· U C ·

UNIVERSIDADE DE COIMBRA

Kateline Cristina Mota Piedade

Influence of vancomycin controlled release from heparinized collagen/nanophased hydroxyapatite granules on osteoblast and osteoclast cells

Dissertation submitted to the Faculty of Pharmacy of Coimbra University, as part of the requirements for the Master Degree in Pharmaceutical Biotechnology and under the supervision of Professor Dr. Fernando Jorge Mendes Monteiro from Faculdade de Engenharia da Universidade do Porto (FEUP) and co-guided by Professor Dr. Sérgio Paulo de Magalhães Simões from Faculdade de Farmácia da Universidade de Coimbra.

September, 2014

“Aprender na vida, aprender junto do nosso povo,
aprender nos livros e nas experiências dos outros.
Aprender sempre."

Amilcar Cabral

Acknowledgements

I am using this opportunity to acknowledge everyone who supported me throughout the course of this project.

I would like to express my gratitude to my supervisors Prof. Dr. Fernando Jorge Monteiro and Prof. Dr. Susana Sousa for the opportunity, guidance, constructive criticism and friendly advices through the learning process of this master thesis. I would also like to thank my master thesis external guide Prof. Dr. Sérgio Simões.

I am very thankful to Nilza Ribeiro, who kindly shared her precious time helping and guiding me in a critical step of my work. My sincere thanks also goes to Catarina Coelho for all the patience, guidance, advices and friendship. These words are not enough to say how thankful I am as you helped me in most part of this work. Thank you so much.

I also want to thank Liliana Grenho for helping me with microbiology subjects and Joana Barros for the availability to help me with bioactivity assays. To Angela Carvalho and Marta Ribeiro for the friendship. Without all these people, an important part of my work would not be possible. Thank you to all members of Biocomposites group for the friendship and kindness.

Foremost, I would like to express my sincere gratitude to Prof. Dr. Maria Helena Fernandes for the opportunity to develop part of my work at FMDUP and to Dr. João Rodrigues, also from FMDUP, for all the help, guidance and know-how with cell cultures. I am very thankful.

From FMUP, I would like to thank Dr. Nuno Alegrete for the opportunity to assist him in the *in vivo* studies.

From INEB, I want to acknowledge Ricardo Vidal for all the technical help and also to Dr. Paula Magalhães for the help with NanoDrop 2000 spectrophotometer.

From IBMC, I want to thank Dr. Paula Sampaio for all the support related with CONFOCAL analysis.

From CEMUP, I acknowledge Daniela Silva for all the help related with SEM analysis.

From FEUP, I express my gratitude to Dr. Aurora Futuro for the sieves she kindly lent me.

From UFP, I want to thank Prof. Maria Pia Ferraz and the technician Ricardo Silva for all their assistance during the work I developed at CEBIMED laboratory.

I greatly acknowledge Fluidinova S.A. for the provision of nanoHA powder.

I want to acknowledge my friends for all the friendship and support they gave me along this journey. A special thanks to Maira Etelvino who put up with me through the whole

process, for all good advices in the worst moments and for being such a good friend since forever. I want also to thank Romine Semedo for the help and support, for all the kindness and friendship along these years. Thank you to Lídia Dias for being always caring about my work and for all the wise advices. Your friendship is priceless.

I would like to thank to my Portuguese family, José Plácido Santos, Maria Filomena Santos, Tiago and Andreia, for having welcomed me so warmly, for the unconditionally support, for being always worried about my well-being and for making everything easier so I could dedicate myself to this project. I will be grateful forever for your love.

To my mom Margarida, my dad Carlos and my little sister Karen, who have encouraged my academic interests from day one, I want to express my eternal gratitude for the big support, for always believe in me and never let me give up on my purpose, even though being so far. To all my family for the support during this journey, especially to my grandfather Pedro Mota and my grandmother Maria Piedade Mota. This work is dedicated to you.

Last but not the least, I would like to thank to José Diogo Santos, who has become a rock of stability in my life in the past years, for all the patience, for being so understanding and supportive. And most of all, for keeping me harmonious and helping me putting pieces together.

