation.

1.1 BMP-2 mimetic peptide

During bone formation, osteogenic growth factors are continuously produced [22]. Among osteogenic growth factors bone morphogenetic proteins (BMPs) play a key role in osteogenesis regulation during bone remodelling. BMPs are multifunctional cytokines, which are members of the transforming growth factor-β (TGF- β) superfamily. Polypeptides include TGF- β, activins/inhibins, and BMPs. BMPs stimulate signalling molecules, which can induce *de novo* bone formation at orthotopic, and heterotopic sites and their osteoinductive potency make them clinically valuable as alternatives to bone

graft [23]. The BMPs are also widely distributed in non-skeletal tissues such as nerve, heart, kidney, gastrointestinal tract and lungs. Moreover, BMPs have a pivotal role in vertebrate and non-vertebrate organogenesis [24]. In fact, several studies have demonstrated that BMPs exert neuro, cardio and reno-protective actions. The term "bone morphogenetic protein" (BMP) was introduced to describe the substance(s) in the demineralized bone matrix. "Morphogenesis" means generation of form, the process of tissue and organ construction and assembly [25]. 15 BMPs are currently recognised (BMPs 1-15). The osteoinductive properties of endogeneous BMPs of various origin (*e.g.* murine, ovine, bovine, reindeer, primate and human) have widely been studied both *in vitro* and *in vivo* systems [26]. Human BMPs are now available more readily and in larger quantities due to the advent of recombinant DNA technology [27]. Bone morphogenetic proteins (BMPs) can be associated with scaffolds and/or cells for use in bone tissue engineering. The presence of BMPs has been found in extracts of demineralized bone matrix and they have the ability to induce ectopic bone formation in subcutaneous and intramuscular pockets of rodents [28-29]. However, BMPs induce differentiation of multipotent mesenchymal stem cells into both osteochondrogenic and osteoblast phenotypes [30-31]. BMPs can be classified into subgroups depending on their sequence homology, *e.g.,* BMP-2 and BMP-4 belong to the BMP-2/-4 subgroup, BMP-5, -6, -7, and -8 belong to the osteogenic protein-1 (OP-1) group. Members of the BMP family promote the formation of bone and cartilage *in vivo*. Especially, BMP-2 and BMP-7, also called OP-1, are under evaluation for tissue engineering application and available as clinically approved recombinant human proteins [32]. BMP family proteins bind to type I and type II serine/threonine kinase receptors which can phosphorylate the cytosplasmic proteins. As doother members of the TGFβ superfamily, BMP signals activate the intracellular signalling molecules, Smad1, Smad5 and Smad8, through serine phosphorylation. Activated Smad proteins form heterodimers with the co-Smad and Smad4, and translocate to the nucleus, where they regulate target gene transcription [33]. BMP2 stimulates transcription of *Runx2*, the master regulator of osteoblast commitment and BMP2-activated Smad proteins collaborate with Runx2 to induce expression of other genes in differentiating osteoblasts, including osterix (Osx/Sp7), another bone-specific transcription factor. Several studies report that bone morphogenetic proteins (BMPs) initiate, promote and maintain osteogenesis and chondrogenesis [34]. It has been demonstrated that BMP-2 based treatments induce osteogenic differentiation, whereas BMP-7 stimulates a chondrogenic phenotype of MSCs [35]. Therefore, MSCs with BMPs

provide a tool for bone and cartilage tissue engineering. [36]. Protein-based biomaterials have emerged as powerful tools for tissue engineering applications. It is possible to incorporate a peptide derived from bone morphogenetic protein-2 (BMP-2) into a single material in order to assess its effect on different phenotype cells such as human mesenchymal stem cells. Several means of delivering BMPs to patients are undergoing evaluation including systemic administration, gene transfer and local matrix delivery vehicle implantation. Indeed, BMP-2 peptide, within the context of genetically engineered proteins, is a central morphogenetic regulator of osteogenesis able to accelerate osteogenic differentiation. The incorporation of BMP-2 peptide within the context of modular proteins is a successful strategy for engineering biomaterials for applications in bone tissue engineering. BMP-2 growth factor is a potent cue for promoting osteogenic differentiation and is approved by the Food and Drug Administration (FDA) for clinical use in the regeneration of bone defects. An attractive alternative in the use of soluble BMP-2 growth factor is incorporating BMP-2 peptide, which is known to promote bone regeneration into a scaffold material. BMP-2 peptide can be immobilized by covalent or specific binding on the surface of the material. Previous studies have shown that BMP-2 peptide in modular proteins accelerates osteogenic differentiation and supports hMSC proliferation.

1.2 Angiogenesis and bone fracture repair

Angiogenesis represents an important area of scientific research due to its involvement in various physiological and pathological processes [37]. Investigations into angiogenic mechanisms require *in vitro* experimental models that simulate the important steps of angiogenesis to assess therapeutic agents' efficacy (that either up-regulate or down-regulate specific angiogenic mechanisms). Currently, the interest in anti-angiogenic agents for the treatment of cancer is particularly prominent. Several *in vitro* models have been developed to study the angiogenesis process [38]. These models involve methods for the study of angiogenic biological response in terms of proliferation, migration and differentiation of endothelial cells. The establishment of a functional vascular system is crucial during organ development as well as during tissue repair. In fact, in newly forming bone blood vessels not only does it represent a source of oxygen and nutrients but it also supplies calcium and phosphate, the building blocks for mineralization.

Additionally, blood vessels in the bone marrow appear to perform a crucial role as a niche for both the bone-forming skeletal stem cells and blood-forming hematopoietic stem cells [39]. The connection between angiogenesis and osteogenesis is also evident during the healing of bone fractures, as a timely and coordinated angiogenic response is of vital importance for successful bone repair [40]. Increasing insight into the molecular and cellular processes orchestrating the angiogenic cascade may help in the development of new treatments for fracture healing, especially for clinical situations with a limited angiogenic host response such as large bone defects or fractures with severe soft tissue trauma. When an organism or tissue grows beyond a size where passive diffusion for the exchange of oxygen, nutrients and metabolic waste products becomes insufficient, the need for the development of a vascular system emerges. Angiogenesis is the process by which the organism establishes new blood vessels from pre-existing ones. Recent studies have highlighted the fundamental aspects of vessel formation, including vasculogenic assembly, vessel sprouting, lumen formation and vascular remodelling [41]. The sprouting of endothelial cells (EC) is the first event in a sequence of events during angiogenesis [42]. In a resting vessel, both endothelial and mural cells form a basement membrane around the vessel tube, preventing resident EC to migrate. When new blood vessels are needed in a growing tissue, local production of angiogenic growth factors is enhanced, which trigger the EC to degrade the basement membrane extracellular matrix, a process mediated by matrix metalloproteinases (MMPs). The action of these MMPs further fortifies the angiogenic response by releasing proangiogenic factors that were stored within the matrix. Attracted by the angiogenic signals, some EC become motile and express filopodia. These so-called tip cells form the leading front of the newly forming vessel; the trailing EC are called stalk cells. The key angiogenic factor, Vascular Endothelial Growth Factor (VEGF) stimulates tip cell induction and filopodia formation via VEGF receptor (VEGFR) signalling [43]. The filopodia on the tip cells ‘sense’ the environment for attractive cues, guiding the sprouts into stromal tissue. Stalk cells are equipped to form tubes and branches, mediated by their high proliferative capacity and the ability to stabilize the newly formed lumen. The priming of EC into tip or stalk cells is also dependent on the Notch signalling pathway, in close intimacy with VEGFR signalling. In tip cells, VEGF signalling enhances Delta-like protein 4 (Dll4) expression, a Notch ligand. Dll4 activates Notch signalling in the neighbouring EC, thereby preventing their switch to a tip cell phenotype and favouring a stalk cell phenotype. New vessel circuits are created via the interaction of two neighbouring tip cells (*i.e.*

anastomosis). These connections are then stabilized by several processes including the deposition of extracellular matrix, the recruitment of pericytes, reduced endothelial cell proliferation and increased formation of cell junctions [44]. In addition to the maturation of the endothelium, vascular branches are remodelled to match their rigidity to the local tissue needs. The timely formation of new blood vessels is a critical process during embryonic and foetal development [45]. In adults, angiogenesis occurs during physiological processes like wound healing, the menstrual cycle, and pregnancy but also in specific diseases such as intraocular neovascular disorders and tumourigenesis. As opposed to soft-tissue healing, bone can regenerate itself without the formation of fibrous scar tissue and hereby maintains its physiological and mechanical characteristics. Normal fracture healing in adults occurs through intramembranous or endochondral bone formation, closely mimicking skeletal development in the embryo [46]. Intramembranous ossification is characterised by the direct formation of bone by committed osteoprogenitor cells and mesenchymal stem cells (MSC) from the periosteum. During endochondral ossification, MSC differentiate into chondrocytes, which in turn produce a cartilaginous matrix. Consecutively, this matrix undergoes calcification and is eventually replaced by bone [47]. The scar-less regeneration of fractured bones through the endochondral pathway might be attributed to the avascular nature of the cartilage template. In fact, chondrocytes are metabolically well adapted to survive in poorly oxygenated regions and still produce the extracellular matrix needed for mineral deposition, and hereby likely contribute to optimal fracture healing. Despite the remarkable regenerative capacity of bone tissue, fracture healing fails in about 10% of the cases leading to delayed union or non-union. Adequate vascularisation has been shown to be critical for successful bone healing, along with the presence of osteoprogenitor cells and mechanical stabilization. Indeed, inappropriate blood vessel supply is a major cause of delayed union or non-union during fracture healing [48]. More precisely, when fracture is associated with large vascular injuries, the rate of impaired healing is as high as 46%, exceeding by far the global 10% non-union rate [49]. In addition, inhibition of angiogenesis during fracture repair in animal models resulted in the formation of fibrous scar tissue, resembling human atrophic non-union [50]. Therefore, treatments promoting tissue vascularisation provide a central strategy to accelerate the healing response and tissue regeneration. It is evident that an adequate vascular response is needed for normal bone healing and the concept of therapeutic angiogenesis has been appreciated for many years. For successful tissue regeneration, it is necessary to understand the relations

established between different cell types involved in newly forming tissue [51, 52]. Indeed, most of the tissues require proper vascularisation and blood supply to support their functions. Alterations in the vascularisation process cause hypoxia at a cellular level, lack of nutrients, accumulation of waste products, impaired biochemical signalling and altered cell recruitment. This leads to the failure of tissue homeostasis and the inhibition of tissue regeneration [52, 53]. Therefore, the study of co-culture *in vitro* models consisting of osteogenic cell precursors and endothelial cells received more attention, in order to clarify vascularisation mechanisms. In the context of bone tissue engineering, endothelial cells, endothelial progenitors, osteoblasts or mesenchymal stem cells interactions are currently studied [52-54]. Indeed, the interaction between human umbilical vein endothelial cells (HUVECs) with primary osteoblasts demonstrated the formation of a vascular tissue-like network *in vitro* [55-56]. To provide successful substitutes for tissue and improve vascularisation in the local environment of the implant, pre/vascularised or endothelialised constructs were used [57]. Another valid possibility is the use of smart proangiogenic biomaterials, able to recruit endothelial progenitor cells. Angiogenesis and osteogenesis are intimately linked and it has been demonstrated that the processes need to be correctly regulated when bone regeneration occurs. This is also true for muscular tissue, given that its function depends on blood supply. In the specific case of bone, the formation of matrix starts with osteoblast-mediated collagen I deposition by forming osteoid, with the angiogenesis process occurring almost simultaneously. The matrix is gradually mineralized acquiring properties of mature bone [58]. Therefore, the angiogenesis process plays an important role in skeletal development and bone fracture repair. Indeed, the processes include the coordination of events such as migration, proliferation, differentiation and activation of many cell types (endothelial cells) [59]. The importance of blood vessels in the formation of the skeleton and in bone repair was documented in the 1700s [60]. Angiogenesis is the formation of new blood capillaries from pre-existing vessels [61]. Specifically, it is a physiological process for embryonic development, growth of organs and tissue repair. During development, wound healing and pregnancy, blood vessels respond to pro and anti-angiogenic factors involved in the process. An impairment of the process leads to abnormal growth of blood vessels and several human diseases, including tumour growth and metastasis, proliferative retinopathies, age-related macular degeneration, neo-vascular glaucoma, ischemia disorders and rheumatoid arthritis [62]. Since the function of three-dimensional (3D) scaffold tissue constructs depends on vascularisation after implantation, the analysis of

angiogenesis in tissue engineering is under investigation. For this purpose, in order to evaluate the effect on angiogenesis process, several *in vitro* and *in vivo* models were carried out. Notably, *in vitro* models often attempt to mimic one or more steps of the angiogenic process, in order to assess whether and how materials can affect endothelial cell function. In this chapter, the interest was in evaluating the effects of chitosan scaffold materials on the angiogenesis process in terms of proliferation, migration and differentiation of endothelial cell precursors. In other words, the aim of this part of the thesis was to explore the angiogenic potential of chitosan scaffolds on endothelial cells. The materials previously described have been studied to assess their ability to induce an angiogenic cell response using HUVEC cells.

2. Aim

The aim of the first part of this thesis was to bioactivate chitosan-based scaffolds, prepared in collaboration with Dr. Christian Demitri of University of Salento, using two different approaches based on inorganic (deposition of hydroxyapatite nanoparticles) and organic (BMP-2 peptide functionalisation) components for promoting stem cells' osteogenic commitment and preventing inflammatory response. Additionally, the osteoinductive properties of scaffolds on MSC and their angiogenic potential on *in vitro* model obtained by using HUVECs, a well-known endothelial cell line, were evaluated.

3. Materials and methods

3.1 Scaffold preparation

Scaffold preparation was performed in collaboration with Dr. Christian Demitri (University of Salento) [20]. Briefly, CS scaffolds were prepared using a new method based on two different steps. In the first step of the process, chitosan solution or CS (Medium molecular weight, DD 75-85%) was prepared by dissolving chitosan in 0.1 M Acetic Acid (AA, 99%) solution (1.5%wt). The mixture was stirred for 1.5 hours, in order to obtain a homogeneous viscous polymer solution; then, fixed amounts of PEGDA (20 and 40%w/V) (average molecular weight of 700 g/mol) were added to the CS solution and stirred for 1.5 hours. In this step, 2.2-Azobis [2-(2-imidazolin-2yl)propane] dihydrochloride was used as a thermoinitiator (TI). A selected physical blowing agent

such as Pluronic F127 (0,4% w/w), was added and mixed at 800 rpm for 30 minutes, so that a homogeneous distribution of air bubbles in a stable form was obtained [62]. In the second step, the porous structures of the foaming at different concentrations of PEGDA were chemically stabilized via radical polymerization induced by heating the sample in a microwave (MTS 1200 Mega, Milestone Inc., Shelton, CT 06484). Some of the resulting samples were washed in acetone to evaluate the possible removal of unreacted PEGDA and dried in a vacuum oven at 45°C. The samples were described and summarized in Table 1. Different percentages of CS and PEGDA allowed CS-based scaffolds to be acquired at different compositions.

Table 1. Composition of chitosan (CS)-polyethylene glycol diacrylate (PEGDA) foams

SAMPLES	% CS	% PEGDA
80CS20P	80	20
60CS40P	60	40
70CS30P	70	30

3.2 Scaffold bioactivation by inorganic signals

The semi-interpenetrating CS-PEGDA scaffolds (80CS20P, 60CS40P and 70CS30P) were bioactivated using a modified method described by Kokubo and co-workers [50]. The treatment is based on the use of supersaturated SBF solutions to stimulate nuclei formation and growth (Figure 2). In the present study, the pH solution was fixed at 7.4 in order to select interactions between decarboxylated groups (COO^-) and Ca^{2+} ions. The SBF volume was calculated with reference to the total material surface using a specific ratio of the exposed surface to SBF volume, as reported in the literature [63]. After the incubation time, all materials were gently rinsed in distilled water to remove excess ions and, then, dried overnight under a laminar flow hood.

3.3 Scaffold bioactivation by organic signals

The second approach for scaffold bioactivation is based on the use of a bioactive peptide of Bone Morphogenetic Protein (BMP-2). The 67-87 residue of the "knuckle" epitope of hBMP-2 (NSVNSKIPKACCVPTELSAI) was synthesized using microwave-assisted Fmoc solid phase peptide synthesis (SPPS). The resin support and all amino acids (AA)

were obtained from Inbios S.r.l. (Naples, Italy). Rink amide linker (0.4 mmoles; Iris Biotech GmBH) was attached to a Tentagel-S-NH_2 resin (Iris Biotech GmBH) in a glass vial; 1mL of 0.45M HBTU in N,N-dimethylformamide (DMF) and 0.5mL of 33% DIPEA in DMF were added to the reaction vessel to activate the exposed amino groups of the resin and after each coupling step to activate the deprotected group of the coupled AA. The AA coupling was performed after stirring the reaction suspension for 7 min in a Microwave synthesizer (Power 13W; Temperature 60°C; stirring rate 900 rpm); the solvent was removed and the mixture was washed with DMF (Aldrich). The Fmoc-protecting group was removed from the coupled AA by incubating the resin with 20% v/v piperidine in DMF for 3 and 7 minutes. Following the final AA coupling, the resin-bound BMP-2 peptide was washed several times with dichloromethane, methanol, and diethyl ether. Finally, BMP-2 peptide was cleaved from the resin support through the exposure to a solution composed by TFA 95%-TIS 2.5%-dH_2O 2.5% mixture for 3 hours and precipitated in a cold diethyl ether by centrifugation for 5minutes at 6,000 rpm. The cleaved products were characterised by analytical HPLC and mass spectrometry (micro-TOF; Burker), before and after purification by preparative HPLC. In our systems, peptide immobilization occurred between NH_{2_CS} and $COOH_{BMP2}$ with a molar ratio 1:0.007; 3.6 and 1.4-µg peptide/mg scaffold was used for 80CS20P and 60CS40P, respectively. Moreover, for 60CS40P a molar ratio of 1:0.018 of NH_{2_CS} and $COOH_{BMP2}$ was also used to obtain scaffold with the same amount of peptide used in 80CS20P (3.6 µg peptide/mg scaffold) (see Table 2). In this study, the peptide amount was determined based on preliminary biological experiments performed on BMP-2 peptide at different concentrations.

Table 2: Scaffold composition and amount of peptide loaded for bioactivation

	NH_{2_CS}: $COOH_{BMP2}$	
	1:0.007	**1:0.018**
80CS20P_BMP	3,6µg peptide/mg scaffold	-
60CS40P_BMP	1,4µg peptide/mg scaffold	*3,6µg peptide/mg scaffold
60CS40P_BMP*		

3.4 Morphological investigation: Scanning electron microscopy (SEM)

For each sample, the morphology of the cross-section was analysed with scanning electron microscopy (ZEISS EVO 40 (Carl Zeiss, Milano, Italy)) in low vacuum modality with an applied voltage of 25 kV. The dried samples were cut into slices using a surgical blade. The samples were placed on the SEM sample holder using double-sided adhesive tape and were observed without any further manipulation.

3.5 *In vitro* degradation test

The samples were incubated at 37°C for 6 weeks in phosphate buffered saline (PBS) solution (KH_2PO_4 0.0087 M, Na_2HPO_4 0.0304 M, NaCl 0.154 M, pH 7.5). After 1, 2, 4 and 6 weeks, the samples were washed in distilled water and dried in a vacuum oven at 45°C for 12 hours. At the end of each time-point, the weight loss percentage of each samples was measured using equation weight loss percentage (%) = $[(w_0 - w_t)/w_0] \times 100$ where w_0 is the initial dry weight, w_t is the dry weight after t time of degradation. CS scaffold surfaces, before and after biomineralisation, were analysed by Scanning Electron Microscopy (SEM, JEOL 6310). For SEM analysis, the materials were mounted using double adhesive tape to aluminium stubs. The stubs were sputter-coated with gold to a thickness of around 15-20 nm. SEM analysis was performed at different magnification at a voltage of 10 keV. Energy dispersive x-ray spectroscopy (EDAX, Genesis 2000) analysis was used for a qualitative estimation of the Ca/P ratio.

3.6 *In vitro* release study

The peptide release profiles from scaffolds at different compositions (80CS20P, 60CS40P) were determined *in vitro* by High Performance Liquid Chromatography system (HPLC, Agilent). The bioactive scaffolds with BMP-2 peptide were dipped in 200µL sterile Tris buffer solution (pH=6.8) and kept in a shaking incubator (37 °C, 40 rpm) for various time periods of up to 4 weeks. At specific times, supernatant was collected and an equal amount of fresh medium was added to each sample. A quantity equal to 20 µL of the obtained supernatant was injected in a chromatograph equipped with a UV detector and a reversed phase column (Reprospher C18, 150 mm×4.6 mm, DR. MAISCH, GmbH). The mobile phase systems consisted of 90% water (A) and 10%

acetonitrile (B). The flow rate was 1.0 mL/min and the wavelength was set at 220 nm. All experiments were triplicated for each sample.

3.7. Biological investigations

3.7.1. *In vitro* cell culture

Cell proliferation assays were performed on human mesenchymal stem cells (hMSC) obtained from LONZA (Milano, Italy). hMSC were cultured in 75 cm^2 cell culture flask in Eagle's alpha minimum essential medium (α-MEM) supplemented with 10% foetal bovine serum, antibiotic solution (streptomycin 100 μg/ml and penicillin 100U/ml, Sigma Chem. Co) and 2 mM L-glutamine. For all experimental procedures hMSCs at passages 3, were used. Cells were incubated at 37 °C in a humidified atmosphere (5% CO_2, 95% air). For cell proliferation, hMSC were plated at a concentration of 1.6 x 10^4 in triplicate onto CS scaffolds sterilized with ethanol for 60 minutes. The cell proliferation was evaluated with Alamar Blue assay for 3, 7, 14, and 21 days of culture. Fluorescence was measured at 540 and 600 nm. During the experiment, the culture medium was changed every two days.

3.7.2 Cell viability test on CS scaffolds

The cell biocompatibility was evaluated by seeding hMSCs (5,000 cells) in triplicate onto neat scaffolds (80CS20P and 60CS40P) after biomimetic treatments (80CS20P_bio, 80CS20P_BMP, 60CS40P_bio, 60CS40P_BMP, 60CS40P_BMP*). The medium in cell-load scaffolds culture plates was removed after cultures for 3, 7, 14 and 21 days and *in vitro* cell viability was checked using Alamar Blue assay, in accordance with the manufacturer's protocol. During the experiment, the culture medium was changed every 2 days. The cellular attachment is the first step in evaluating the biocompatibility of hMSCs onto CS-based scaffolds with and without biomimetic treatment. Biological assays were performed using human Mesenchymal Stem Cells line (hMSCs) purchased from LONZA (Milano, Italy). hMSCs were cultured in 75 cm^2 cell culture flask in Eagle's alpha Minimum Essential Medium (α-MEM) supplemented with 10% Foetal Bovine Serum (FBS), antibiotic solution (streptomycin 100 μg/ml and penicillin 100U/ml, Sigma Chem. Co) and 2 mM L-glutamine, without osteogenic factors. hMSCs

of passage 4 for all the experimental procedures were used and incubated at 37°C in a humidified atmosphere with 5% CO_2 and 95% air.

3.7.3 BMP-2 peptide biological activity

The bioactivity of BMP-2 peptide was evaluated using solutions at different concentrations starting from 2.70 µM to 270 pM, on hMSCs (5,000 cells at 4 passage) in order to investigate their effect on cellular behaviour, in terms of proliferation and osteogenic differentiation. The analysis were performed at 1, 3, 7 and 14 days in α-MEM supplemented with 10% foetal Bovine Serum (FBS), antibiotic solution (streptomycin 100 µg/ml and penicillin 100U/ml) and 2 mM L-glutamine. The cell proliferation was determined using an Alamar Blue assay (AbD Serotec, Milano, Italy) based on the metabolic activity of live cells. In the meantime, the osteogenic differentiation was evaluated measuring the alkaline phosphatase activity (ALP) in cultures of scaffold materials after 7, 14 and 21 days (SensoLyte pNPP ALP assay kit, ANASPEC, Milano, Italy). At the end of each interval, cultures were washed gently with PBS, followed by washing with cold 1X assay buffer (BD Biosciences, Milano, Italy). The ALP activity was evaluated onto the cell lysates (50µl), in that case the cultures were treated with 1X lysis buffer with 0.2% of Triton X-100. To correct the ALP values for the number of cells present on each scaffold, double stranded DNA (dsDNA) was measured using a PicoGreen®_dsDNA quantification kit (Invitrogen). First, 100µl of diluted PicoGreen®_dsDNA quantification reagent was added to 100µl of cell lysates in a flat-bottomed, 96-well plate. Following 10 minutes of incubation, the fluorescence of PicoGreen® was determined at a wavelength of 520 nm, after excitation at 585 nm using a spectrophotometer (Victor X3, Perkin-Elmer, Italy). dsDNA was quantified using a calibration curve of l-dsDNA standard in 10 mM Tris, 1 mM EDTA, pH 7.5, buffer. Each experiment was performed three times in triplicate. The results of ALP activity were reported as nanograms of ALP normalized to the micrograms of total DNA content. ALP experiments were repeated twice, and three materials were used in each experiment.

3.8 Effect of scaffolds on hMSC: osteogenic differentiation

3.8.1 Alkaline phosphatase expression

Differentiation of hMSC was tested measuring alkaline phosphatase (ALP) activity in the cultures of CS-based scaffolds after 7, 14 and 21 days (SensoLyte pNPP ALP assay kit, ANASPEC, Milano, Italy). This pNPP assay is based on the ability of phosphatase to catalyse the hydrolysis of p-nitrophenyl phosphate (pNPP) to p-nitrophenol (Sensolyte pNPP ALP assay Kit, AnaSpec). The phosphatase activity was analysed on the lysed cell following the manufacturer's protocol. In fact, at the end of each time point, cultures were washed gently with PBS followed by double washing with cold 1X assay buffer (BD Biosciences, Milano, Italy). For the extract cell layers, the cultures were lysed with 1X lysis buffer with 0.2% of Triton X-100. The ALP activity was analysed on the lysed cell (50µl). Sample absorbance was measured in a 96-well plate at 405 nm. To correct the ALP values for the number of cells present on each scaffold, double stranded DNA (dsDNA), as a marker for the cell number, was measured using a PicoGreen®_dsDNA quantification kit (Invitrogen). First, 100µl of 200µl diluted PicoGreen®_dsDNA quantification reagent was added to 100 µl of cell lysates in a flat-bottomed, 96-well plate. Following 10 minutes of incubation, the fluorescence of Picogreen was determined at a wavelength of 520 nm after excitation at 585 nm using a plate reader (multilabel counter 1420 Victor, Perkin-Elmer, Italy). The quantification of dsDNA occurred according to a calibration curve of l-dsDNA standard in 10 mM Tris, 1 mM EDTA, pH 7.5, and buffer. Each experiment was completed three times in triplicate. The results of ALP activity were reported as nanograms (ng) of ALP normalized to the micrograms (µg) of total DNA content. ALP experiments were repeated twice and three scaffolds were used in each experiment. The alkaline phosphatase activity of the hMSC seeded onto biomimetic, non-biomimetic 80CS20P and 60CS40P scaffolds was determined.

3.8.2 Osteocalcin levels

The effect of CS-based scaffolds on osteogenic differentiation of hMSCs was evaluated by measuring a later marker of differentiation, such as osteocalcin (OCN). Osteocalcin levels were measured at days 14 and 21 using a commercially available kit (Quantikine Human Osteocalcin Immunoassay R&D system, Italy) following the manufacturer's

instructions. hMSC cells were cultured on control scaffolds (80CS20P and 60CS40P) and after biomimetic treatments (80CS20P_bio, 80CS20P_BMP, 60CS40P_bio, 60CS40P_BMP, 60CS40P_BMP*) in basal medium at 21 days of culture time. Quantitative levels of OCN secreted into the culture medium were determined using an enzyme-linked immunoassay kit following the manufacturer's instructions.

3.8.3 Effect of CS-based-scaffolds on inflammation

In the present study, the effect of CS-based scaffolds on inflammatory response using an *in vitro* experimental model was investigated. To this end, hMSCs were plated onto scaffolds and in tissue culture plates at a density of $1*10^4$ cells/well in 96 multi-well plates. After 24 hours, inflammation was induced by using LPS at a concentration of 1 μg/ml. At day 3 of stimulation interleukin 1β (IL-1β), interleukin-10 (IL-10) and nitrite levels were measured. Interleukin-1β (IL-1 β), interleukin-10 (IL-10) levels in cell supernatants were quantified using commercial ELISA kits (Affymetrix, eBioscience Srl, Milano) according to the manufacturer's instructions. Nitrite levels (stable metabolites of nitric monoxide) were analysed using Griess assay. For this purpose, 100 μl of supernatant was transferred into a 96-well plate and an equal volume of Griess reagent was added to each well. The samples were incubated for 1 hour at RT and absorbance was detected at 550 nm using a microplate reader (VICTOR^{TM} X3 Multilabel Plate Reader, PerkinElmer).

3.9 Effect of CS-based-scaffolds on angiogenesis

This study was developed during my time at IBEC under the supervision of Prof. Elisabeth Engel.

3.9.1 Isolation and maintenance of human umbilical vein endothelial cells (HUVECs)

Human umbilical vein endothelial cells (HUVECs) were isolated from the umbilical cord and cultured according to van Wachem *et al*. [3]. After harvesting, cells were cultured to the third passage in tissue culture polystyrene flasks T75 cm^2 pre-coated with a solution

of partially purified human fibronectin (Fnc, 2 mg/ml) in a humidified atmosphere of 5% CO_2 at 37°C and 95% air. EndoGRO^{TM} medium for human endothelial cell culture was used. The medium contained 0,2% ENDOGRO-LS supplement, 5ng/ml rhEGF, 10 mM L-glutamine, 1.0 mg/ml hydrocortisone hemisuccinate, 0.75 U/ml heparin sulfate, 50 mg/ml ascorbic acid, 5 % FBS. HUVECs, used for subcultures and for experiments were detached by incubation with trypsin (Gibco, 0.05 % trypsin with 0.02% EDTA in PBS). Residual trypsin was inactivated by addition of culture medium.

3.9.2 HUVECs proliferation

To evaluate effects on angiogenesis process, human umbilical vein endothelial cells (HUVECs) at passage 5 were used for experiments. 70CS30P based scaffolds were prepared for cell seeding by soaking them in 70 % ethanol (24 hours) under UV-ray and then pre-wetting them in medium (2 hours). The experiments were performed using EndoGRO^{TM} medium products for human endothelial cell culture. The cell proliferation was checked by using Quanti-IT PicoGreen® D*s*DNA reagent and kits (Invitrogen). HUVECs were cultured at density of $1x10^3$ cell/well onto CS-based materials for 1, 3 and 7 days of cell culture.

3.9.3 Live-dead assay

An *in vitro* qualitative analysis of cell viability with live/dead assay (Invitrogen) was performed. HUVECs were seeded at a density of 1 x 10^3 cells in a 48-well plate and incubated at 37 °C in a humidified atmosphere with 5 % CO_2 and 95 % air. The assay allowed living cells to be distinguished from dead cells. The staining was done using calcein AM/propidium iodide. Calcein AM stained living cells in green, propidium iodide stained dead cells in red. Chitosan 70CS30P samples were put in contact with ENDOGRO medium for an incubation time of 24 hours. After this incubation period, conditioned medium was added to cells for 24 hours. At the end, the non-attached cells were removed and carefully rinsed with PBS 1X three times. The cells were incubated with 2.5µl of calcein AM and 10 µl of propidium iodide for 15 min 37°C. Subsequently cell culture was washed with phosphate buffered saline (PBS1X) three times. The cell morphology and cell spreading pattern interaction of HUVECs in contact with materials

was evaluated with confocal laser scanning microscopy (LSM510, CarlZeiss) or a fluorescent microscope. Images of two sites per well with microscope and digital camera with a 10X phase contrast objective were taken.

3.9.4 Calcium release

To evaluate calcium release, 70CS30P materials scaffolds were washed three times with phosphate buffered saline (PBS) and put in 96 multi-well plate with EndoGRO^{TM} and DMEM 1X medium. The day after, CS conditioned media were collected and cleared with centrifugation. Resulting supernatants were assessed with *Ortho-Cresolphthalein complexone* (CPC) method to investigate released calcium concentrations at 1 and 4 days of incubation time as previously described [52].

3.9.5 Cell migration: scratch wound assay

The migration capability of human vein endothelial cells (HUVECs) was assessed using a scratch-wound assay, which measures the migration of cells in a wounded area. To evaluate the effect of different conditioned media on cell migration, a scratch-wounded HUVECs monolayer was employed. Briefly, cells were seeded into 12-well tissue culture to confluence using ENDOGRO medium. Confluent HUVECs were scrape-wounded using a sterile p200 pipette tip for one perpendicular linear scratch. A blank line perpendicular to the scratch, painted under the bottom of the plate, ensured recording of the same wound area per well. Wells were washed with phosphate buffered saline (PBS 1X) to remove floating and dead cells and conditioned media was added. Wound closure was monitored collecting digitalised images immediately after the scratch and 4 and 18 hours afterwards. Images of two sites per well with an inverted microscope (Nikon TE200) and digital camera (Olympus DP72) with a 5X phase contrast objective lens were captured. Three representative images from each well of the scratched areas under each condition were photographed to estimate the migration cells. The reduction of wound areas was analysed using *Image J*1.48i software. Four replicates per condition were used and the experiment was repeated three times.

3.9.6 *In vitro* Matrigel-based formation assay

In vitro tube formation assay is considered as representative of a later stage of angiogenesis and is widely used to assay potential stimulators or inhibitors of angiogenesis. Tube formation was determined in 48-well plates coated using Matrigel (Corning®) for 1 hour at 37 °C. HUVECs of passages 5 were harvested by trypsinization and seeded onto or within Matrigel. At the same time point, CS scaffolds conditioned media was added to the wells. The 48-well plates were incubated and the tubes formed at 4 and 8 hours were observed under inverted phase contrast microscope (Leica). The presence of tube like-capillary structures indicated angiogenesis formation.

3.10. Statistical analysis

All quantitative experiments were performed in triplicate and the results were expressed as mean ± standard deviation (SD). Statistical analysis of the data was conducted using one-way ANOVA. Differences between the groups of $p < 0.05$ were considered statistically significant.

4. Results

4.1 Scaffold preparation

The foaming method used to prepare CS scaffolds by Dr. Demitri's group [20], allowed an interconnected and homogeneous structure with pores of different size from 20 μm to 300 μm and a hierarchical structure of the scaffolds to be obtained. This property allows the cells to migrate in the internal part of scaffold, while the micropores, due to solvent evaporation, manage nutrient flow and exchange of oxygen, avoiding the necrosis of cells. However, the presence of a second more rigid non-porous phase represented by PEGDA determines a decreasing of porosity [63]; in fact, an open porosity of 78% and 60% for 80CS20P and 60CS40P, respectively. Measurements of water adsorption capacity report that there was no significant improvement in water absorption capacity by increasing CS concentration, probably owing to the higher capillary absorption effect, resulting from a homogeneous porous structure in each composition. The maximum swelling ratio was approximately 10.5 g/g, which was measured for sample 30P70CS. Rigidity of the scaffold, measured through a compressive test (carried out using the Ares

rheometer), shows that scaffold with 40% PEGDA (60CS40P) has a higher Young's modulus because of the presence of a second more rigid non-porous phase. Young's modulus increased with the CS concentration, except in this sample, where the higher polymer density led to the presence of physical entanglements and a higher degree of cross-linking [64].

4.2 Scaffold bioactivation by inorganic signals: morphological analysis

Following the scaffold production step, a bioactivation process was performed. Bioactivation is a simple two-step biomimetic approach, which consists of a nucleation phase obtained by incubation of scaffolds in a supersaturated SBF solution for 3 days, followed by a crystallisation and growth of apatite nuclei in a modified solution without inhibitors of crystallisation, such as hydrogen carbonate and magnesium ions. SEM images suggested the presence of calcium phosphate deposits on the scaffold surface and on the internal pore walls, after only 7 days of treatment (Figure 3). This behaviour was due to the use of two specific parameters represented by a supersaturated concentration (5xSBF) and pH value, in fact, at 7.4 the CS polymer shows a negative charge due to the deprotonation of COOH groups (pKa_{CS} = 6.5). In this way, interactions between two COO^- groups and bivalent Ca^{2+} were favoured.

4.3 Scaffold bioactivation by organic signals and *in vitro* release study

The microwave-assisted Fmoc solid phase peptide synthesis allows a peptide to be obtained in a shorter time than through the conventional synthesis process, with a molecular weight of 2074,4 Da and high purity (>98%), as demonstrated by chemical characterisation through mass spectrometry and analytical HPLC, respectively (Figure 4A). The pure peptide was immobilized on CS scaffolds (80CS20P and 60CS40P) (Figure 4B) by carbodiimide reaction mechanism, where the $-NH_2$ groups of CS and -COOH groups of BMP-peptide were involved. This covalent bond guarantees an *in vitro* release for an extended time. Particularly, the results onto 80CS20P scaffolds demonstrated a burst release of about 8-10% the first time (Figure 4C), due to an early scaffold degradation; however, the release was sustained up to 4 weeks where 80CS20P

released about 94.5% of peptide. In the case of 60CS40P_BMP scaffolds, with a higher amount of BMP2 mimic peptide (3.6μg/mg scaffold), a slower release in the first 48 hours was obtained, probably due to a much more stable structure for the higher amount of PEGDA, compared to the 80CS20P samples; however, up to 4 weeks 60CS40P_BMP and 60CS40P_BMP* did release 97.3% and 92.9% of peptide, respectively.

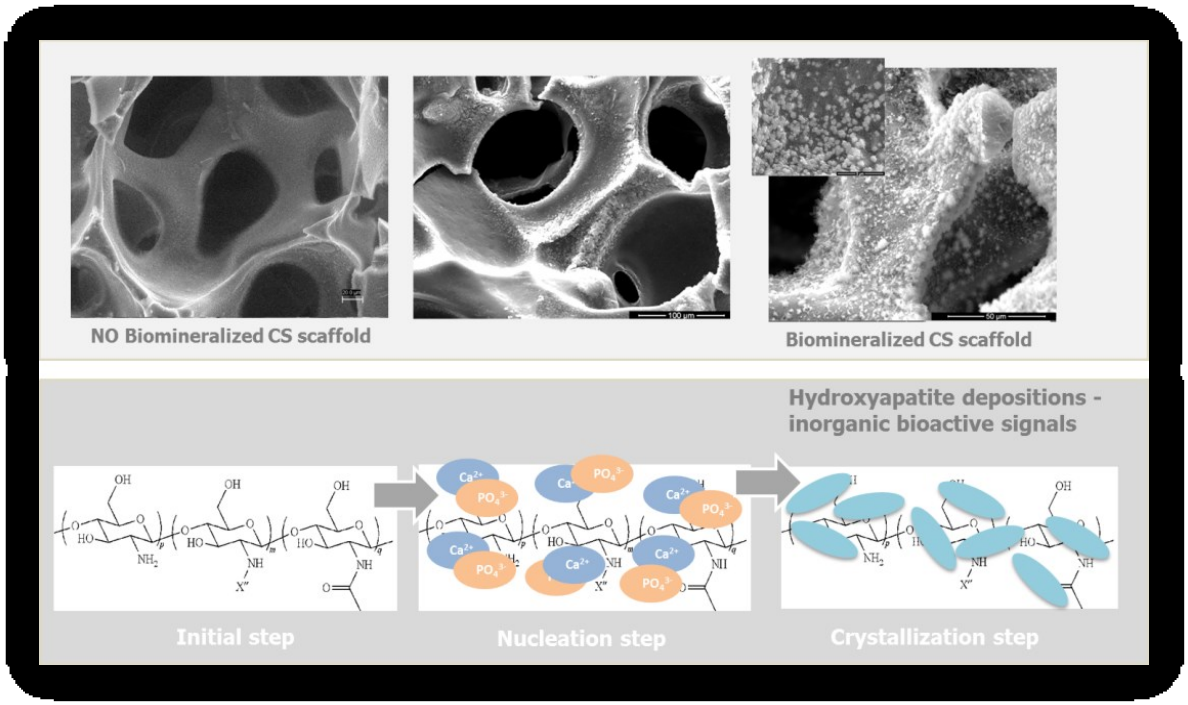
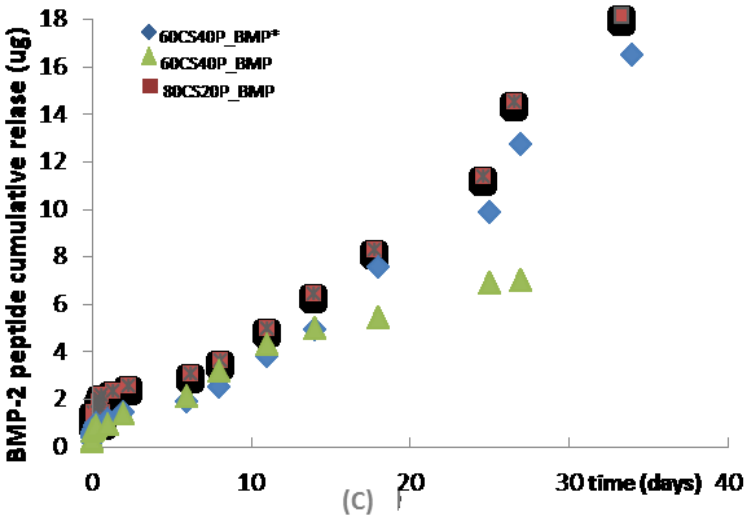


Figure 3. Representation of biomineralization of CS-based-scaffolds. The process consists of nucleation and crystallization. Biomineralization obtained with hydroxyapatite (HA) deposition was performed using a supersaturated solution (5xSBF).



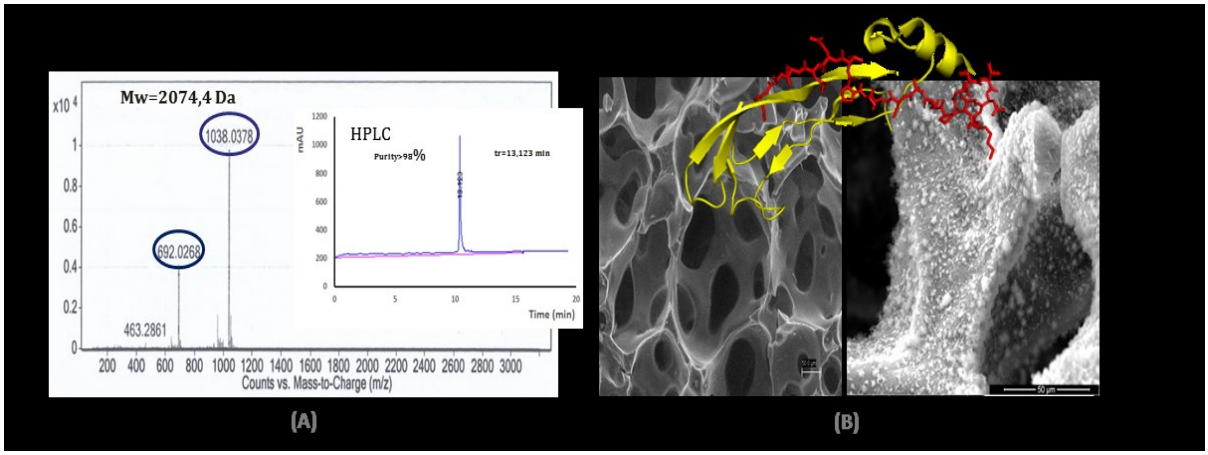


Figure 4. **(A)** Chemical characterisation of BMP2-peptide by HPLC and TOF mass spectrometry; **(B)** SEM images show the covalent immobilization of peptide on scaffold surface; **(C)** Peptide release profiles from 80CS20P and 60CS40P scaffolds.

4.4 Biological properties

4.4.1 Biological activity of BMP-2 mimic peptide

The bioactivity of neat peptide was investigated by measuring its effect on hMSCs behaviour in terms of proliferation and differentiation. First, the effect of peptide at different concentrations on hMSCs adhesion was determined after 1 day of cell culture. The LSCM images (Figure 5A) demonstrated that the cells in contact with solution at a higher concentration (2.7μM) showed a polygonal morphology typical of phenotype more similar to osteoblast than the control (CTR), whereas at lower concentration (270 pM) the cells showed a fibroblast-like morphology as compared to the control. Furthermore, quantitative analysis about the cell proliferation (Figure 5B) proved an increased proliferation in the first 3days, followed by a decrease, due to initial osteogenic differentiation. In fact, a higher ALP value at 7 days (Figure 5C) was observed, particularly for the hMSCs in contact with solution at higher concentrations. This preliminary study was used as a screening to help determine the optimal concentration of BMP-2 peptide necessary for scaffold bioactivation; the results showed that 2.7μM solution had the best biological performance.