Abstract

Bone is one of the most dynamic tissues of the human body, characterized by its great ability to self-regenerate and self-remodel after injury. Bone tissue can be exposed to some diseases that compromise its self-renewal, such as osteomyelitis.

Osteomyelitis is a bone infection that can be caused by many species of microorganisms, being *Staphylococcus aureus* (*S. aureus*) the most commonly isolated organism. Vancomycin is an important glycopeptide antibiotic known by its efficacy against *S. aureus* and thus, frequently used in cases of osteomyelitis. Although vancomycin is able to eradicate the bacteria, it has no ability to induce bone regeneration itself, so a combined treatment including a biomaterial which has the ability to induce tissue regeneration would be a good strategy.

Nanohydroxyapatite/collagen (nanoHA/collagen) composites have been used for bone regeneration because of its similarity to bone, bioactivity, biodegrability and osteoconductivity and, thereforem, may stimulate bone regeneration. Taking advantage of these properties, nanoHA/collagen composites can be used as a controlled Drug Delivery System by associating them with heparin. Heparin is a linear polysaccharide produced in mast cells that can control the diffusion of vancomycin from the scaffold.

The main purpose of this work was to assess bone cells behavior, namely osteoblasts and osteoclasts, cultured in contact with vancomycin continuously released from heparinized nanoHA/collagen granules. Based on different renewal kinetics, a sustainable release of antibiotic from the granules in PBS was confirmed and the amount of vancomycin released was enough to inhibit bacterial growth, remaining below MTC values for *S. aureus*. In both cell type tested, vancomycin did not reveal to be cytotoxic but instead it seemed to have cytostatic effect, according to TRAP and ALP activity assay performed. Nevertheless, TRAP and ALP activity results revealed that heparinized nanoHA/collagen granules mimetize bone tissue better than all other materials tested once cells were able to adhere, proliferate and differentiate even after being exposed to vancomycin effects. Therefore, heparinized nanoHA/collagen composite might be a good choice regarding osteomyelitis treatment as they are capable of inhibit pathogen growth, promote osseointegration and tissue regeneration.

Keywords: Osteomyelitis, *S. aureus*, vancomycin, nanohydroxyapatite, collagen, heparin, osteoblasts, osteoclasts.

Resumo

O osso é um dos tecidos mais dinâmicos do corpo humano, caracterizado pela sua capacidade regenerativa e de remodelação após lesão. O tecido ósseo pode ser alvo de doenças que o destroem e comprometem a sua auto-regeneração, como por exemplo a osteomielite.

A osteomielite é uma infeção que afeta o tecido ósseo, tendo como principal causador da doença a bactéria *Staphylococcus aureus* (*S. aureus*). A vancomicina é um importante antibiótico pertencente à classe dos glicopéptidos, bem conhecido pela sua eficácia contra *S. aureus* e, portanto, frequentemente utilizado no tratamento da osteomielite.

Compósitos de nanohidroxiapatite (nanoHA) e colagénio têm sido utilizados na regeneração óssea graças à sua semelhança com o osso, bioactividade, biodegradação e osteocondutividade. Tendo em conta essas propriedades, compósitos de nanoHA e colagénio podem ser utilizados como sistemas de libertação controlada, associados à heparina. A heparina é um polissacarídeo linear produzida pelos mastócitos e é utilizada, neste caso, para controlar a difusão da vancomicina a partir do scaffold.

O principal objetivo deste trabalho era avaliar o comportamento de células ósseas, nomeadamente osteoblastos e osteoclastos, em contacto com a vancomicina continuamente libertada a partir de grânulos de nanoHA e colagénio heparinizados. Baseando-se em diferentes cinétcas de renovação de fluidos, confirmou-se uma libertação controlada do fármaco dos granulos em PBS , e a quantidade libertada mostrou-se suficiente para inibir o crescimento bacteriano, permanecendo abaixo dos valores considerados tóxicos. A vancomicina não revelou efeito citotóxico em nenhum dos tipos celulares testados. Ao invés disso, revelou um aparente efeito citostático. Apesar disso, os granulos heparinizados demonstraram mimetizar melhor o osso do que os restantes materiais testados, uma vez que as células revelaram uma melhor aderência, proliferação e diferenciação na presença desses grânulos. Portanto, grânulos de nanoHA/colagénio heparinizados poderão ser uma boa escolha no tratamento da osteomielite, uma vez que demonstraram ser capazes de inibir o crescimento bacteriano e, ao mesmo tempo, promover osseointegração e possível regeneração óssea.