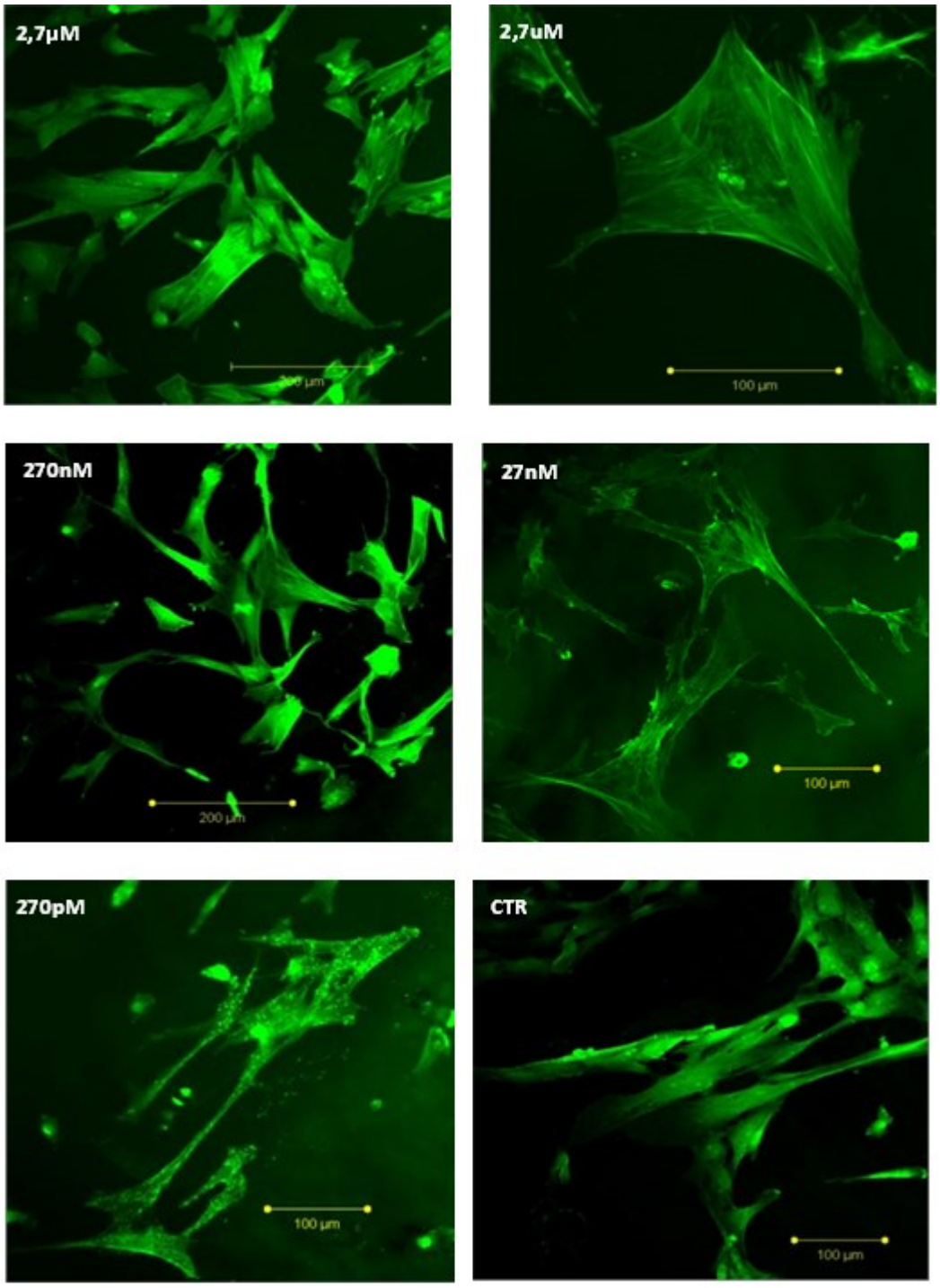


Figure 5. Confocal images of hMSCs after 24 hours of cell culture with BMP-2-peptide at different concentrations. BMP-2 at concentration of 2,7 µM induces the best biological response in terms of cell morphology. Polygonal morphology suggests osteogenic cell differentiation.

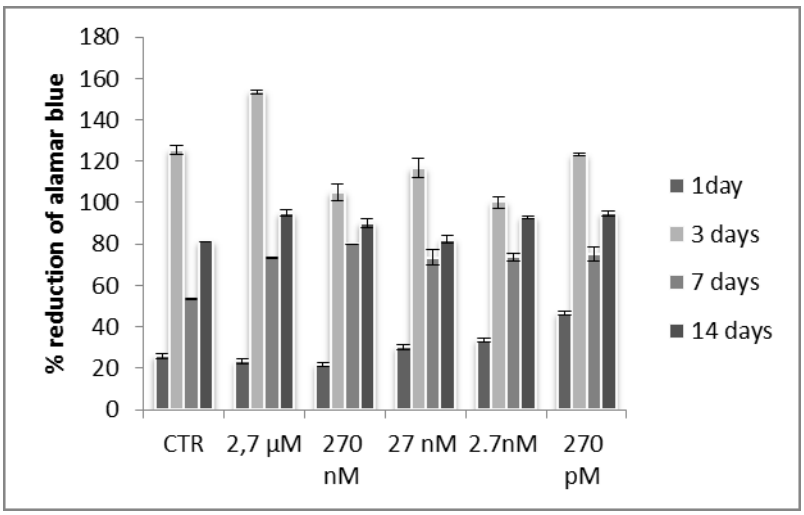
4.5 Viability test of bioactive CS-based scaffolds

The effect of 80CS20P and 60CS40P scaffolds with and w/o BMP-2 mimic peptide (2.7 μM) on hMSCs was also evaluated. The analysis demonstrated that, at 24 hours, there was good cell attachment on the scaffold surface with higher values for the biomimetic scaffolds at different compositions (Figure 6). After this time, the proliferation increased over culture time. This evidence confirmed that the scaffolds support adhesion and cell migration inside the scaffold structure. The proliferation showed higher values at day 14 (Figure 7). On the other hand, at day 21 a slight decrease for the scaffold with the highest amount of BMP peptide (60CS40P_BMP*) was observed.

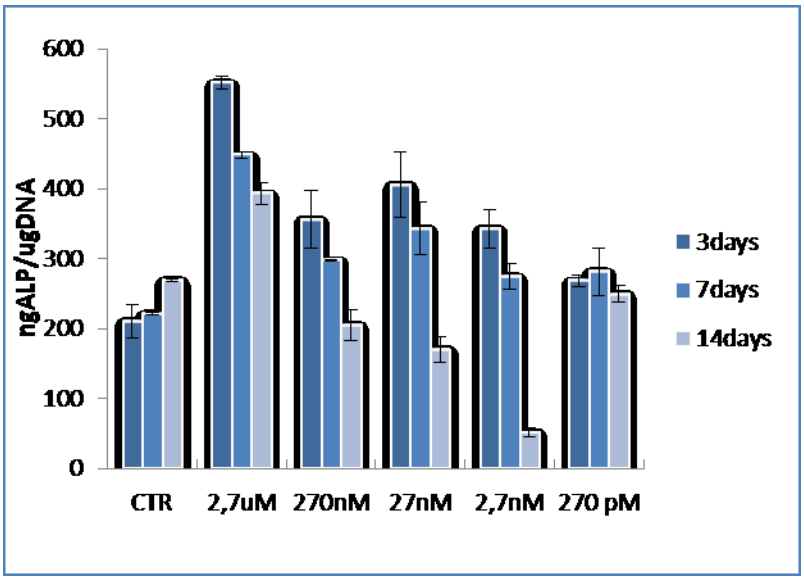
4.6 Osteogenic differentiation

4.6.1 Alkaline phosphatase and osteocalcin expression

To study the effect of CS-based-scaffolds on osteogenic differentiation, the expression of alkaline phosphatase (ALP) and osteocalcin (OCN), was evaluated. The early osteogenic differentiation of hMSCs was tested measuring alkaline phosphatase (ALP) activity in cultures of scaffold materials after 7, 14 and 21 days (SensoLyte pNPP ALP assay kit; ANASPEC, Milan, Italy). The experiments were performed in basal medium considering the inorganic and organic bioactive signals as osteoinductive factors. Results showed that scaffolds with BMP-2 like peptide (80CS20P_BMP) do induce higher ALP values than biomineralized scaffolds (80CS20P_bio), even if 80CS20P_bio showed better results than neat 80CS20P. Furthermore, the scaffolds with lower amount of CS bioactive with BMP-2 like peptide (60CS40P_BMP and 60CS40P_BMP*) showed highest ALP values at day 3, probably due to the lower release profile of peptide in the first 48 hours and a higher scaffold stability (as demonstrated in the kinetic release study reported in the Figure 4). However, a higher ALP expression for the full set of scaffolds and, particularly, 80CS20P_bio was observed. Moreover, 80CS20P_BMP showed a similar behaviour to 60CS40P scaffolds (both 60CS40P_bio and 60CS40_BMP) at day 3. 60CS40P_BMP* showed the best results over the culture time (Figure 9B). This behaviour was also observed for the expression of non-collagenous bone ECM protein (i.e. osteocalcin) used as later marker of osteogenic differentiation (Figure 10).



(A)



(B)

Figure 6. (A) Cell proliferation and **(B)** alkaline Phosphatase activity at different time points up to 14 days of cell culture. Results on cell proliferation and differentiation confirmed that the optimal concentration of BMP-2 is 2,7 µM.

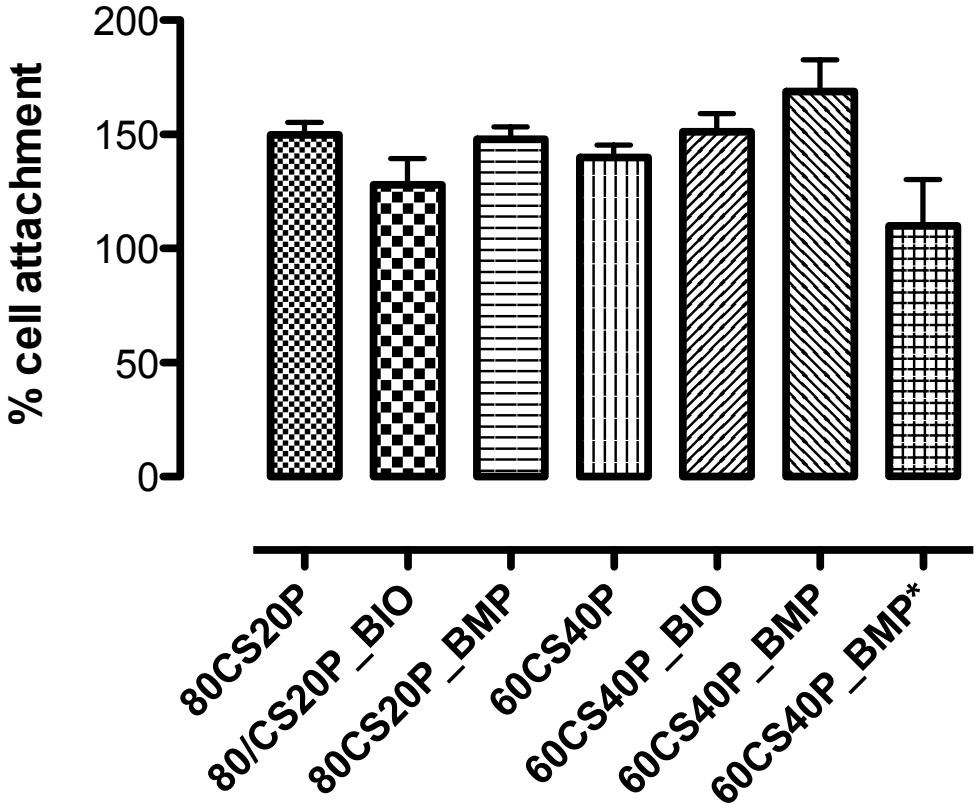


Figure 7. Cell adhesion, expressed as percentage of control, of hMSCs seeded onto CS-based-scaffolds (80CS20P and 60CS40P w/wo BMP-2 mimic peptide) after 24 hours of cell culture.

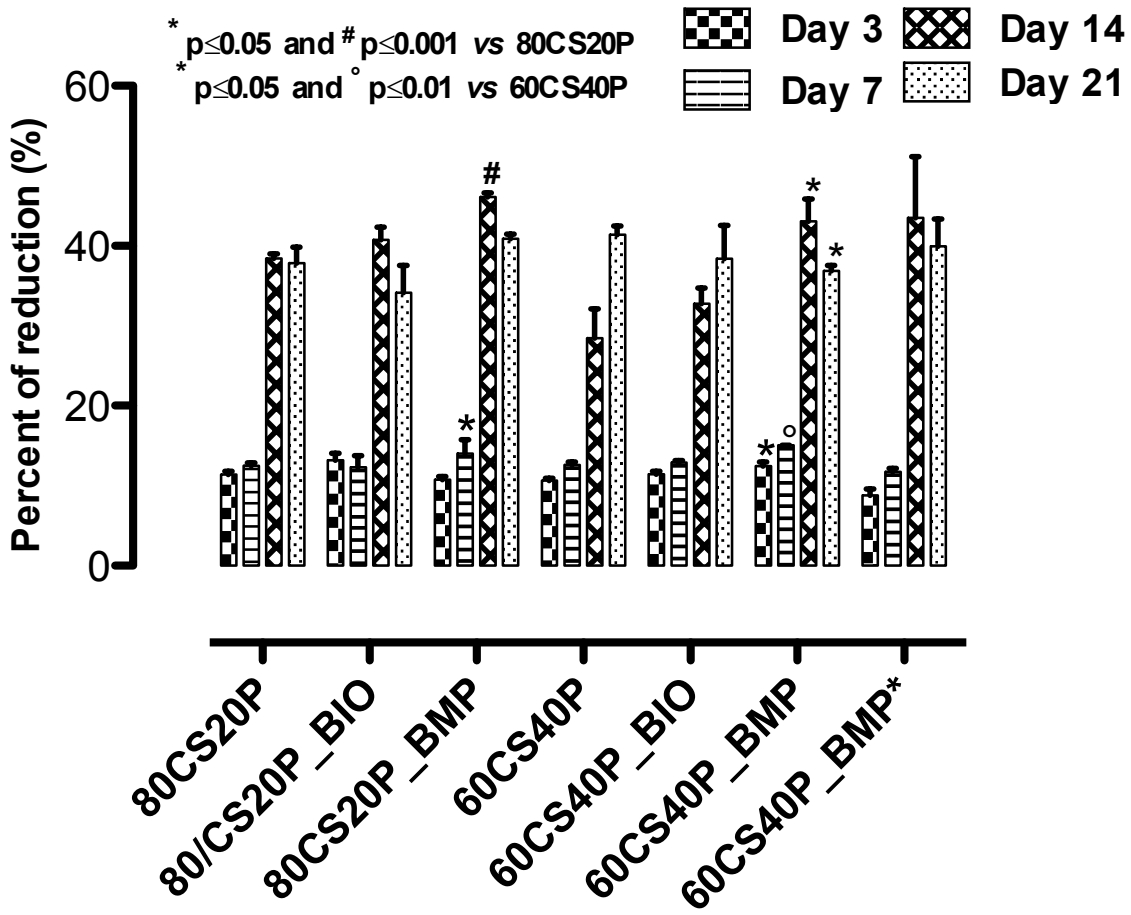
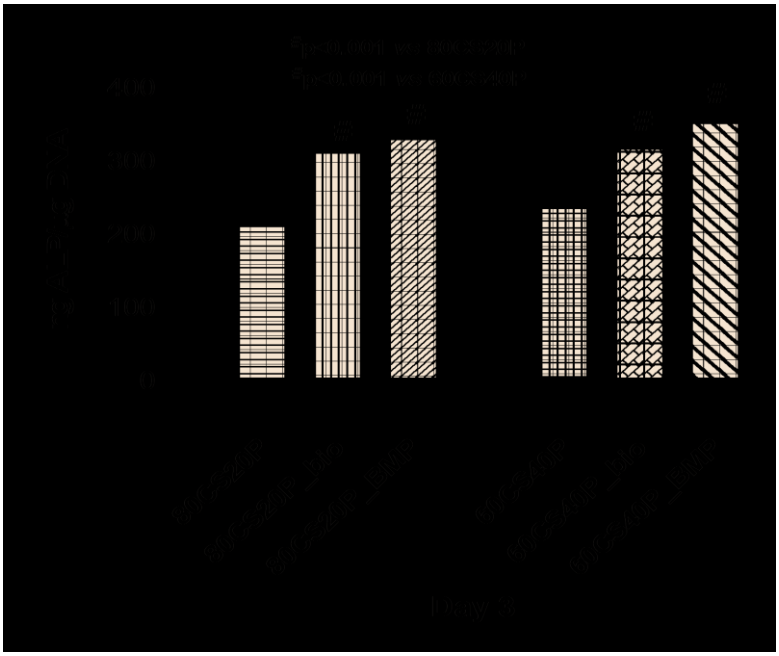
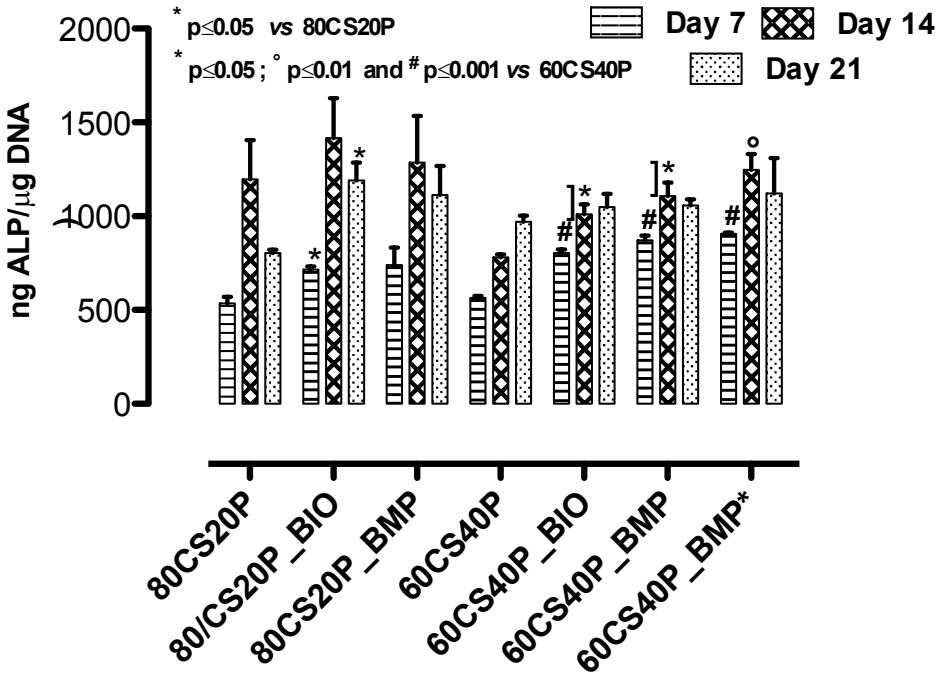


Figure 8. Cell proliferation evaluated through Alamar Blue test. An increase in cell proliferation for bioactivated and non-bioactivated CS-based-scaffolds for up to 21 days of cell culture was obtained. Results, expressed as a percent reduction of Alamar Blue solution, are the mean ±SEM of three experiments. $^{*}p<0.05$, $^{\#}p < 0.01–0.001$ *vs.* control (80CS20P-60CS40P).



(A)



(B)

Figure 9. Alkaline Phosphatase activity normalized to micrograms of DNA (ngALP/μgDNA) at short **(A)** and long-time **(B)**. Results are mean ±SEM of three experiments. *p<0.05, #p < 0.01–0.001 *vs.* control.

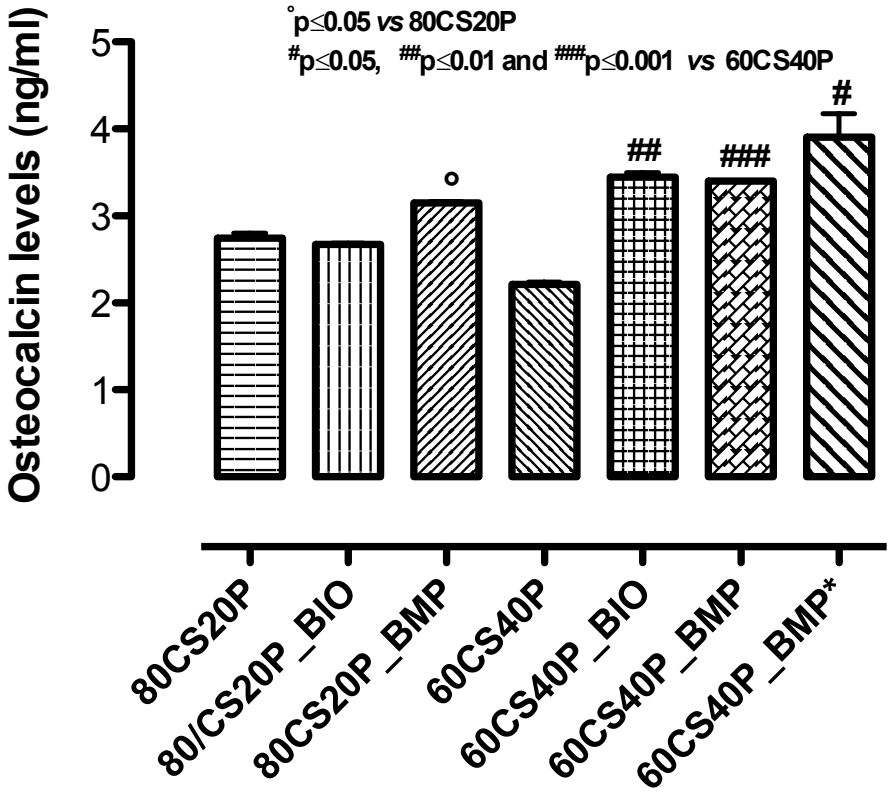


Figure 10. Osteocalcin expression of CS-materials at day 21 of culture time. Results, expressed as osteocalcin levels (ng/ml), are the mean ±SEM of 3 experiments. $^{*}p<0.05$, $^{\#}p < 0.01–0.001$ *vs.* control.

4.7 Effect of CS materials on inflammation: *in vitro* model

LPS significantly increased the levels of IL-1β, a pro-inflammatory cytokine at the concentration of 1μg/ml. A pre-treatment of CS-based scaffolds with and without two (inorganic and organic) types of bioactivation counteracted the changes in IL-1β observed in cells treated with LPS alone (Figure 11A–B). In contrast, the levels of IL-10, an anti-inflammatory marker, were significantly increased only in presence of the biomineralized 80CS20P scaffold (Figure 11C-D). Furthermore, LPS (1μg/ml for 72h) stimulation caused a significant increase in the nitrite production (Figure 12A-B). However, a pre-treatment with biomineralized 80CS20P induced a significant reduction in nitrite production. The inhibitory effect of the biomineralized 80CS20P scaffold on nitrite production in LPS-treated hMSCs was accompanied by a reduction in IL-1β and an increase in IL-10 levels. These results suggested that the biomineralized 80CS20P showed the best effect in terms of anti-inflammatory response on a model of bone inflammation.

4.8 Effect of CS on angiogenesis

4.8.1 Calcium release from CS-based-scaffolds

Calcium released from CS-based-scaffolds by using *O-Cresolphthalein complexone* method was assessed. Preliminary study suggested that CS materials do not contain cytotoxic calcium concentration for HUVECs. In addition, data showed that CS bioactive and non-bioactive scaffolds contain a lower calcium amount than DMEM 1X (Figure 13A) and ENDOGRO medium (Figure 13B). This behaviour could be explained by chitosan's high adsorptive capacity.

4.8.2 Live-dead assay

In vitro qualitative analysis of cell viability with live/dead assay (Invitrogen) was performed. Preliminary, ENDOGRO medium by 70CS30P based-scaffolds through 24 hours of incubation was conditioned and added to HUVECs. Microscopy images of 70CS30P based-scaffolds showed that medium conditioned with bioactive CS materials does not compromise HUVECs survival thus inducing a better cell spreading compared

to medium conditioned with neat chitosan scaffolds (Figure 14). Moreover, a difference in HUVECs morphology between different CS samples was observed. In particular, CS bioactivated with BMP-2 peptide confers a different HUVECs morphology than other samples. This effect is probably due to the activation of proteins involved in cell differentiation such as VE-cadherin which induces an increase in HUVECs permeability [65].

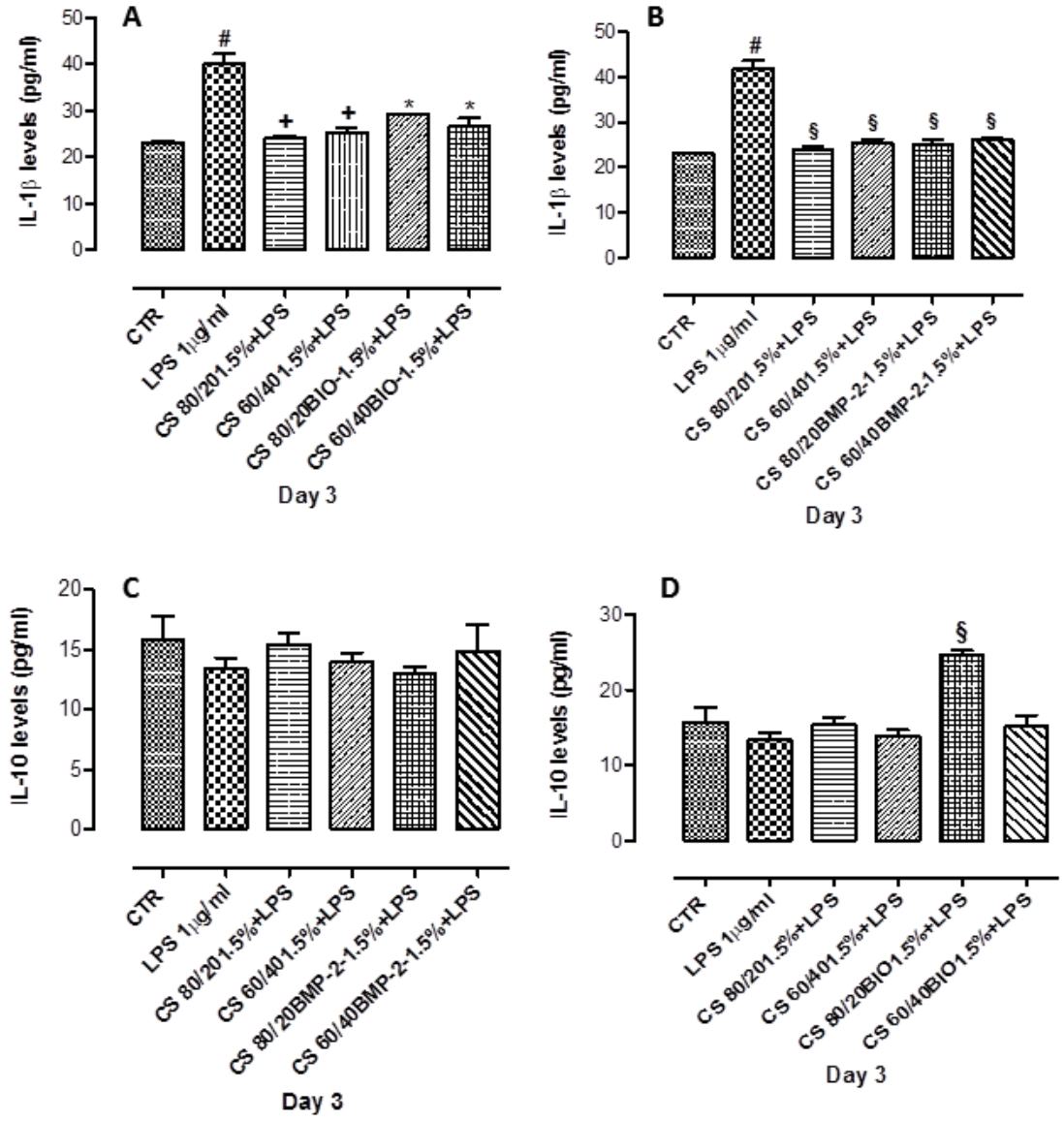


Figure 11. Effect of bioactive and non-bioactive CS-based-scaffolds on IL-1β **(A-B)** and IL-10β **(C-D)** levels in hMSC cells treated with LPS (1μg/ml). Measurements were performed 3 days after LPS (1μg/ml) stimulation. The exposure of hMSCs to CS-based scaffolds started 24 hours before the inflammatory insult. Results, expressed as picograms per ml of supernatant, are the mean ±SEM of three experiments. $^{\#}p < 0.01–0.001$ *vs.* control and $^{\S}p < 0.001$ *vs.* LPS alone.

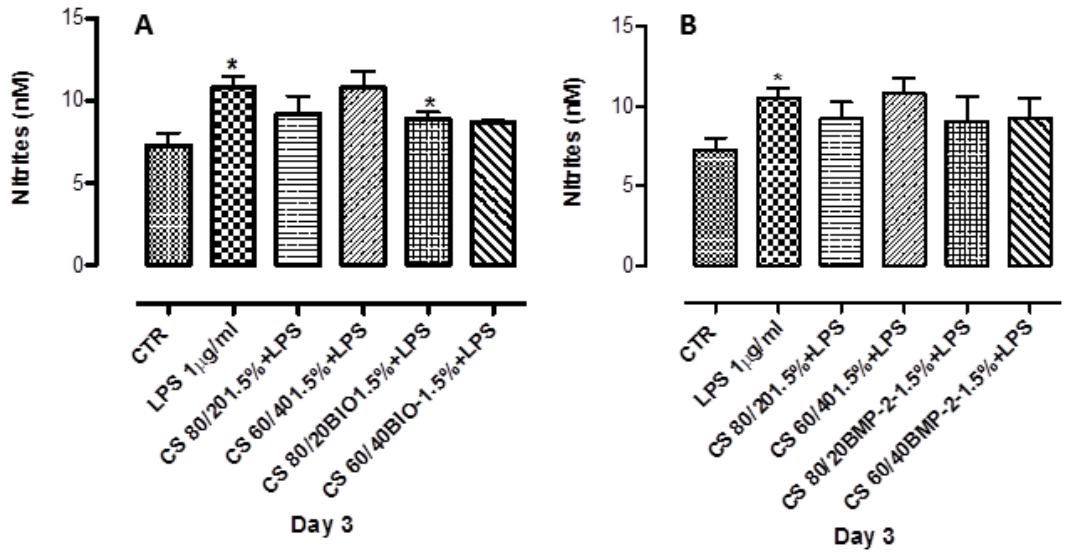
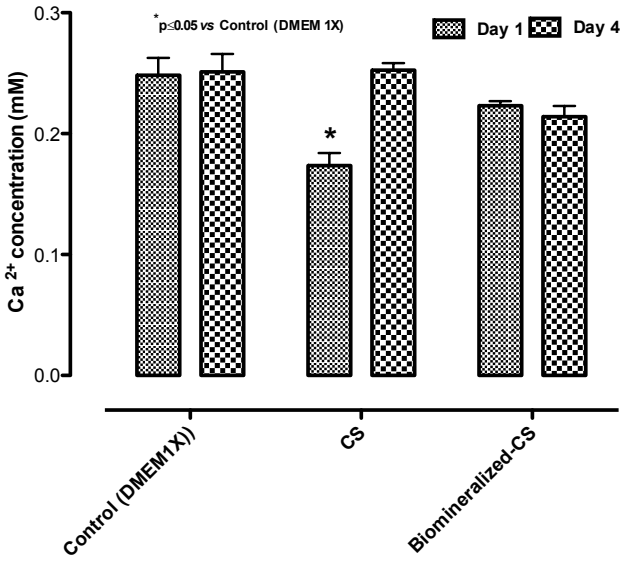
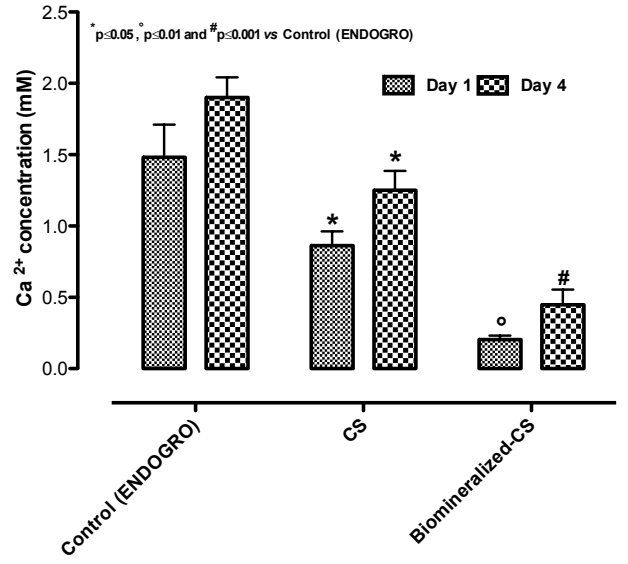


Figure 12. Effect of bioactive and non-bioactive CS scaffold on nitrite levels **(A-B)** in the cell medium of hMSCs incubated with lipopolysaccharide (LPS, 1 μg/ml) for 72 hours. The exposure of hMSCs to chitosan-based scaffold started 24 hours before LPS challenge. Results, expressed as nitrite concentration (nM), are the mean ± SEM of three experiments (in triplicates). $^{*}p<0.05$ *vs.* control and $^{*}p < 0.05$ *vs.* LPS alone.



(A)



(B)

Figure 13. Calcium concentration (mM) release from CS-based scaffolds using cell medium. The materials were conditioned with DMEM 1X **(A)** and ENDOGRO **(B)** medium for an incubation time of 1 and 4 days. The results, expressed as Ca^{2+} concentration (mM) are the mean ± SEM of three experiments.*$p<0.05$, °$p < 0.01$ and $^{\#}p < 0.001$ *vs.* control (ENDOGRO).

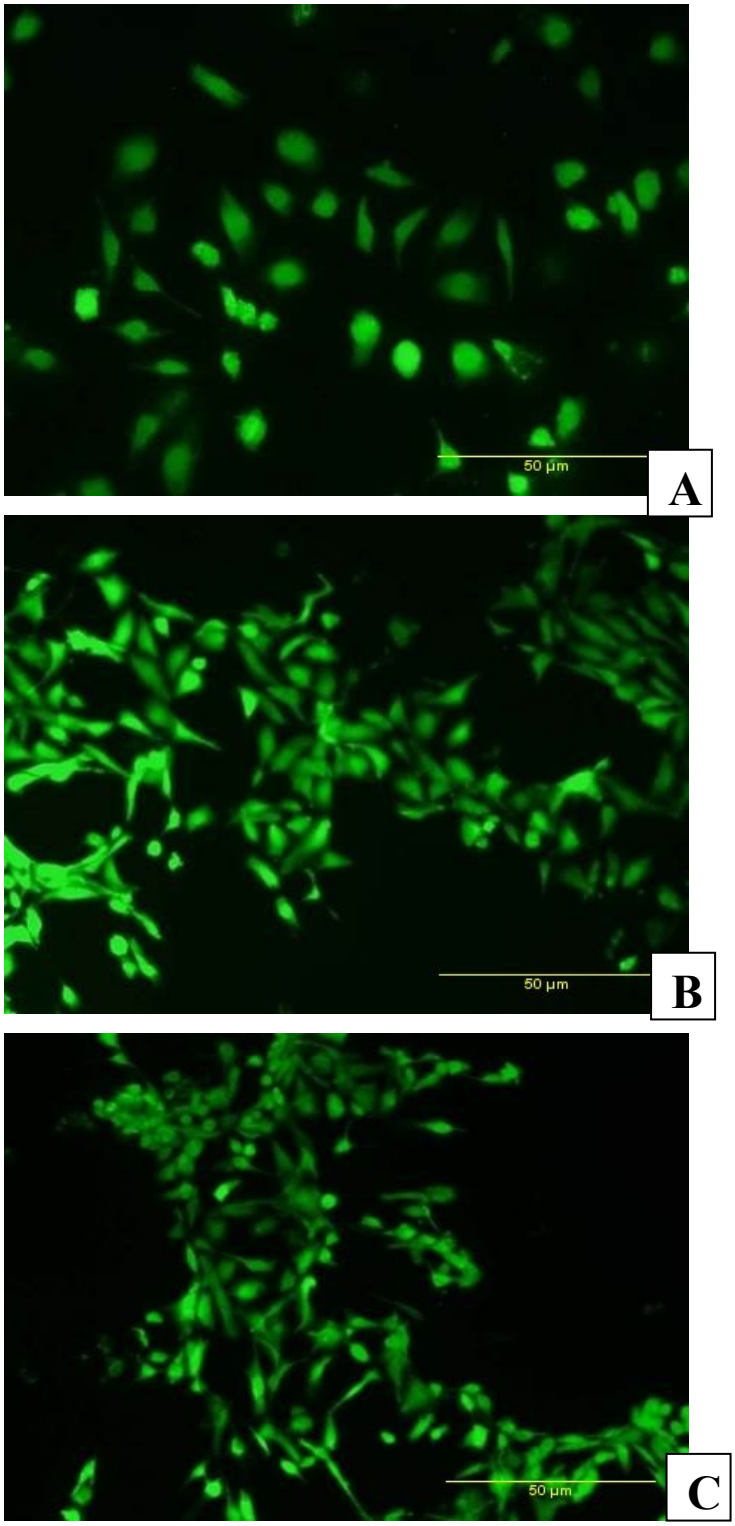


Figure 14. Fluorescent microscopic images of live-dead assay of HUVEC cells after 24 hours of incubation on CS without bioactivation **(A)**, biomineralized chitosan **(B)** and BMP-2 chitosan **(C)**. Scale bar, 50 µm.

4.9 HUVECs proliferation

Preliminary results in cell proliferation demonstrated that 70CS30P based-scaffolds increase HUVECs proliferation over culture time. However, the values are lower than the control plate, suggesting the potential effect of scaffolds on differentiation mechanisms (Figure 15).

4.10 Cell migration: scratch-wound assay

To evaluate the wound healing effect of 70CS30P samples on HUVECs an *in vitro* model was used. The experiment was performed with *Mitomycin* 10 μg/ml, as proliferation inhibitor, to promote migration rather than cell proliferation. Results obtained showed that the scratch assay is a convenient and inexpensive first method to measure cell migration, even if the scratch assay cannot substitute *in vivo* studies as a final proof of the efficacy in wound healing. This study confirmed the potential effect of CS biocomposites on HUVECs migration. Moreover, our results were also in line with tube formation data, which confirmed the effect of CS-based scaffolds on the angiogenesis process. Indeed, scaffolds ability to stimulate cell migration has been confirmed. These results indicated that both bioactive and non-bioactive scaffolds stimulate and support HUVECs migration (Figure 16).

4.11 Tube formation quantification

Angiogenesis involves the proliferation, migration, and remodelling of endothelial cells in the process of tube formation. HUVECs offer an important *in vitro* model to study these processes. Cultures of HUVECs on Matrigel, an extract of endothelial basement membrane, result in the formation of honeycomb-like structures that simulate tube formation of endothelial cells *in vivo* (19). Studying the effects of hypoxia, VEGF, FGF, and transforming growth factor-β and inhibitors of angiogenesis on the formation and organization of honeycombs by HUVECs has helped elucidate the mechanisms by which these factors regulate both pathological and therapeutic angiogenesis and the role of small GTP-binding proteins in this process. An important example has been the demonstration that 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors,

cholesterol lowering drugs, exert a dose dependent effect on honeycomb formation and signalling pathways in HUVECs suggesting that under appropriate conditions and at different concentrations, statins might both decrease the progression of atherosclerosis and stimulate the revascularization of ischemic tissues through its effect on angiogenesis [66]. A second model system for the study of angiogenesis has been the growth of human dermal microvascular endothelial cells (HDMECs), which are derived from the foreskins of newborns. When cultured on a thick collagen gel these cells form tube like structures that penetrate the collagen surface resembling capillary-like structures. To evaluate the later stages of angiogenesis *in vitro* tube formation assay was performed. Tube formation assay provides an *in vitro* model of tubulogenesis, which mimics *in vivo* conditions. For this reason, human umbilical vein endothelial cells (HUVECs) and Matrigel were used. As mentioned before, results demonstrated that chitosan 70CS30P based scaffolds induce tube formation in HUVECs at 4 and 8 hours of cell culture. Qualitative analysis confirms the possible use of chitosan materials for the promotion tube formation as shown in Figure 17. The potential angiogenic effect of the materials is enhanced by quantitative evaluation of HUVEC tube formation. In fact, chitosan scaffolds have a good effect on angiogenesis in terms of angiogenic parameters such as total tube length, branches and junctions quantification (Figure 18). Figure 17 shows the typical appearance of tubules on Matrigel for each CS-based-scaffolds at 4 and 8 hours of cell culture (CS alone, BMP-2 and biomineralized CS) as compared to control and positive control (HUVECs and VEGF 20 ng/ml). Tubule development at 4 and 8 hours was observed and the extent of tubule formation was measured quantitatively using Angiosys 2 analysis program.

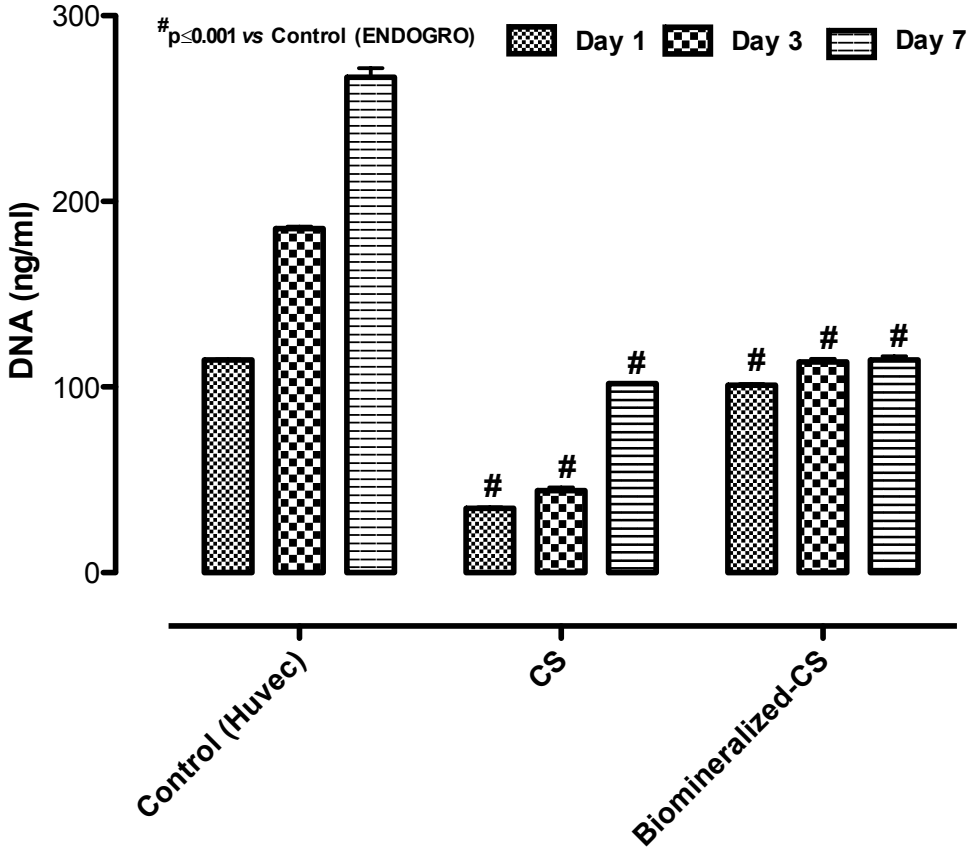


Figure 15. HUVEC proliferation was evaluated with PicoGreen assay. Cells were cultured on CS scaffolds for 1, 3 and 7 days of cell culture. Results, reported as DNA amount (ng/ml), are the mean ±SEM of three experiments. #p < 0.001 *vs.* control (HUVECs).

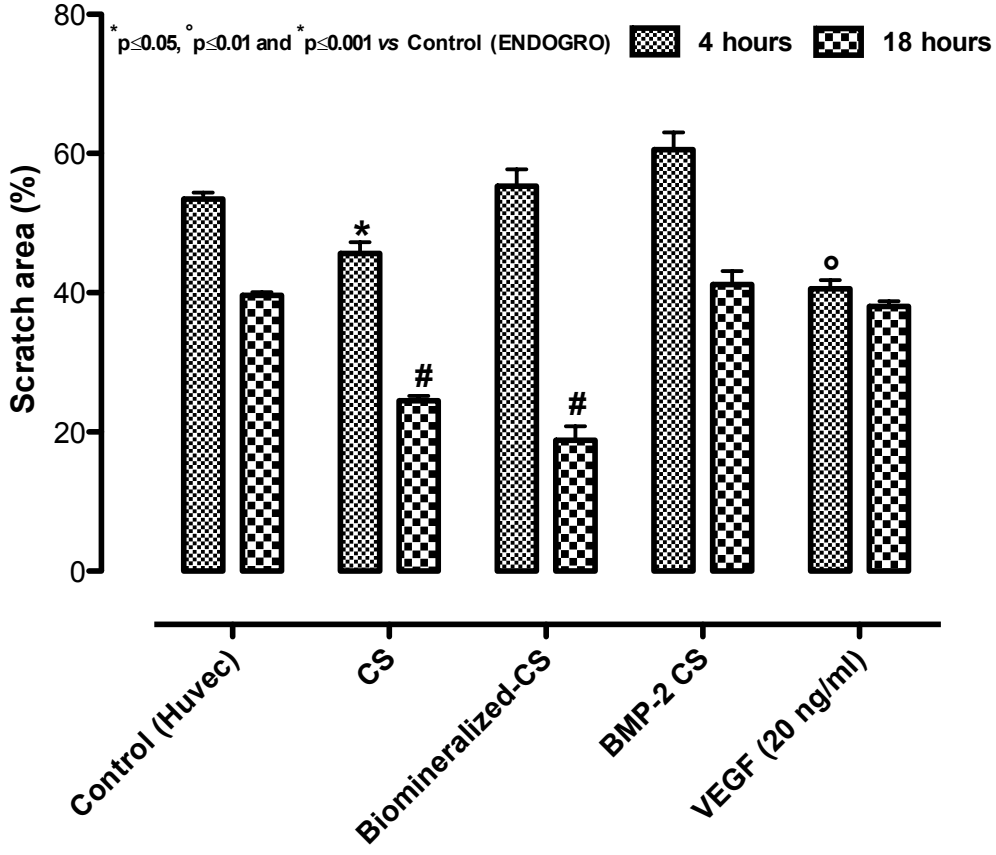


Figure 16. Effect of CS scaffolds on the migratory activities of HUVECs in the scratch assay in the presence of 10 μg/ml of antimitotic mitomycin C after 4, 8 hours of incubation (37◦C; 5% CO_2) in ENDOGRO medium. Quantitative analysis of migration of human umbilical vein endothelial cells, in contact with CS scaffolds conditioned media, as compared to control (HUVECs), positive control (VEGF 20 ng/ml) was reported. Data are expressed as scratch area percentage (%) at 4, 8 hours in the wounded area normalized to 0 hours. Bars represent the mean ± S.E.M. of three experiments. #$p < 0.001$ *vs.* control (HUVECs).