Palavras-chave: Osteomielite, *S. aureus*, vancomicina, nanohydroxyapatite, colagénio, heparina, osteoclastos, osteoblastos

Contents

List of figures

List of tables

Abbreviations

α-MEM – alpha Minimum Essential Medium

Abs – Absorbance

ALP – Alkaline phosphatase

ATR-FTIR – Attenuated Total Reflectance Fourier Transformed Infrared

BSA – Bovine Serum Albumin

CFU – Colony-forming Unit

CLSM – Confocal Laser Scanning Microscopy

EDC – N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide

DAPI - 4',6-Diamidino-2-phenylindole dihydrochloride

DDS – Drug delivery system

dsDNA – double-strained DNA

ECM – Extracellular matrix

FBS – Fetal Bovine Serum

HA – Hydroxyapatite

HSC – Hematopoietic Stem Cell

NAG – N-acetylglucosamine

NAM – N-acetylmuramic acid

M-CSF – Macrophage Colony Stimulating factor

MES - 2-mofpholinoethane sulfonic acid

MIC – Minimum Inhibitory Concentration

Micro-CT – X-Ray Micro-Computed Tomography

MRSA – Methicillin-resistant *Staphylococcus aureus*

MSC – Marrow Stem Cell

MSCRAMM – Microbial surface components recognizing adhesive matrix molecules

MTC – Maximum Toxic Concentration

NA – Nutrient Agar

nanoHA – Nanohydroxyapatite

NHS – N-hydroxysuccinimide

NO – Nitric Oxide

OPG – Osteoprotegerin

PBMNC – Peripheral Blood Mononuclear Cells

PBS – Phosphate Buffer Solution

pI – Isoelectric point

pNPP – p-Nitrophenyl Phosphatase

PTH – Paratiroid hormone

PU - polyurethane

RANK - Receptor activator of nuclear kB

RANKL – Receptor activator of nuclear kB Ligand

RT – room temperature

SEM – Scanning Electron Microscopy

TAE – Tris-acetate EDTA

TCPS – Tissue culture Polystyrene

TGF-β– Transforming Growth Factor beta

TSB – Tryptic Soy Broth

TNF – Tumor Necrosis Factor

TRAP – Tartrate-resistant acid phosphatase

TSST-1 – Toxic Shock Syndrome Toxin 1

VRSA – Vancomycin- resistant *Staphylococcus aureus*

Chapter 1: Introduction

Bone is one of the most dynamic tissues of the human body, characterized by its high vascularization, rigidity, hardness and great ability to self-regenerate and self-remodel after injury without leaving a scar. (1-3) This tissue is constantly in adaptation to preserve skeletal integrity and to regulate mineral homeostasis.

Bone tissue serves a variety of functions from structural support for the rest of the body, which allows movement and locomotion, protection of vital internal organs such as lungs, brain and heart, maintenance of pH balance, to mineral reservoir for calcium, phosphorus growth factors and cytokines for balanced metabolism and also by providing a suitable environment for hematopoiesis in bone marrow. (4, 5) Therefore, due to all the functions described above, body balance can be considerably disturbed by any change in bone composition, structure or function.

1.1. Bone matrix composition

Bone matrix is mainly constituted by two different components:

- The inorganic component, consisting of calcium phosphate mineral apatite - essentially hydroxyapatite crystals (70 wt%) - which is responsible for bone hardness and rigidity, and a low percentage of ions. (3) Crystalline hydroxyapatite [$Ca_{10}(PO_4)6(OH)_2$], with traces of other ions such as F^-, Mn^{2+} Mg^{2+}, CO_3^{2-} and many others, is the main mineral component of bone, making up about a quarter of the volume of normal adult bone. (3)
- The organic component, which is essentially composed by type I collagen (90% - 95%) and noncollagenous structural proteins, namely proteoglicans, sialoproteins and glicoproteins (osteopontin and osteocalcin). (3)

Collagen forms fibrils of aligned protein helices (with 50 to 70 nm of diameter) where hydroxyapatite crystals (with 10 to 50 nanometer in length) are stuck. These crystals are sized and oriented according to collagen template, conferring a well- arranged nanoscale structure to bone. (6)

This structure of bone is critical to reach its specific properties. So, the tissue can maintain a reasonable balance, i.e., nor extremely flexible (if the organic component would be removed) nor extremely hard and thus brittle (if the inorganic component would be removed).

1.2. Bone architecture

As shown in figure 1, bone's organization is a highly hierarchical structure. (5)

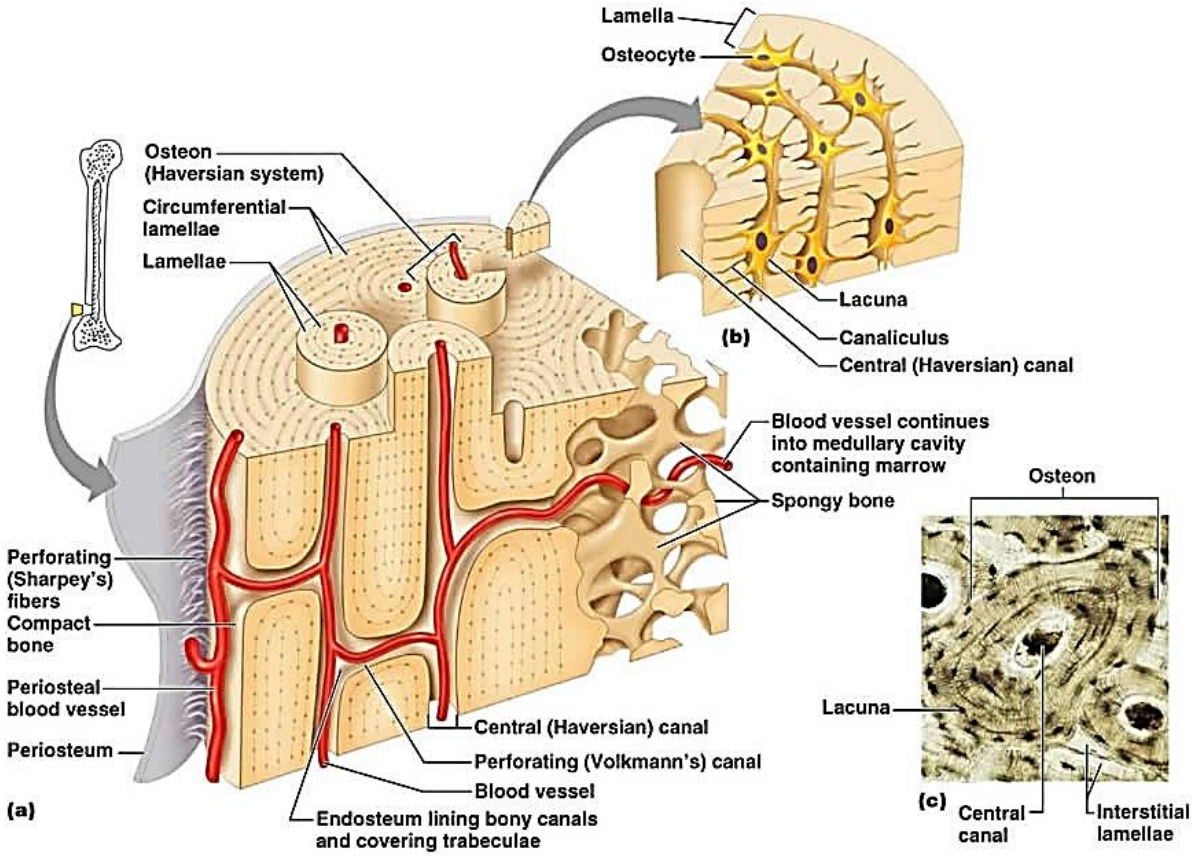


Figure 1. Bone microscopic composition. (7)

Bone is usually classified as cortical (compact or dense) and/or trabecular (cancellous or spongy).(1, 4) It is estimated that 80% of an adult human skeleton is composed by cortical bone and 20% is trabecular bone.(1, 5)

Cortical bone is found mainly in the periphery of long bones, and in short and flat bones. It is dense and massive (only 10% porous) (1) and surrounds the marrow space containing a great deal of blood vessels. (3, 5)

Cortical bone also has an inner and an outer surfaces: periosteum and endosteum, respectively. The first one is a non-calcified connective tissue layer that covers the endosteum, except at joints. Periosteum is constituted by blood vessels, nerve fibers, osteoblasts and osteoclast, and it has important features in bone growth and fracture repairs once it protects, nourishes and supports bone formation.