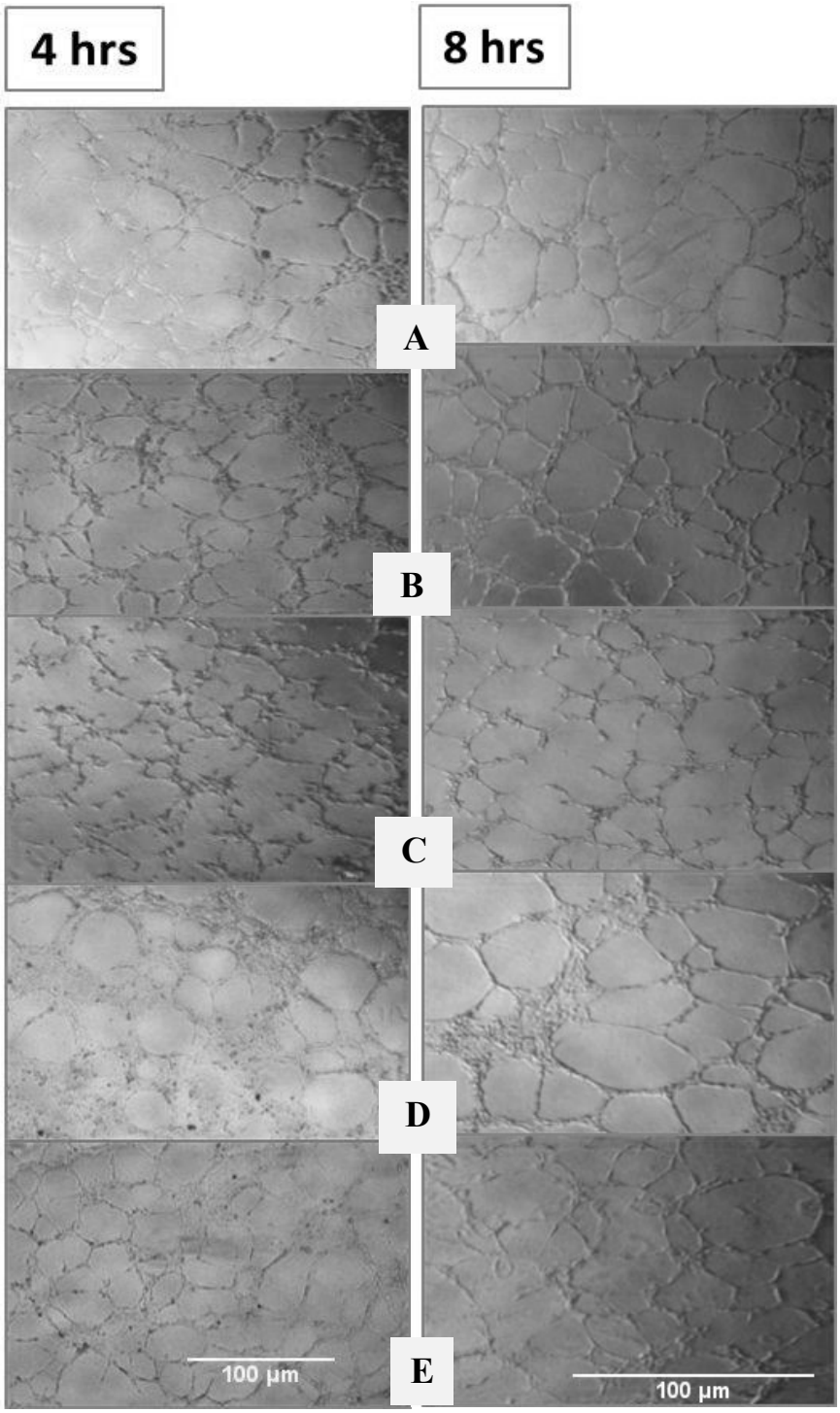


Figure 17. Microscopy phase contrast images of HUVECs tubules at 4 and 8 hours of cell culture of CS-BMP-2 **(A)**, CS control **(B)**, CS biomineralised with HA **(C)**, HUVECs **(D)**, VEGF 20 ng/ml **(E)**. Scale bar, 100 µm.

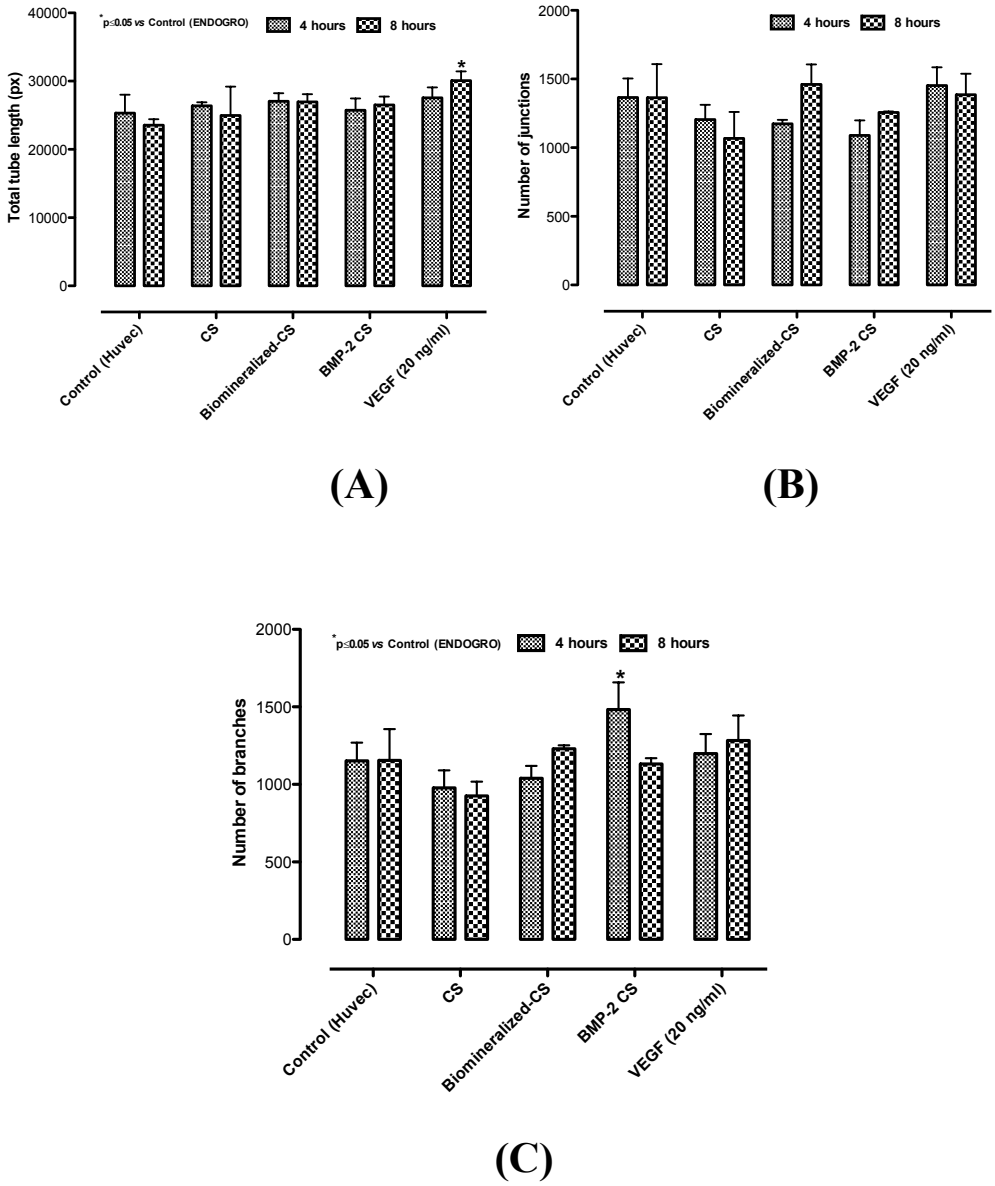


Figure 18. Quantification of HUVEC tube formation in CS-based scaffold conditioned media. Results, relative to cells growing on Matrigel, are expressed as a complete average of tube length **(A)**, number of junctions **(B)** and branches **(C)**, after 4, 8 hours of cell culture. Results are the mean ± SEM of three experiments (in triplicates). *p<0.05 *vs.* control (HUVECs).

5. Discussion

In bone tissue engineering the scaffold should provide necessary support as an artificial extracellular matrix that allows cells to proliferate and maintain their differentiated functions. Essentially, the scaffold acts as a temporary template to guide the formation of new tissue. In this context, an ideal scaffold is characterised by excellent biocompatibility, controllable biodegradability, cytocompatibility, suitable microstructure (pore size and porosity) and mechanical properties. Additionally, the scaffold must be capable of promoting cell adhesion and retaining the metabolic functions of the attached cells [49].For these reasons, the aim of this section of the thesis was to study the effect of biomimetic functional scaffolds obtained by modifying polymer scaffolds with osteoinductive signals including inorganic components, such as hydroxyapatite depositions and organic signals, such as BMP-2 mimetic peptide immobilized on the scaffolds with a covalent binding. Therefore, this research could help develop biodegradable scaffolds that are also carriers of osteogenic signals that promote positive cellular behaviour in terms of osteogenic commitment. To perform a covalent peptide immobilization on the scaffolds with different chitosan concentrations (80CS20P_BMP and 60CS40P_BMP) a low percentage of burst release, 20% and 10% in the first 48 hours, respectively is determined; whereas at longer intervals, a prolonged sustained release of up to 4 weeks has an important effect on cellular behaviour. The early effect of BMP in the first days of cell culture is due to the burst release of peptide, which continues over time, demonstrating the positive effect of prolonged delivery on cellular behaviour, because of the high stability of the scaffold structure (prepared by physical foaming combined with microwave curing) [55]. *In vitro* degradation tests showed a gradual dissolution of scaffolds over time, while maintaining their 3D morphology and integrity even after 6 weeks of incubation [20] and allowing an extended release of organic bioactive signal. This extended release potentially promises the successful infiltration of cells and new tissue formation. In fact, the biological results demonstrated that the materials act as support for hMSCs adhesion and proliferation. A consistent migration of cells into the scaffold is evident, as demonstrated in cross-section SEM images. Moreover, the effect of the organic and inorganic bioactive solid signals on osteogenic differentiation it was also evaluated and it was found that *in vitro* and *in vivo* may be characterised in three stages: (a) cell proliferation, (b) matrix maturation, and (c) matrix mineralization [56]. In *vitro*, matrix maturation and mineralization are usually enhanced by growing the cells to complete confluency and by adding specific osteogenic

factors. After the proliferation, the matrix maturation phase is characterised by an expression of alkaline phosphatase (ALP). ALP is a well-known early marker of osteogenic differentiaton and plays a key role in the mineralization of bone. As such it's considered a useful biochemical marker of bone formation [57]. Finally, during matrix mineralization (c) other genes for proteins such as OC, BSP, and OPN are expressed. Analysis of bone cell specific markers like Procollagen, ALP, OPN and OCN, is used to characterise later osteogenic differentiation phases *in vitro* of hMSC [58]. The mineralization process of osteoblasts in *in vitro* culture has also been used as a model for testing the effects of peptide loading on bone cell differentiation and bone formation. In this project, the expression of ALP and OCN were analysed; in particular, it was observed that at 3 and 7 days no difference was observed among the group of bioactivated scaffold and related controls (60CS40P and 80CS20P). Meanwhile, after 14 days the best expression of ALP for the 80CS20P_BMP scaffold was observed, where a peptide release of approximately 65-75% (12-14μg) was obtained. In parallel, the 60CS40P_BMP* showed the highest ALP expression at 21 days. On the other hand, biomineralized scaffolds developed through biomineralization treatment (80CS20P_bio and 60CS40P_bio) showed an increased ALP expression compared to materials that have not been biomineralized after 3 days of incubation. At the same point in time, similar cell differentiation behaviour, between 80CS20P_bio and 60CS40P_bio, was observed. At longer incubation times (7, 14 and 21 days), no significant difference between the two-biomineralized groups and the BMP-activated scaffolds was observed. In particular, 80CS20P_BMP showed better ALP expression after 14 days than 60CS40P_BMP/BMP*. After 21 days, the 60CS40P_BMP plateaued compared to levels at 14 days. On the contrary, the 60CS40P_BMP* sample at 21 days presents an increased ALP value compared to both 60CS40P_BMP and 80CS20P_BMP. The same behaviour was also detected for Osteocalcin expression (OCN) where the highest level was observed for scaffold with BMP2 mimic-peptide at day 21. This different behaviour could be explained by considering the difference in the ratio between the chitosan free amino groups ($-NH_2$), and the induced carboxylic groups of BMP2 (-COOH). In fact, even if the cumulative release profiles showed no significant differences between the tested samples, the effect of the bioactivation appears in early osteogenic differentiation. This behaviour suggests that, by increasing the $COOH/NH_2$ ratio, a positive effect on cellular behaviour is achieved. This trend can be explained by the specific interaction of peptide with the receptor on the cellular membrane which has significant effect on the

hMSC differentiation in the osteoblast phenotype. The phenomenon demonstrated that CS-PEGDA scaffold was a good carrier of peptide allowing a prolonged release over time, and maintained the local concentration of BMP2 mimic peptide at a good level, thus overcoming the drawbacks of some polymers used as carriers for the therapeutic agents delivery [59]. However, all samples showed significant positive effects on hMSC behaviour in terms of proliferation and osteogenic differentiation in basal medium, as evidenced by the expression of early (ALP) and later signals (OCN) of osteogenic differentiation in biomimetic and non-biomimetic scaffolds. Results have demonstrated that biomimetic scaffolds showed higher expression of OCN level and the best result was obtained for the scaffold bioactivated with BMP mimic-peptide, specifically for 60CS40P_BMP*. Several clinical studies reveal that inflammation is a relevant disease as relates to osteoporosis , characterised by the production of inflammatory mediators including cytokines, histamine, bradykinin, prostaglandins, leukotrienes, PAF, biological response modifiers and the family of cell adhesion-promoting molecules [60]. Thus, immunological dysfunctions are determinant factors in the development and progression of osteoporosis and osteoarthritis. In fact pro-inflammatory cytokines such as (TNF)-α, interleukin (IL)-1β and interleukin (IL)-6 are elevated in pathological bone conditions and are responsible for cartilage and bone destruction [60]. Here we investigated the effectiveness of bioactive chitosan-based-scaffold on immune response modulation. To this end, not only pro-inflammatory cytokines such as interleukin (IL)-1β but also anti-inflammatory cytokines such as IL-10 and stable metabolites of nitric monoxide as oxidative stress markers were assessed. Our studies suggested that bioactivated scaffolds were able to direct osteoinductive processes by preventing immune response induction. However, the best results in terms of anti-inflammatory effect were observed in biomineralized 80CS20P scaffold. The biomineralized 80CS20P scaffold demonstrated a complete profile as an anti-inflammatory agent inducing the inhibition of nitrite production (oxidative stress markers) in LPS-treated hMSCs accompanied by reduction of IL-1β (pro-inflammatory cytokine), while the biomineralized 80CS20P scaffold induced IL-10 production (anti-inflammatory cytokine) promoting bone formation. The engineering of bone tissue offers new therapeutic strategies for the treatment of many diseases, such as osteoporosis. Many scaffold constructs have been employed in the development of tissue-engineered bone; however, an active blood vessel network is an essential requisite for these to survive and integrate with existing host tissue. In recent years, generous attention was paid to processes such as angiogenesis, osteogenesis and

tissue engineering strategies, which could offer future therapeutic opportunities for skeletal repair and regeneration. For this purpose, the aim of this work was to assess, as preliminary study, the effect of different CS-based materials on angiogenesis. Angiogenesis is a complex multistep process consisting in the formation of new blood vessels from an existing vasculature. Furthermore, angiogenesis involves the degradation of extracellular matrix proteins and activation, proliferation, migration of endothelial cells and pericytes. It plays a pivotal role in physiological processes involving neovascularization, such as ovulation, placentation and embryogenesis. In addition, angiogenesis is correlated to several pathological processes, such as tumour growth and metastasis. A great interest was given to the study of molecules which can affect the process of angiogenesis. To this end, human vein endothelial cell (HUVEC) based *in vitro* models were used. In fact, we have used the HUVEC cell line as our model in order to study the potential effect of different CS samples bioactivated by different approaches on angiogenesis. In particular, the study is based on the evaluation of the effect of CS porous scaffolds on HUVECs proliferation and viability. The angiogenic property of CS scaffolds composed of 70 % CS and 30 % PEGDA was evaluated in terms of cell proliferation, migration and tube formation. Angiogenesis was monitored *in vitro* by measuring the growth and migration of human vein endothelial cells (HUVECs) with media conditioned by CS-based scaffolds. Indeed, the work reports the *in vitro* cytotoxicity assessment of CS scaffold through live/dead staining of HUVECs. First, live/dead test was done as preliminary study in order to assess the cytotoxic effect of these samples on HUVECS. Results obtained from this study suggested that both neat and bioactivated scaffolds were not cytotoxic for HUVECs. Indeed, the materials do not compromise cell viability. Furthermore, HUVECs morphology changed in the presence of CS-BMP-2. This behaviour could be explained by the activation of many proteins involved in cell differentiation. Literature on the subject holds that BMP-2 stimulates endothelial cells promoting differentiation and tube formation. Indeed, BMP-2 induces phosphorylation of Erk-1/2, Smad 1/5, and increases Id1 expression. Erk-1/2 regulates several critical cellular functions in endothelial cells including proliferation and tube formation with expression of VEGF, epidermal growth factor, fibroblast growth factor, and angiogenin [61]. Additionally, many papers reported that BMP-2 and their receptors promote hyperpermeabilization of HUVECs by inducing internalization and c-SrC-phosphorylation of VE-cadherin which causes an increase in endothelial cell permeability [65]. Therefore, these data suggested that all CS materials were not toxic for cells and

that BMP-2 changes HUVECs morphology by stimulating the activation of cells and angiogenesis process. Starting from these data, our aim was to assess *in vitro* proliferation of HUVECs on all CS-based-scaffolds at 1, 3 and 7 days of cell culture. Results obtained by cell proliferation suggested that all CS scaffolds (with and without bioactivation) supported cell proliferation over culture time. The values for CS and CS biomineralized scaffolds were lower than for the control plate, because we compared two different systems: 2D (HUVECs) and 3D (scaffolds). Moreover, CS biomineralized scaffolds showed a higher proliferation than CS neat. This behaviour, probably, was due to a potential differentiation in HUVECs [63]. However, the preliminary results demonstrated the potential role of CS scaffold in the angiogenesis process in terms of HUVEC migration and tube formation. In fact, all CS materials conditioned media with Matrigel component were able to induce tubes formation. However, chitosan-BMP2 conditioned medium can also induced tubes formation without Matrigel after 18 hours of culture time. As reported in literature [64], bone Morphogenetic Protein-2 (BMP-2) has a crucial role in angiogenesis. In fact, it is highly over-expressed in the majority of patient-derived lung carcinomas and enhances the neovascularization of developing tumours. In fact, BMP-2 induced tube formation in both HAEC and HUVECs. Moreover, BMP-2 also stimulated proliferation of HAEC. The ability of BMP-2 to activate endothelial cells was further demonstrated by its ability to phosphorylate Smad 1/5/8 and ERK-1/2 and to increase the expression of Id-1 [65].

6. Conclusions

This study has demonstrated the possibility of producing biomimetic structures with highly interconnected and homogeneous structures with pores of different sizes from about 20 μm to 300 μm in agreement with the dimensional characteristics required for bone regeneration scaffold. The proposed technique holds strong potential for applications in the field of custom-made bone substitute. Moreover, the presence of bioactive signals on the scaffold surface allows an osteoinductive effect on hMSC to be obtained. Furthermore, the anti-inflammatory effects of bioactivated scaffold on a model of bone inflammation make the proposed scaffold a promising candidate for material for bone tissue regeneration as it also prevents a foreign body reaction (FBR). Finally, the materials also have a desirable effect on angiogenic response in terms of HUVECs

proliferation, cell migration and tube formation. In future studies, the synergic effect of combining organic and inorganic approaches to bioactivation could enhance every signal.

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CHAPTER III

Injectable ionic liquid-hydroxyapatite based biocomposites for bone defects

1. Introduction

An ideal device aimed at regenerating injured bone should possess a dual function as to stimulate the newly forming bone tissue and prevent bacterial infections. At present devices that are able to inhibit implant infections associated with biofilm formation have received considerable attention in the field of orthopaedic surgery [1]. This chapter describes the possibility of developing a multifunctional injectable material with antimicrobial and osteoinductive properties by using Ionic Liquids (ILs) as antimicrobial agents.

1.1. Ionic liquids

Ionic liquids are large organic cations, such as *imidazolium* or *pyridinium*, with alkyl chain substituents, which alter the molecules' hydrophobicity. This class of chemicals is convenient for many applications, including the substitution of traditional industrial solvents and eradication of a wide range of microorganisms thanks to its antimicrobial activity. Particularly, ionic ILs are composed by cationic derivatives of the neutral imidazole heterocycles combined with (in) organic anions; this combination confers to these compounds a remarkable flexibility appropriate for designing systems with excellent physicochemical and biological properties. For this purpose, ILs can be applied as antimicrobial, antifungal, antitumour, antioxidant and anti-fibrous agents [2]. In addition, ILs can be used as solvents, catalysts in both industrial and academic sectors and can be combined with a wide variety of anions (inorganic or organic) thus considered task specific, because of their ability to be applied in a variety of manners. Substantially ILs, defined as green compounds, represent an alternative to common organic solvent due to low vapour pressures. In addition, several studies demonstrated the beneficial effects of ILs due to their features as vanishingly low vapour pressures, liquid state at ambient condition, and inability to evaporate as air pollution control [3]. Several studies reported that the introduction of a cleavable ester functional group in the side chain leads to a

significant increase in the biodegradability of the ILs molecules in comparison to those containing simple alkyl chains [4]. Long-chain *imidazolium* and *pyridinium* based ionic liquids consist of a charged hydrophilic head group and hydrophobic tail and consequently possess an inherent amphipathic nature. Previously, *imidazolium* and *pyridinium* derivatives with long alkyl chains have shown surface activity and biological activity. Also, ILs functionalized with ester chemical group seem to promote their biodegradability [5]. In the present study, ionic based liquids containing different carbon chain lengths were synthesized in order to investigate effects on cellular behaviour and antimicrobial activity. This research is expected to contribute to the further development of biodegradable improved ionic liquids as multifunctional compounds as well as to an assessment of their fate in the environment. Current investigations are focused on the antibacterial and antifungal effects of ILs-based compounds. Notably, these recent studies have demonstrated that an increase in the alkyl chain substituent in *pyridinium* or *imidazolium* leads to bacterial toxicity. In fact, it is possible to observe a higher toxicity in a wider spectrum of bacteria by increasing alkyl carbon chain. This toxic effect of several *pyridinium* and *imidazolium* compounds is explained by the hydrophobicity of the cation [6] and 1-n-hexadecyl-3-methylimidazolium methanesulfonate (C_{16}MImMeS) have been identified as a good antimicrobial choice in hospital practice due to their effectiveness in inhibiting Candida biofilm formation when compared to chlorhexidine [7]. According to previous studies, ILs based compounds inhibit the biofilm formation of Staphylococcus Epidermidis and Aspergillus Niger in addtion to Candida Albicans thus resulting to be highly effective in therapies for keratitis [8]. The design of bone substitutes must combine antimicrobial and anti-inflammatory substances with bone precursors and it's a considerable task. In order to achieve good osteogenic activity without the complications associated with bacterial infections many attempts have been made using a simple surface modification with biomaterials that mimic bone components [9]. Experimental evidences of P. Aeruginosa *in vitro* and *in vivo* have clearly demonstrated that biofilm bacterial cells are significantly more resistant to antibiotics and host immune defence than their planktonic counterparts. Aggressive and intensive antibiotic treatment is usually helpful in controlling the exacerbations of chronic biofilm infections induced by dispersed bacteria and reducing the biofilms, but cannot eradicate biofilm infections, because the minimal concentration of antibiotic for the eradication of mature biofilm is difficult to reach *in vivo*.

1.2 Biofilm formation

Once a bacterial biofilm infection established, it becomes difficult to eradicate. Bacterial biofilm formation (Figure 1) is widely found in natural environments with water, and in human diseases, especially in the patients with indwelling devices for the purpose of medical treatments. With the progress of medical sciences, more medical devices and/or artificial organs are applied in the treatment of human diseases. However, as a consequence, bacterial biofilm infections have also become frequent. It is reported that most of medical devices or prostheses which include intravenous catheters, vascular prosthesis, cerebrospinal fluid shunts, prosthetic heart valves, urinary catheters, joint prostheses and orthopaedic fixation devices, cardiac pacemakers, peritoneal dialysis catheters, intrauterine devices, biliary tract stents, dentures, breast implants, contact lenses and in the dental area caries and periodontitis, and so on may result in biofilm infections, [10]. Additionally, there are also biofilm infections not associated with foreign bodies, such as chronic airway infections in cystic fibrosis (CF) patients or patients with chronic obstructive pulmonary diseases, native valve endocarditis, chronic otitis media, chronic sinusitis and chronic (diabetes) wound infections. It has been estimated that most bacterial infections in humans are correlated with biofilm and about 50% of the nosocomial infections are indwelling device associated. Bacterial biofilms are characterised as highly resistant to antibiotic treatment and immune responses. Although it is well-known that antibiotic treatment is currently the most important and effective measure for the control of microbial infections, however, it is almost impossible to eradicate biofilm infections with antibiotics. *In vitro* and *in vivo* experiments have demonstrated that the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) for biofilm bacterial cells were usually much higher (approximately 10–1 000 times) than for planktonic bacterial cells. The effective antibiotic MBC *in vivo* for biofilm eradication is therefore impossible to reach by conventional antibiotic administrations due to the toxicities and the side effects of antibiotics and the limitation of renal and hepatic functions [11]. Treatment of biofilm infections has become therefore challenging and has attracted significant scientific attention. Numerous clinical investigations have been performed, which for the benefit of the control of biofilm. The work is focused mainly on the clinical treatment of bacterial biofilm infections with injectable materials based on calcium phosphate and ionic liquids. In this context, tissue-engineering based strategies for bone regeneration suggest the incorporation of various therapeutic agents such as antimicrobial, anti-inflammatory drugs and osteoinductive molecules in injectable

biomaterials [12]. Injectable biomaterials' employment ensures advantages as compared to the use of non-injectable materials because of their ability for drug delivery thus promoting antibiotic and growth factors' local release. Moreover, injectable biomaterials allow bone regeneration in a minimally invasive manner thanks to their remarkable plasticity [12]. Currently, the sol-gel method represents an effective technique for producing injectable bone substitutes and encapsulating therapeutic agents simultaneously. The method is very interesting because it allows encapsulating a wide class of thermosensitive compounds in biomaterials because precursors are mixed at lower temperatures than traditional methods [13]. Furthermore, organic-inorganic hybrid materials can be realized by using sol-gel approach. In particular, the sol-gel approach is very suitable for the synthesis of hydroxyapatite (HA)-based injectable materials due to the possibility to mix at molecular-level calcium and phosphorous precursors in order to obtain calcium phosphate (CaP) materials [14]. CaP based biomaterials are similar to bone in composition and form a uniquely strong interface with bone. Consequently, CaP triggers the newly forming bone as a precursor of biological apatite [15]. In general, biofilm forming on surface implant induces resistance to antibacterial agents, thereby contributing to bone destruction [16]. Indeed, the prevention of bacterial colonization and biofilm formation recieves considerable attention. It is evident from numerous studies of different systems that the use of antiseptic surface coatings is one approach to reduce the incidence of implant infection. Many previous investigations suggest implant modifications including the covalent attachment of polycationic groups, impregnating or loading nanoparticles with antimicrobial agents such as antibiotics, and coating the surfaces with quaternary ammonium compounds, iodine, and silver (Ag) ions [17].

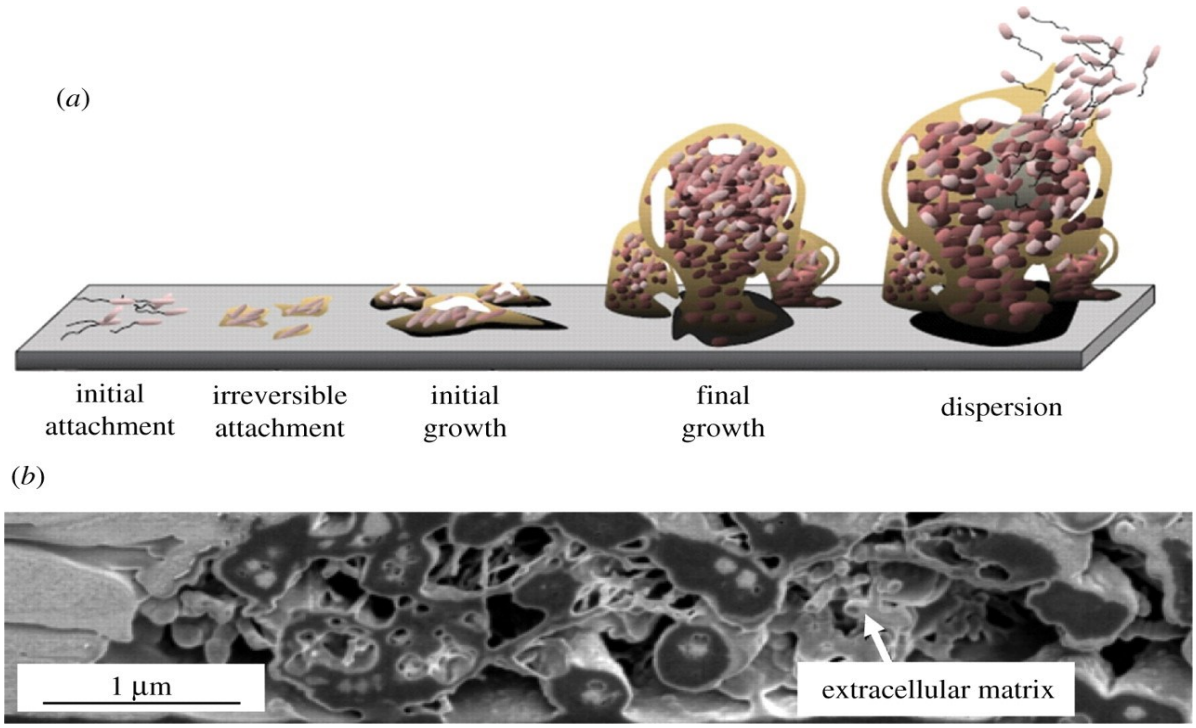


Figure 1. Microbial biofilm formation.

1.3 Calcium phosphate-ionic liquids injectable gels and tissue engineering applications

An ideal bone substitute is aimed at regenerating injured bone should possess a dual function: to stimulate the newly forming bone tissue and to prevent bacterial infections. Many current researchers give more attention to those devices that are able to inhibit implant infections associated with biofilm formation in the field of orthopaedic surgery [8]. The structured consortium attached on living or inert surfaces formed by microbial cells and surrounded by the self-produced extracellular polymeric matrix is known as biofilm. Biofilm formation is considered an adaptation of microbes to hostile environments [8]. A typical development of biofilm includes several stages, *i.e.*, attachment to a surface; formation of microcolonies; development of biofilm; differentiation of structured mature biofilm, and dispersal of mature biofilm.

Biomaterials that possess both excellent osteogenic and antibacterial properties are thus crucially needed to overcome implant failure. Several approaches have been attempted in order to obtain antibacterial and osteogenic effects by using biomaterials functionalized with antimicrobial and osteoinductive agents. For example, Zn/Ag co-implanted titanium showed both excellent osteogenic and antibacterial activities and has strong potential for orthopaedic implants [9]. Currently, within these new approaches chemical engineers have been synthetizing a wide class of ionic liquids (ILs) as antimicrobial agents. Implant failures occur due to implant-associated bacterial infections and inflammation, which inhibit adequate osseointegration [8]. Furthermore, local bacterial infections may induce systemic pathologies, thus increasing the costs of hospitalization for patients. In fact, the costs for bacterial infection care raise the cost of implants used in many orthopaedic surgical procedures and complicate health management [10]. A higher toxicity in a wider spectrum of bacteria was observed. This toxic effect for several pyridinium and imidazolium compounds is due to the hydrophobicity of the cation [11]. In order to obtain antibacterial and osteoinductive effects, ILs can be combined with bone precursors. Recently, several studies have demonstrated that some biomaterials such as Poly (lactic acid) PLA can be improved by adding 1-methyl-3-n-pentylimidazolium and 1, 2-dimethyl-3-n-pentylimi-dazolium ionic liquids on the surfaces. In the context of Candidemia, ILs-based materials showed antifungal activity against isolates of C. Tropicalis, C. Parapsilosis, C. Glabrata, and Trichosporon Asahii and these beneficial effect depend largely on the N-alkyl chain-length. Among these compounds, 1-n-

hexadecyl-3-methylimidazolium chloride (C_{16}MImCl) and 1-n-hexadecyl-3-methylimidazolium methanesulfonate (C_{16}MImMeS) have been identified as an excellent antimicrobial choice for hospital practice, due to their effectiveness in inhibiting Candida biofilm formation when compared to chlorhexidine [12]. According to previous studies, ILs based compounds inhibit biofilm formation not only for C. Albicans, but also for Staphylococcus Epidermidis and Aspergillus Niger, therefore resulting to be highly effective in the therapy for keratitis [13]. In the design of bone substitutes, it is important to combine antimicrobial and antinflammatory substances with bone precursors. In order to achieve good osteogenic activity without the complications associated with bacterial infections, many attempts have been made using a simple surface modification of biomaterials, which mimic bone components [14]. Tissue-engineering-based strategies for bone regeneration suggested the incorporation of various therapeutic agents, such as antimicrobial, anti-inflammatory drugs and osteoinductive molecules in injectable biomaterials [10]. The use of injectable biomaterials ensures advantages as compared to the use of non-injectable materials, because of their ability for drug delivery, as well as promoting antibiotic and growth factors local release. Moreover, these injectable biomaterials allow minimally invasive bone regeneration, thanks to their remarkable plasticity [10]. Nowadays, the sol-gel method represents an effective technique for producing injectable bone substitutes and for encapsulating therapeutic agents simultaneously. This method also allows encapsulating in biomaterials a wide class of thermosensitive compounds, because precursors are mixed at lower temperatures than with traditional methods [11]. Furthermore, organic-inorganic hybrid materials can be realized using the sol-gel approach. Particularly, the sol-gel approach is very useful for the synthesis of hydroxyapatite (HA)-based injectable materials, due to the possibility of mixing at molecular-level calcium and phosphorous precursors in order to obtain calcium phosphate (CaP) materials [12]. It is known that CaP based biomaterials are similar to bone in composition, forming a uniquely strong interface with bone. On this basis, CaP triggers the newly formed bone as a precursor of biological apatite [13]. Generally, in implant infections biofilm induces resistance to antibacterial agents, thereby contributing to bone destruction [14]. Hence, the prevention of bacterial colonization and biofilm formation has garnered considerable attention. It is evident from numerous studies of different systems that the use of antiseptic surface coatings is one approach to reduce the incidence of implant infection. Many previous investigations suggest implant

modifications, including the covalent attachment of polycationic groups, impregnating or loading nanoparticles with antimicrobial agents, such as antibiotics, and coating the surfaces with quaternary ammonium compounds, iodine, and silver (Ag) ions [15]. The main goal of orthopaedics is the capacity for pain-relief as derived from function restoration and the correction of patients' deformities through less invasive techniques. Pain is an important consequence of inflammation and infection induced by body-implant surface interaction. Innovation in the orthopaedic material sector can solve this issue, even if these new procedures might cause an increase in healthcare costs [16]. In order to obtain a multifunctional product able to inhibit infection and, at the same time, to induce osteogenic activity, in the present study we developed CaP based injectable materials containing a variety of 1-n-alkyl-3-methylimidazolium chlorides ILs (CaP, CaP_C_4, CaP_C_{10}, CaP_ $(C_{10})_2$, CaP_C_{16}) obtained with the sol-gel method. Specifically, the present work suggests the possibility of combining osteogenic enhancement and antibacterial ability in a single injectable material. The importance of this combination will be decided by its impact on future studies focused on the synthesis of more complex medical devices for bone tissue regeneration and the simultaneous prevention of biofilm formation.

2. Aim

The aim of second part of this thesis was to develop injectable biomaterials consisting of ionic liquid loaded hydroxyapatite obtained with the sol-gel approach. The effect of ILs alkyl-chain length on crystallization of hydroxyapatite were tested. Biological effects in terms of osteogenic differentiation of hMSC and microbial proliferation were also evaluated.

3. Materials and Methods

3.1 Synthesis of nanostructured IL-loaded calcium phosphates

The CaP/IL gel injectable systems were obtained at room temperature using sol-gel technique. For the sol-gel synthesis, calcium nitrate tetrahydrate Ca $(NO_3)_2 \cdot 4H_2O$ (99% A.C.S. Reagent, Aldrich) and ammonium phosphate dibasic $(NH_4)_2HPO_4$ (A.C.S. reagent, Aldrich) were chosen as precursors of Ca^{2+} and PO_4^{3-}, respectively with a molar ratio Ca/P in the range 1.5-1.67. Before the synthesis process, IL $[C_nMI_m]Cl$ (1-n-alkyl-3-methylimidazolium) with different single chain-lengths (C_n= $C_nH_{(2n+1)}$; n= 4, 10, 16) and double chain $(C_{10})_2MI_mCl$ were dried under vacuum for 3 hours at 60°C and dissolved in a solution of distilled water (dH_2O). Particularly, C_4MI_mCl and $C_{10}MI_mCl$ were completely dissolved in dH_2O, in the meantime $C_{16}MI_mCl$ and $(C_{10})_2MI_mCl$ were dissolved in a mixture of dH_2O:DMSO (1:1), in order to obtain a complete dissolution of ILs. When $Ca(NO_3)_2 \cdot 4H_2O$ was completely dissolved in dH_2O, $[C_nMI_m]Cl$ solution at a different composition (1 and 2wt%) was added to Ca^{2+} solution and stirred for 2 hours at 40°C. Then, a PO_4^{3-} solution was added dropwise to the system and pH was adjusted at alkaline value (pH equal to 11) by NH_4OH. Gel transition took place after 1 hour of magnetic stirring at room temperature; furthermore, it was observed that the presence of $[C_nMI_m]Cl$ induces shorter gelification times in comparison with the neat CaP system. After gelification, an ageing step of 2 days at 60°C for all systems with and without $[C_nMI_m]Cl$ was performed.

3.2 Morphological characterisation: TEM analysis

The morphological distribution of ILs in CaP matrix and their effect on nanoparticle size were evaluated by Transmission Electron Microscopy (TEM) investigation. The analysis

was performed by a TEM FEI Tecnai G12 Spirit Twin model instrument operated at an accelerating voltage of 100 kW. Samples for TEM imaging were prepared by placing a drop of the aged IL/CaP suspensions (the IL-CaP gels were dissolved in dH_2O and the obtained suspensions were diluted and dispersed by ultrasonic waves before use on carbon coated copper grids, dried in air and loaded into the electron microscope chamber).

3.3 Antifungal and Antibacterial tests

-Antifungal tests

The Candida Albicans (ATCC 14053) and Candida Tropicalis (ATCC 13803) strains were grown in Sabouraud agar plates for 24 hours at 36.6 °C. Aliquots of 150 µL of 106 colony-forming units per millilitre (CFU mL^{-1}) were diluted in to 5mL of saline solution and then aliquots of 100µL of the second solution were again diluted to 2mL of RPMI medium. For the antifungal tests 500µL of the third solution were added to 500µL of a suspension (1 mg mL-1 in RPMI) of the investigated materials and incubated for 5 hours at 36.6 °C. The percentage calculation of surviving fungi was done by CFU counting after seeding 20 µL (drop method) of each test in Sabouraud agar plates, which were incubated for 24 hours at 36.6 °C.

-Antibacterial tests

A single colony of E. coli DH5α was taken from an agar plate grown overnight (18-20hours) at 37 °C and transferred to 5 mL of Luria Bertani (LB) broth (containing 10 $g{\cdot}L^{-1}$ peptone and NaCl and 5 $g{\cdot}L^{-1}$ yeast extract) and incubated overnight. The bacteria concentration was then determined by turbidimetry (OD_{600}) and the solution was diluted to 108 CFU.mL^{-1}. The antibacterial tests were performed via the overnight incubation of 980 µL of LB broth, 20 µL of the 108 CFU.mL^{-1} bacteria solution and 1000µL of the materials' aqueous suspension (1 mg.mL^{-1}). The pure substance controls were performed by the addition of 1000µL of a 20ug.mL^{-1} of each ionic liquid. After incubation, the OD_{600} was measured for the positive control (bacteria growing without any material or substance) and all the tests were diluted to obtain a concentration of 2000 CFU mL^{-1} based on the control. From the diluted solutions, 100µL were seeded to LB agar plates using automated plating equipment (Easy Spiral Dilute Interscience) and incubated

overnight at 37°C. The antibacterial effect was evaluated by CFU counting that was performed using *ImageJ* software.

4. Biological properties

4.1 *In vitro* cell culture

Biological assays were performed using human Mesenchymal Stem Cells line (hMSC) obtained from LONZA (Milano, Italy). hMSC were cultured in 75 cm^2 cell culture flask in Eagle's alpha Minimum Essential Medium (α-MEM) supplemented with 10% Foetal Bovine Serum (FBS), antibiotic solution (streptomycin 100 µg/ml and penicillin 100U/ml, Sigma Chem. Co) and 2 mM L-glutamine, without osteogenic factors. hMSCs at passages from 4 to 6 were used for all the experimental procedures and incubated at 37°C in a humidified atmosphere with 5% CO_2 and 95% air.

4.2 Cell adhesion and proliferation

Cell adhesion was evaluated at day 1 using a quantitative colorimetric test, such as Alamar Blue assay. hMSCs (5,000 cells/well) were re-suspended in 50ul medium and plated on the surface of sterilized gel materials with and without ILs at different concentrations (1 and 2wt%). After 2 hours of cell culture, 500µl of medium was added and the gel materials were incubated overnight at 37°C. After this time, the medium was replaced with 500 µL of Alamar Blue solution (obtained by dilution 1:10 of alamarBlue™ reagent in DMEM w/o red-phenol) and added to each well and incubated for 4 hours at 37°C, 5% CO_2. Then, 200µL of solution was transferred into a 96-well plate, in order to evaluate the absorbance at 540 and 600nm (Victor X3 spectrophotometer, Perkin Elmer). Wells without any cell were used to correct any background interference from the redox indicator. The cell proliferation at longer intervals (3, 7, 14 and 21 days) was evaluated by quantifying the DNA amount by using PicoGreen dsDNA quantitation assay. PicoGreen® dsDNA Quantitation Reagent is an ultra-sensitive fluorescent nucleic acid stain used to quantify double-stranded DNA (dsDNA); fluorescence was measured in black 96-well plate at 480 nm. The dsDNA amount was determined by a standard calibration curve according to the manufacturer's protocol. The culture medium during the experiment was changed every two days.

4.3 Alkaline Phosphatase and Osteocalcin expression as markers of osteogenic differentiation

The effect of IL-CaP gels on osteogenic differentiation of hMSCs was evaluated by measuring early and later markers of differentiation such as Alkaline Phosphatase (ALP) and Osteocalcin (OCN), respectively. The phosphatase activity levels were tested on cell lysates (50µl) by measuring the activity of ALP enzyme, which catalyses the cleavage of a phosphate group and releases p-nitrophenol from p-nitrophenyl phosphate using the SensoLyteTM pNPP Alkaline Phosphatase Assay Kit (AnaSpec, DBA, Italy), according to the manufacturer's instructions. The absorbance was measured in a 96-well plate at 405 nm on a plate reader to determine enzyme concentrations per construct at 3 and 7 days. The ALP values were corrected for the number of cells present on each material. In that case, double stranded DNA (dsDNA), used as a marker for cell number, was measured by using a PicoGreen_dsDNA quantification kit (Invitrogen, USA). The ALP results were reported as nanograms of ALP normalised to the micrograms of total DNA content (ng ALP/µg DNA). Furthermore, Osteocalcin (OCN) levels were measured at days 14 and 21 using a commercially available kit (Quantikine Human Osteocalcin Immunoassay R&D system, Italy), following the manufacturer's instructions.