On the other hand, endosteum is a membranous thin layer of connective tissue that covers the marrow cavities. This structure is responsible for bone growth and repair once it provides osteoprogenitor cell and osteoblasts.

The periosteum is strongly connected to the outer cortical surface of bone by thick collagenous fibers, called Sharpeys' fibers. (4)

Cortical bone is also composed by units called osteons (or Haversian systems) which cannot be found in trabecular bone. Osteons are concentric cylinders of lamellae formed around the Haversian canal and they are considered the primary histological unit of bone. (4) In turn, each Haversian canal holds a blood vessel which supplies nourishment to the bone cells. (8) Osteons can communicate with each other through Volkmann's canals that are vascular spaces disposed obliquely or perpendicularly to them. (9)

Trabecular bone is found within cortical bone, in medullary spaces and in the interior of short bones. It is composed of a set of polygonal network of trabecular plates and sticks highly interconnected, giving this type of bone a spongy-like appearance. (4, 5) Unlike cortical bone, trabecular bone presents 50–90% of porosity, as seen in figure 2. (1)

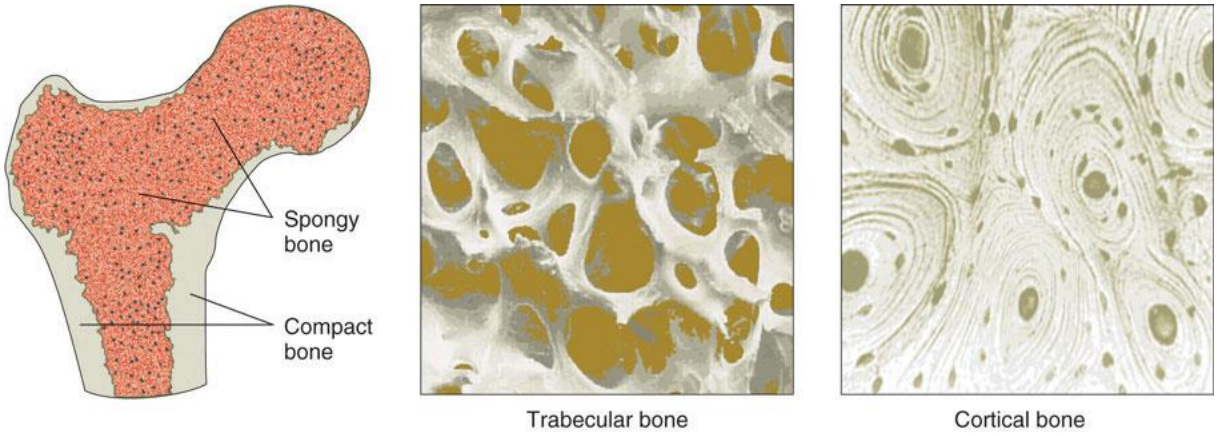


Figure 2. The two basic types of bone, trabecular and cortical bone. (10)

Mineralized bone could be either woven bone (immature) or lamellar bone (mature). Woven bone is characterized by a random organization of collagen fibers, i.e., collagen fibrils are laid down in a disorganized way while lamellar bone is characterized by a parallel orientation of collagen into sheets, which confers to bone its significant mechanical strength. (3, 9)

Bone marrow, in turn, is divided in two different parts: yellow bone marrow (composed essentially of fat) and red bone marrow (also known as myeloid tissue). It has as main functions the formation of blood cells, the removal of particulate matter from the blood and possible production of antibodies and plasma proteins. (11)