4.4 Effect of IL-loaded CaP gels on inflammatory response

The effect of IL-CaP gels on *in vitro* experimental models of inflammation and oxidative stress was also tested. To this end, hMSCs were plated onto scaffolds and tissue culture plate at density of $1x10^4$ cells/well in 96 multi-well plates. After 24 hours, inflammation was induced by using LPS at concentration of 1 µg/ml. At day 3 of stimulation interleukin-10 (IL-10) levels in cell supernatants were quantified using commercial ELISA kits (Affymetrix, eBioscience Srl, Milano), according to the manufacturer's instructions. Nitrite levels (stable metabolites of nitric monoxide) were analysed by using Griess assay. For this purpose, 100 µl of supernatant was transferred into a 96-well plate and an equal volume of Griess reagent was added to each well. The samples were incubated for 1 hour at room temperature and the absorbance was detected at 550 nm using a microplate reader (VICTORTM X3 Multilabel Plate Reader, PerkinElmer). For ROS assay, hMSCs were plated in 96-well black costar plates (Corning, USA) at the density of 1×10^4 cells/well and cultured onto materials for 72 hours at 37°C. The cells

cultured on tissue culture plate were used as a control. After washing, cells were incubated for 1 hour with 200µl of 100 µM H2DCF-DA in HBSS containing 1% FBS. Then cells were incubated with Fenton's reagent (H_2O_2/Fe^{2+} 2 mM) for 3hours at 37°C. The intracellular ROS levels were expressed as fluorescence intensity, which was detected using a fluorescent microplate reader (excitation 485 nm and emission 538 nm).

4.5 Statistical analysis

All quantitative experiments were performed in triplicate and the results were expressed as mean ± standard deviation (SD). Statistical analysis of the data was conducted using one-way ANOVA. Differences between the groups with $p < 0.05$ were considered statistically significant.

5. Results

5.1 Morphological Investigation: TEM analysis

TEM investigations (Figure 2) showed changes in the nanoparticle size of the HA/IL systems and demonstrated the important role of the IL-structure in HA morphology. IL interacts with the growing particles through the hydrogen bond "*co-π-π stacking*" mechanism, which creates an IL-layer on the HA surface. Therefore, differences in the size, geometry, polarity and Coulomb coupling forces between ILs' anions and cations, contribute directly to the final HA particle size and morphology. In addition, the hydrophobic tail-tail interactions of the IL with longer cation side-chains caused the formation of agglomerates larger than those in shorter alkyl chains.

5.2 X-ray diffraction

Figure 3A shows the XRPD patterns of the investigated HA/IL systems: the blue curves are the experimental data, the grey curves are the Rietveld fits and the red curves are the difference between the experimental and calculated profiles. The qualitative analysis of each pattern has been carried out by the program QUALX2 [17] that allowed us to identify the crystalline phases that match the diffraction patterns: Hydroxyapatite (HA,

Ca_{10} $(PO_4)_6(OH)_2$), Monetite ($CaHPO_4$) and Brushite ($CaHPO_4(H_2O)_2$). Through the QUANTO [18] program, performing Rietveld fits of the XRPD data, a quantitative determination of all the crystalline phase weight fractions for each sample was achieved (reported in Figure 3B).

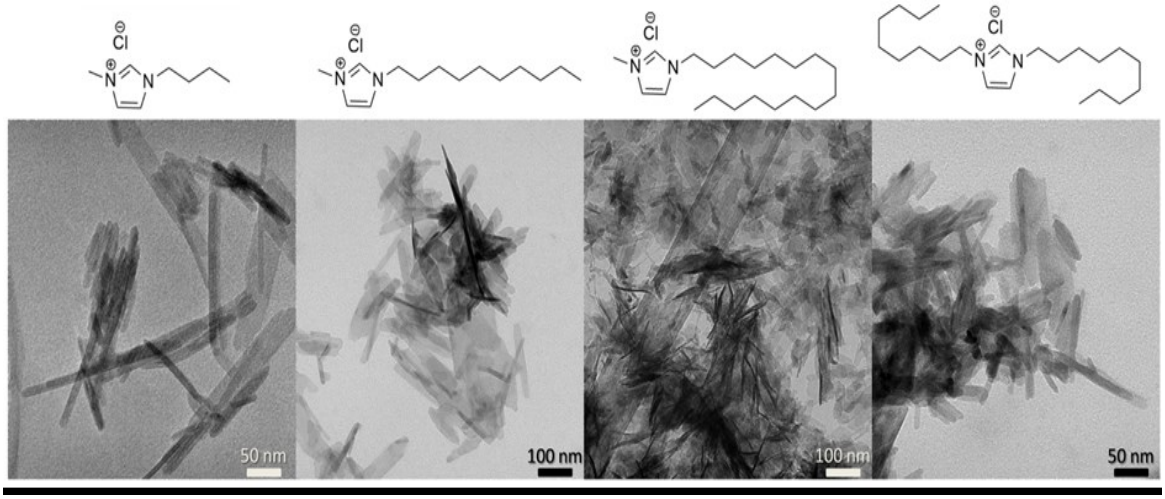
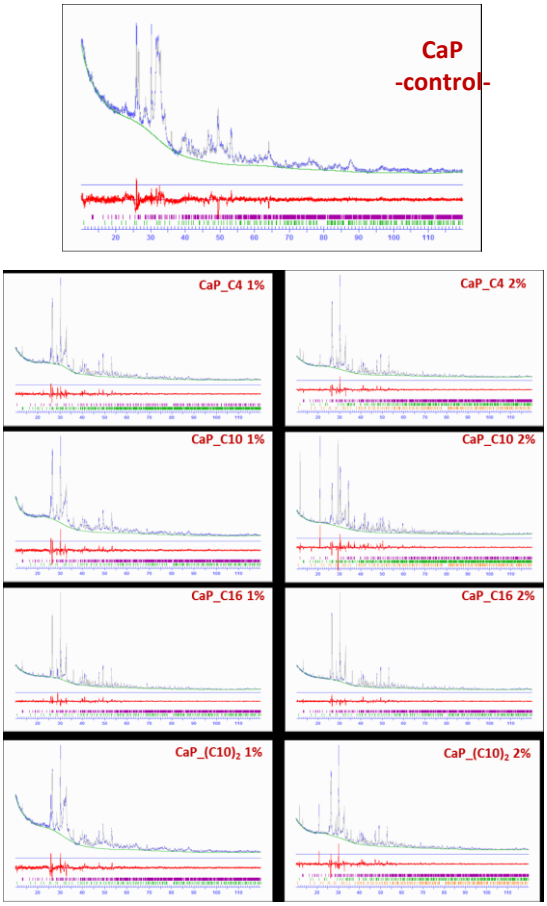
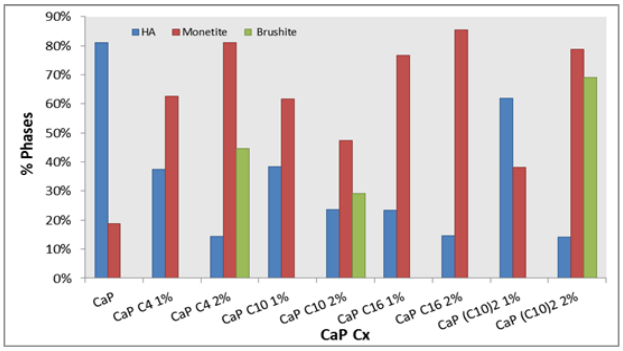


Figure 2. TEM analysis of nanoparticles of HA with ILs at different carbon chain lengths**.**



(A)



	HA [$Ca_{10}(PO_4)_6(OH)_2$]	Monetite [$CaHPO_4$]	Brushite [$CaHPO_4(H_2O)_2$]
CaP	81.149%	18.851%	---
CaP_C4 2%	37.47%	62.53%	---
CaP_C_4 2%	14.411%	81.134%	4.455%
CaP_C_{10} 1%	38.366%	61.634%	---
CaP_C_{10} 2%	23.532%	47.288%	29.180%
CaP_C_{16} 1%	23.419%	76.581%	---
CaP_C_{16} 2%	14.664%	85.336%	---
CaP_$(C_{10})_2$ 1%	38.059%	61.941%	
CaP_$(C_{10})_2$ 2%	14.258%	78.839%	6.903%

(B)

Figure 3. (**A**) Diffraction patterns in CaP-ILs powders. (**B**) Quantitative determination of all the crystalline phase weight fractions.

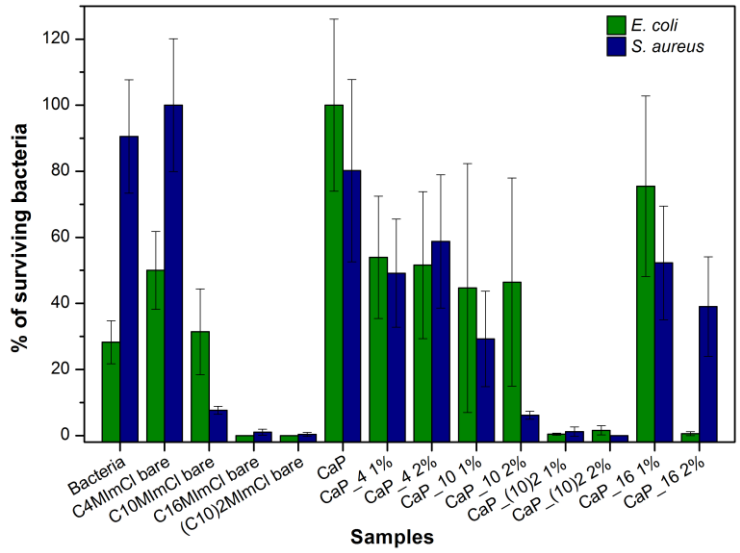
5.3 Antimicrobial investigation

5.3.1 Antifungal and Antibacterial tests

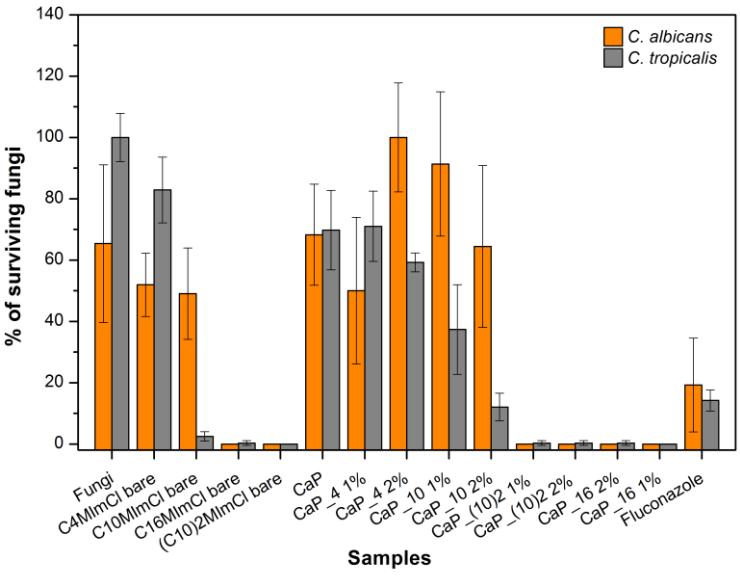
The antimicrobial study of fungi and bacteria highlighted the antifungal activity on *C. Albicans* and *C. Tropicalis* of the biocomposites with $C_{10}MI_mCl$, $C_{16}MI_mCl$ and $(C10)_2MI_mCl$. At the same time, antibacterial tests showed positive properties for use in materials but the best performance in bacteria proliferation inhibition was obtained with $(C_{10})_2MI_mCl$ based biomaterial on *E. Coli* (Figure 4A-B).

5.3.2. Biofilm formation

SEM analysis (Figure 5) demonstrated the presence of biofilm formation on CaP alone and CaP with short-chain ionic liquid with no trace of biofilm on CaP with long alkyl chain as observed in CaP_C_{10};CaP_C_{16}; and CaP_$(C_{10})_2$.



(A)



(B)

Figure 4. (**A**) Antifungal and (**B**) antibacterial tests.

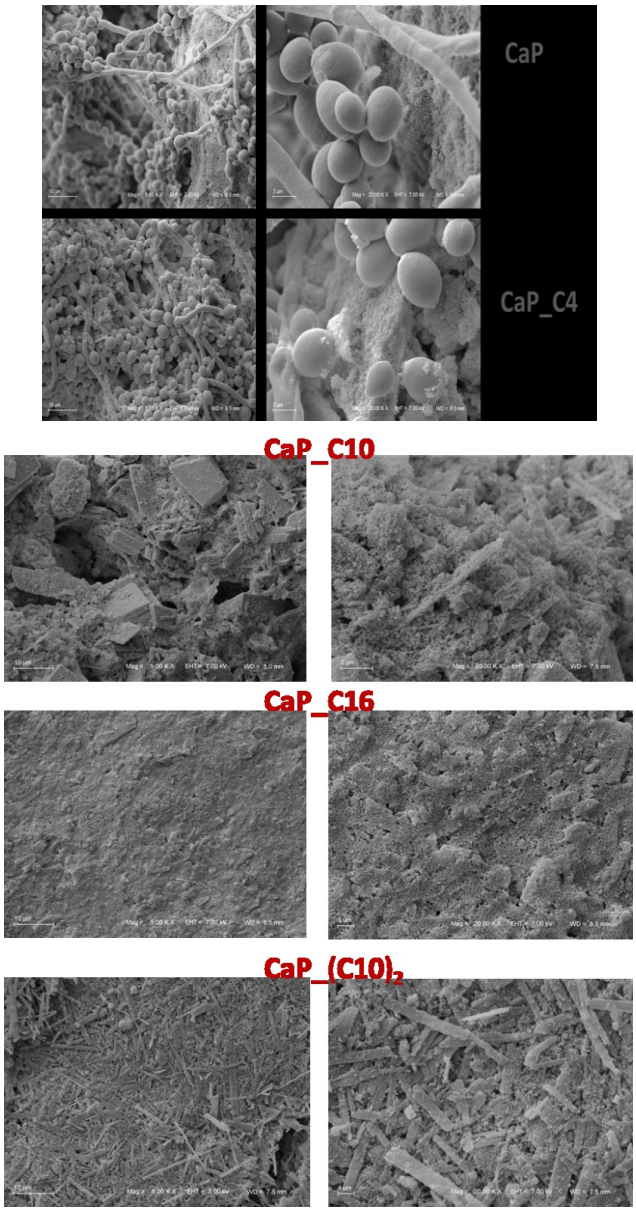


Figure 5. SEM images of biofilm formation in CaP system.

5.4 Biological studies

The effect of IL-loaded calcium phosphate gels on the cell behaviour was evaluated through some investigations concerning adhesion, proliferation, osteogenic differentiation and inflammatory response. Biological analyses were performed using human mesenchymal stem cells (hMSC), undifferentiated cells with the capability to differentiate into many cell phenotypes (i.e. fibroblast, osteoblasts, muscle, chondrocytes). In the research process, our interest was in evaluating the differentiation of hMSC in osteoblast phenotypes. Particularly, in order to evaluate the osteoinductive effect of IL-CaP materials on human cells, experiments in basal medium without the presence of osteogenic factors such as ascorbic acid, dexamethasone and β-glycerophosphate, were performed.

5.4.1 Cell adhesion and proliferation

The results demonstrated that the presence of ILs in the CaP matrix improves the metabolic activity and cell attachment in the first 24hrs of culture time with the best results for ILs with longer and double chain as C_{16}MImCl and $(C_{10})_2$MImCl, respectively (Figure 6A). An increasing of cell proliferation over longer culture time for unloaded-CaP and ILs-loaded CaP was obtained, however unloaded-CaP shows higher values than loaded-CaP (Figure 6B).

5.4.2 Alkaline Phosphatase and Osteocalcin expression as markers of osteogenic differentiation

ILs-loaded CaP showed higher value on ALP expression than CaP at 3 and 7 days. In the meantime, a decrease in ALP value at 14 and 21 days was observed (Figure 7A). The later osteogenic marker as Osteocalcin (OCN) was also analysed. The results demonstrated that ILs-loaded CaP showed higher values of OCN than unloaded-CaP, particularly, the highest values were observed for $C_{16}MI_mCl$ and $(C_{10})_2MI_mCl$-loaded CaP at day14. C_4MI_mCl(2wt%)-loaded CaP showed OCN levels comparable to unloaded CaP; this behaviour might be due to an greater increase in cell proliferation and extra culture time for the two materials as compared to each other (Figure 7B).

5.5 Effect of IL-loaded CaP gels on inflammation response

To assess the effect of gel materials on inflammatory response through oxidative stress and anti-inflammatory cytokine, investigations were performed. LPS (1µg/ml for 72h) stimulation caused a significant increase on nitrite level production with highest values for unloaded-CaP system (Figure 8A). In the meantime, the presence of ILs of short and long chain significantly reduces the nitrites production. The lowest values were obtained for CaP loading ILs at 1wt% with short chain (C_4 and C_{10}), whereas high values were obtained for $(C_{10})_2$MImCl (2%)-loaded CaP. Moreover, a reduction in ROS levels was also observed for all gels compared to LPS stimulation (Figure 8B). The lowest values were obtained for CaP loading ILs at 1wt% with short chain (C_4 and C_{10}) confirming their different behaviour in comparison with the others. The inhibitory effect of the ILs-loaded CaP on nitrites and ROS production in LPS-treated hMSCs was combined with an increase in IL-10 levels (Figure 8C) for CaP loading C_{10}MImCl and C_{16}MImCl (2wt%).

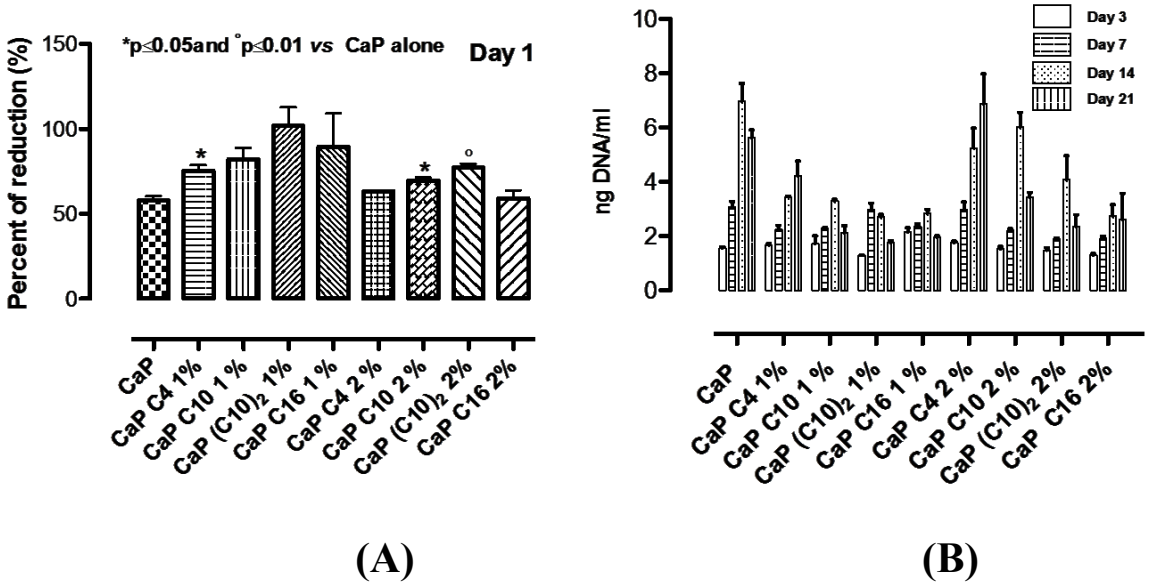
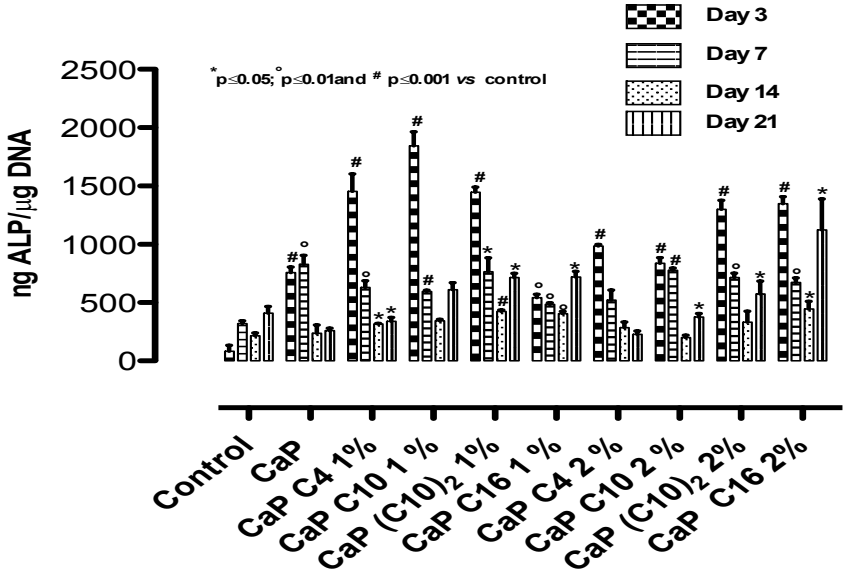
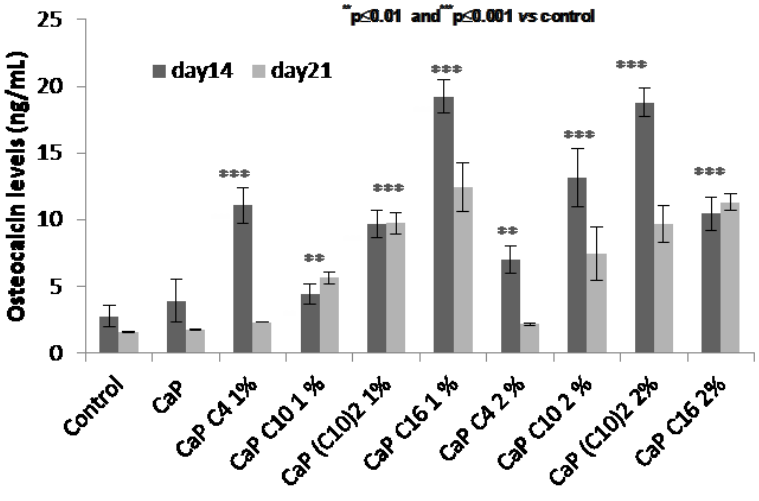


Figure 6. Cell proliferation at day 1 **(A)** and 3, 7, 14 and 21 **(B)** days of cell culture.



(A)



(B)

Figure 7. ALP expression at 3, 7, 14 and 21 days (**A**) and osteocalcin expression at 14 and 21 days of cell culture (**B**).

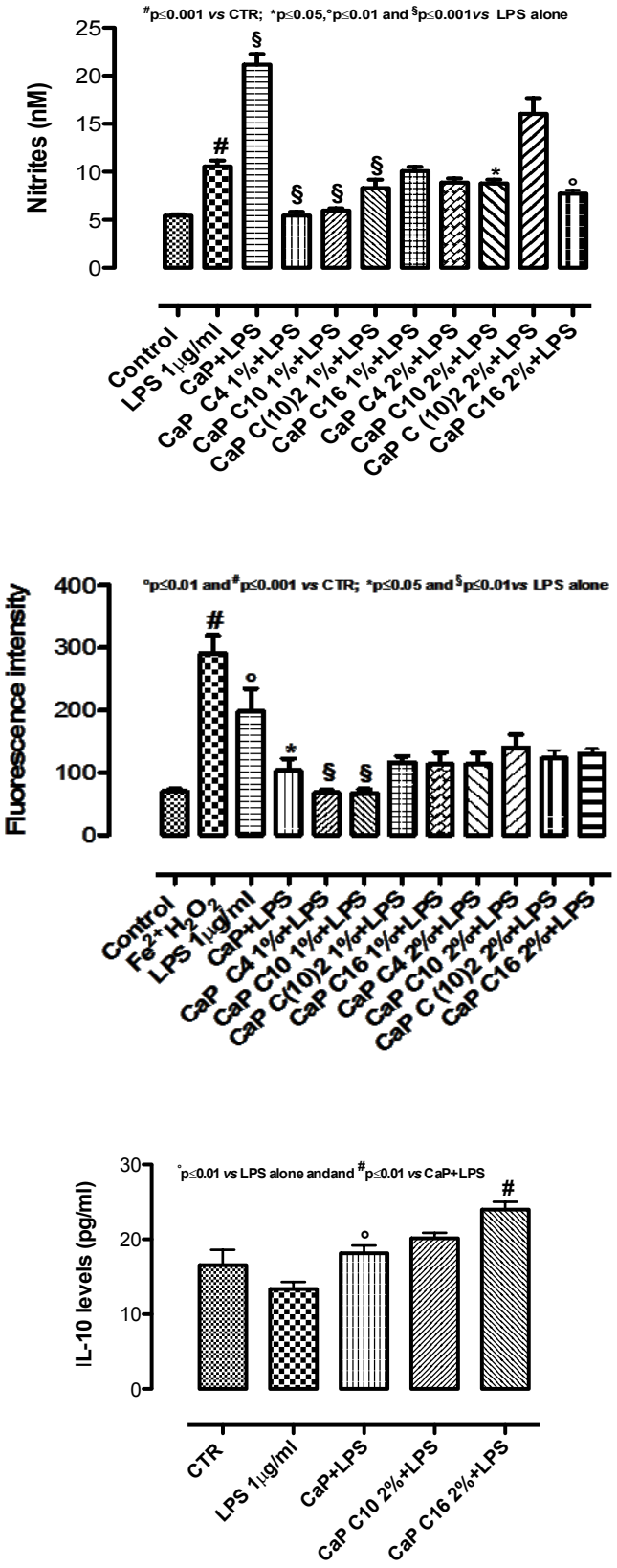


Figure 8. Effects of CaP-ILs plus ionic liquids on anti-inflammatory markers [nitrites **(A)**, ROS **(B)**, IL-10 **(C)** levels].

6. Discussion

Infections associated with *in vivo* implants represent the most severe postsurgical problems of medical device implants, including prosthetic joints (i.e. hip, knee and shoulder) and fracture fixation [20]. The increasing ageing population and increased participation in recreational activities propels growing demands for surgical implantation, which is associated with a continuously increasing of number of infections [21]. These infections are usually produced by microorganisms, which grow in specific structures, known as biofilms [22]. In this case, the pathogenesis of implant-associated infection involves interaction between microorganisms, implant and host. Foreign bodies remain devoid of microcirculation, which is crucial for host defence and delivery of antibiotics. The life within a biofilm represents a basic survival mechanism by which microbes resist against external and internal environmental factors, such as antimicrobial agents (i.e. silver nanoparticles, antibiotics) and the host's immune system [23]. Appropriate diagnosis of infections associated with implants is an important challenge, and recognized infections may not be effectively treated with long-term systemic antibiotic therapy [24]. Consequently, postsurgical infections often require additional surgical procedures such as debridement, prosthesis removal and re-implantation with increasing costs for healthcare systems. The systemic antibiotic treatment increases several issues, such as systemic toxicity, low efficiency and need for hospitalization [25, 26]. Given this context, extended delivery of antimicrobial agents at the site of implantation is highly desirable in order to offer high local antibiotic concentration without systemic toxicity and thereby prevent postoperative, implant-associated infections. However, microbial biofilms are resistant to a variety of antimicrobial agents, including antibiotics and industrial antiseptic biocides. On this basis, in this work the possibility to load Ionic Liquids (ILs) in calcium phosphate (CaPs) materials at different percentages (1-2wt%) and at different alkyl chain lengths (starting from 4 to 16 carbon atoms at single chain and 10carbon atoms at double chain) to obtaining antimicrobial and antifungal biocomposites was demonstrated. CaP based materials were chosen because they are commonly used in orthopaedic and dental surgery as bone void fillers [27]. In particular, Hydroxyapatite (HAP), β-tricalcium phosphate (β-TCP) and biphasic calcium phosphate (BCP) represent typical examples of CaPs minerals, which are known to integrate well with surrounding bone tissue and facilitate bone tissue growth [28]. Moreover, CaPs are generally considered to play a structural role in bone regeneration, acting as a barrier to soft tissue infiltration and as facilitators of the onset and growth of new bone tissue. On

the other hand, all CaP-based compounds are, to different extents, degradable in the physiological environment. However, no conclusive evidence exists yet to explain exactly how free calcium (Ca^{2+}) or inorganic phosphate ions released from these materials play a direct role in the biological responses to these materials [29]. This is in a part because the process of dissolution/reprecipitation of a ceramic is not dependent on one, but on a multitude of material characteristics, including CaP crystal phase and specific surface area. In our work, the loading of ILs at different chain lengths during hydroxyapatite sol-gel synthesis had some effect on morphology and nanoparticles sizes. In fact, morphological investigations by TEM analysis (Fig.1) demonstrated the presence of crystal nanoparticles and nanoplates, which increases in size by increasing ILs chain length. The nanoplates are typical of the monetite phase, which was determined by XRD analysis (Fig.2). X-ray results demonstrate an increasing amount of monetite amount when alkyl chain is increased that causes a reduction of HA phase. Furthermore, the alkyl-chain length and the amount of ILs affect cellular behaviour in terms of osteogenic differentiation. Osteogenic differentiation of the cells was first assessed using ALP enzyme activity. ALP is considered an early marker of osteogenesis that is known for its ability to convert extracellular pyrophosphate, a mineralization inhibitor, into inorganic phosphate [30]. To evaluate the effect of monetite on early and later osteogenic differentiation, we have also plotted the ALP and OCN markers concentrations (ng/mL) in function of monetite %. In this case, it has been evaluated how ALP and OCN expressions change in relation to monetite % for different biocomposites at different alkyl chain lengths and ILs amounts (Figure S1-S3). In detail, at an early time interval (day 3) a correlation between alkyl-chain length and amount of ILs with ALP expression was not observed (Supplementary Information Figure S1). In Fig. S2 (Supplementary Information) we reported the ALP expression, monitored on all the samples after 14 days of incubation, for ILS amounts of 1% (empty circles) and 2% (full circles). We reported the same plots twice as, on the left, we wanted to show how, at day 14, the ALP expression increases with the alkyl-chain (red arrows); while on the right Fig. S2 shows that the ALP expression increases with the ILs amount with the exception of CaP_C4 (yellow arrow), CaP_C10 (blue arrow) and CaP_$(C10)_2$ (red arrow) biocomposites, which show lower ALP levels. However the drop after day 14 is possibly reflective of the cells entering a matrix maturation phase and mineralization, in fact these biocomposites show an increase in OCN expression at the same point in time (Supplementary Information Figure S3). The linear correlation of OCN expression and alkyl-chain length and ILs

amount was observed at day 21, where a stable material was obtained. The effect of ILs on hMSC osteogenic differentiation was due to the presence of monetite phase in synthesized biocomposites. Danoux *et al*, [31] have reported that monetite nanoplates show osteoinductive properties due to higher surface area and roughness than HA nano-crystals. Di Silvio *et al* [32] demonstrated the possibility of producing monetite-based scaffolds able to facilitate differentiation of hMSCs towards osteogenic differentiation in the absence of external additives, that is, non-osteogenic-conditioned medium. Furthermore, the antimicrobial studies confirmed the antifungal activity (*C. Albicans*, *C. Tropicalis*) of the biocomposites with $C_{10}MI_mCl$, $C_{16}MI_mCl$ and $(C10)_2MI_mCl$. In the meantime, results on the antibacterial properties of the materials suggested that the best response on bacteria proliferation inhibition was obtained with $(C_{10})_2MI_mCl$ based biomaterial on *E. Coli*. The bone implant in the osteoporotic area can also induce an inflammatory response associated with microorganism infections. Therefore, the aim of this work was to study the effect of these biocomposites on inflammation processes using *in vitro* experimental models. We evaluated the effect on two different markers of oxide stress, such as nitrite levels and ROS production and quantification of anti-inflammatory cytokines, such as IL-10 levels. Results suggested that all the system composed of calcium phosphate and ionic liquids reduced nitrite levels when compared to the usage of calcium phosphate alone. Moreover, the materials also induced a reduction in ROS levels, such as the control plate. This anti-inflammatory effect was confirmed by the evaluation of IL-10 level quantification, which was higher for calcium phosphate alone compared to lower levels of LPS. At the same time, the materials induced a much better expression of IL-10 than the control plate.

7. Conclusion

In the present study, our aim was to evaluate the effect of CaP-ILs injectable materials (which could be defined as multitasking) on the osteogenesis process. Indeed, these materials provide a positive response to hMSC and microorganisms proliferation. Furthermore, as systems composed of calcium phosphate and ionic liquids can be synthesized at room temperature with the sol-gel approach, it is therefore, possible to conclude that ionic liquid-hydroxyapatite based biocomposites possess antimicrobial,

osteoinductive and anti-inflammatory properties with better performance obtained in systems composed of longer alkyl chain lengths.

Supplementary Information

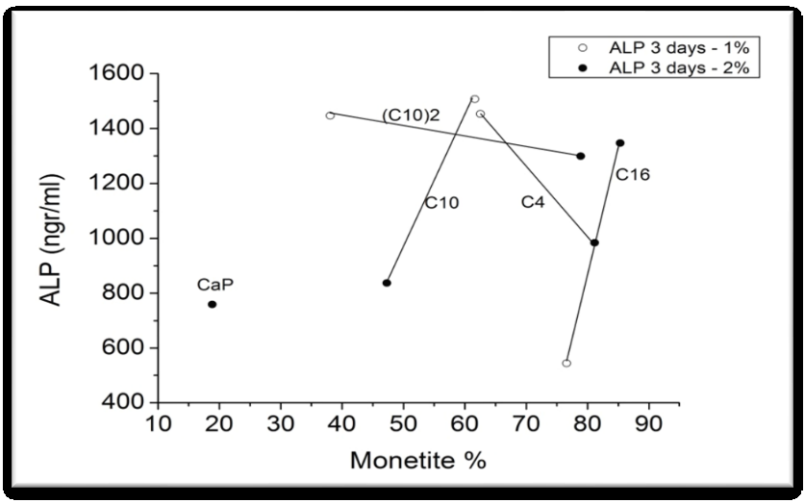
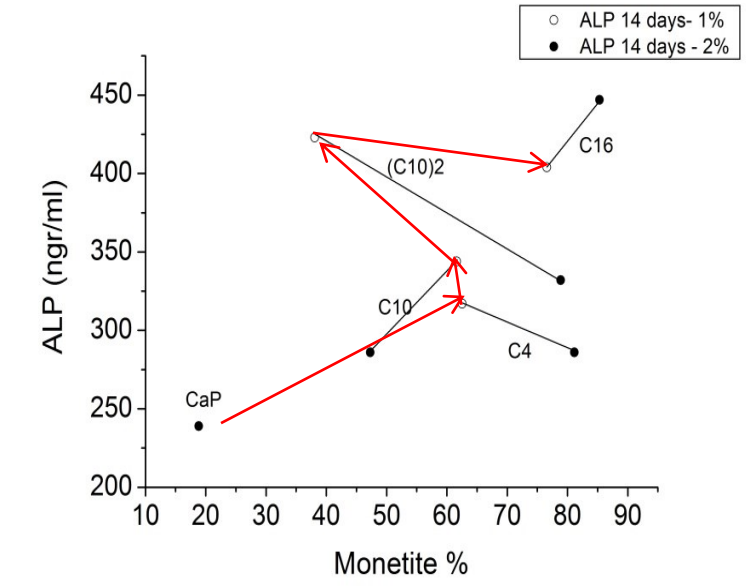
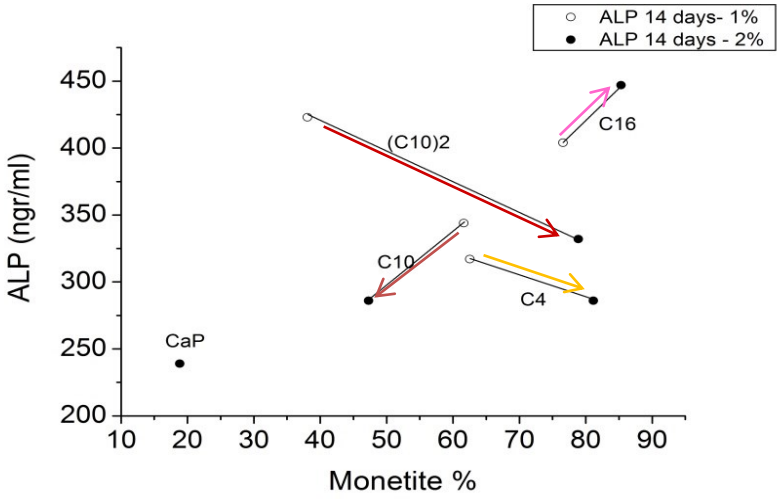


Figure S1: Evaluation of ALP expression at day 3 demonstrated by increasing n-alkyl chain length. ALP does not increase. However higher levels of ALP than compared to CaP alone were observed in presence of C_4, C_{10} and $(C_{10})_2$ at 1 % of ILs. In contrast to CaP plus ILs, the highest values of ALP were observed in presence of C_{16}.

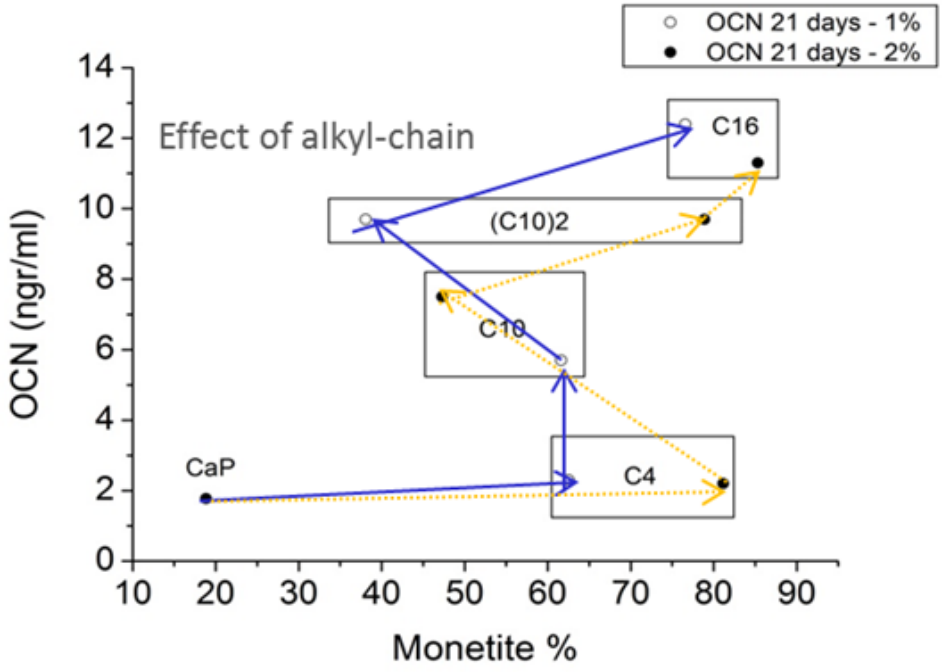


(A)

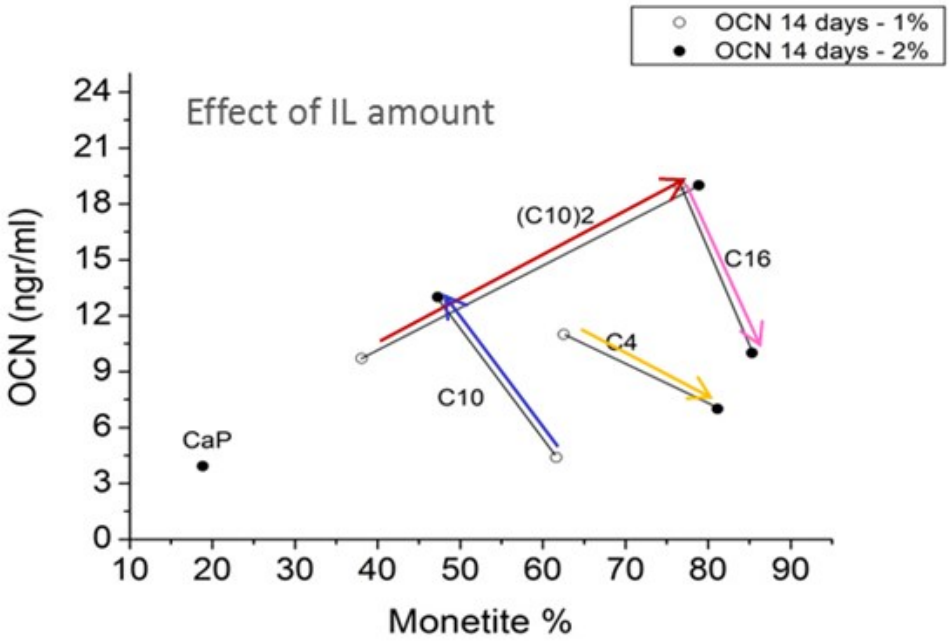


(B)

Figure S2: The evaluation of ALP expression showed that by increasing n-alkyl chain length (C_4, C_{10} and C_{16}) ALP values increase (**A**). Moreover, at day 14 ALP for C_{16} increases not only with the alkyl chain but also with increasing the amount of ILs (2%) (**B**). Meanwhile for C_4, C_{10} and $(C_{10})_2$ increasing ILs decreases the amount of ALP expressions.



(A)



(B)

Figure S3: Evaluation of Osteocalcin (OCN) production after 21 days of cell culture. Demonstrated that increases in n-alkyl chain length increases OCN values. (**A**). In contrast at day 14 OCN levels increase when the ILs amount increases as well (2%) only for C_{10} and $C_{(10)2}$ (**B**).

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CHAPTER IV

Conclusions and Future Perspectives

Tissue engineering strategies are based on the combination of cells, bioactive factors and biomaterials for tissue regeneration. Osteoporosis, the most common bone pathology, is characterised by an imbalance between bone resorption and formation resulting in a high risk of fractures. Thus, the aim of the present thesis was to evaluate the *in vitro* effect of different biomaterials on bone regeneration in terms of osteogenesis and inhibition of inflammation and infection as related to osteoporosis processes in order to identify new alternatives to conventional osteoporosis treatments that cause several systemic side effects. Biological studies on these materials leads to the conclusion that our scaffolds may be a source of a new kind of implants with a potential therapeutic activity in bone defects derived from pathological bone conditions such as osteoporosis. In fact, the materials proved capable of regenerating damaged bone tissue. The present study concerned the bioactivation and the development of two different systems: chitosan-based scaffolds bioactivated with organic and inorganic signals and injectable CaP based materials combined with ionic liquids, at different carbon chain lengths. Results on CS-based scaffolds, obtained through biomimetic treatments, suggested the possibility of using these materials for bone tissue regeneration thanks to their high interconnectivity, homogeneous structure, and pores with different dimensions from 20 μm to 300 μm. The presence of bioactive signals allows for osteoinductive effect on hMSC, making chitosan-based scaffolds a promising material for bone tissue regeneration. Indeed, bioactive and biocompatible chitosan scaffolds promote proliferation and induce differentiation of hMSC thus representing a new clinical prospect in the field of biomedical applications. Furthermore, the combination of chitosan-based scaffolds with different bioactive signals (inorganic and organic) leads to the development of a new class of multifunctional biomaterials in tissue engineering field. Besides, the ability of chitosan-based materials to inhibit inflammatory response and promote the angiogenesis process emphasizes the possibility of making use of this scaffold material as promising candidate for bone regeneration. In conclusion, the present investigations on chitosan materials, not only reveal new and interesting opportunities to obtain substitutes for a complete bone tissue regeneration but they also underline their preliminary effect on the angiogenesis process. Beginning with this evidence, future perspectives could continue the study of

bioactivated-based scaffolds in an *in vitro* co-culture system, composed of osteoblast-like phenotype and endothelial cells, in order to mimic *in vivo* conditions. Meanwhile, the second part of this thesis was based on the synthesis of injectable ionic liquids-hydroxyapatite (CaP-ILs) based biocomposites by sol-gel approach. They represent an advantageous method that allows operating at room temperature while fabricating biocomposites with high levels of osteoinductivity, excellent antimicrobial activity, bioactivity and full injectability. In other words, our interest was to investigate the effect of these biocomposites on cellular behaviour in terms of proliferation and osteogenic differentiation. Materials showed high biocompatibility without cytotoxic effect and a positive effect on cell attachment. Furthermore, results demonstrated that multitasking CaP-ILs gels have a simultaneous opposite responses toward hMSC and microorganisms proliferation inhibition. Particularly, data showed that the loading of ILs at different chain lengths during hydroxyapatite sol-gel synthesis had effect on morphology and nanoparticle sizes with the formation of monetite phase. The presence of monetite increases with the alkyl chain length had effect on biological properties. In fact, the best performances, in terms of early and late osteogenic differentiation of hMSC were obtained in the presence of systems at longer alkyl chain lengths CaP_C_{16} and CaP_$(C_{10})_2$. Hence, CaP-ILs systems appear to be very useful for regenerating bone tissue and reducing microbial infections associated with bone implants. In conclusion, all results obtained by testing chitosan-based scaffolds and injectable CaP-ILs systems in *in vitro* models of remodelling bone tissue make it a possibility to translate these preclinical results to future clinical trials.