1.3. Bone Cells

Four characteristic cell types can be found within the bone homogeneous intercellular material: osteoblasts, osteocytes, osteoclasts and undifferentiated bone mesenchymal stem cells. (9) Each one has specific tasks that together are crucial for the maintenance of a healthy bone tissue. (1)

1.3.1. Osteoblasts and Osteocytes

Found at the bone surface, where they form a tight layer of cells, osteoblasts, as well as adipocytes, chondrocytes, myoblasts and fibroblasts, are originated from multipotent mesenchymal stem cells that have the ability to differentiate into these cells when properly stimulated (Figure 3). They are polarized and present a cuboidal shape. (1, 12)

Osteoblasts are responsible for the synthesis and regulation of new bone extracellular matrix constituents, being their major product the type I collagen and bone apatite. (1, 3, 12) Therefore, active mature osteoblasts present large nuclei, enlarged Golgi systems and extensive endoplasmic reticula. (4) They also express on their membrane surface a glycosylated protein called alkaline phosphatase (ALP). High levels of this protein are found in the blood during bone formation.

Some of these cells are differentiated into osteocytes just before the end of the matrix secreting period, being entrapped in the new bone matrix, while the remained non-differentiated cells are kept at the bone surface. Osteocytes are located in a cavities called *lacuna* and communicate with each other through *canaliculi.* (9)

Osteocytes present a stellate shape and have less organelles than osteoblasts. Their main function is to support bone structure and metabolism but also take part in the calcification of the osteoid matrix and in the blood-calcium homeostasis. (1, 3)

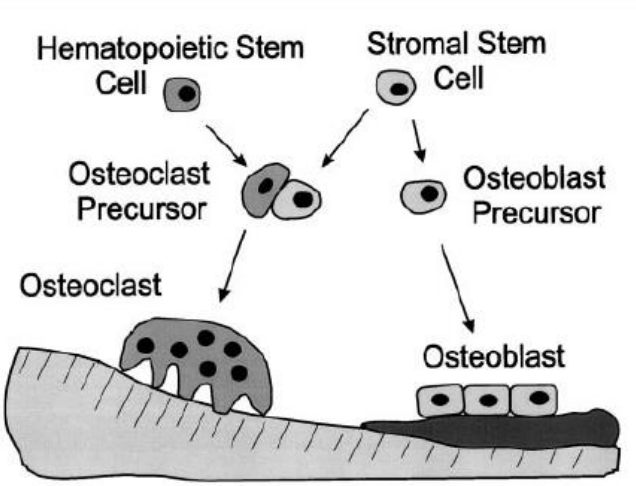


Figure 3. Origin and fate of osteoblasts. (13)

1.3.2. Osteoclasts

Osteoclasts are terminally differentiated myeloid cells with specific characteristics to remove mineralized bone matrix by direct chemical and enzymatical attack. (9) They are the only cells known to be able to resorb bone. (4) Generally, these cells have a typically large multinucleated shape, containing a high number of mitochondria and expressing TRAP (tartrate-resistant acid phosphatase), which is associated with their activation and differentiation, and the calcitonin receptor. (2, 14)

Two cytokines are considered critical for osteoclast formation: RANKL (Receptor Activator of Nuclear kB Ligand) and macrophage CSF (M-CSF). RANKL interacts with its receptor RANK expressed by pre-osteoclasts and therefore, promotes pre-osteoclasts fusion and differentiation into osteoclasts. (4) This interaction can be inhibited by a member of the tumor necrosis factor (TNF) receptor family of molecules - the Osteoprotegerin (OPG). OPG is a soluble protein which is mainly produced by osteoblasts but also by other cells in bone marrow, and acts as decoy (antagonist) receptor for the osteoclast differentiation. (12, 15) On the other hand, M-CSF plays an important role in proliferation, survival and differentiation of osteoclast precursors. (4)

To perform its main function, osteoclasts secrete H^+ ions to dissolve the mineral component of bone and cathepsin K enzyme to digest the proteinaceous matrix, which is mostly composed of type I collagen. (4)

1.4. Bone remodeling process

Bone remodeling is a very important, dynamic and active process of synthesis and destruction that living bone is constantly undergoing in order to allow primary bone substitution in infants (woven bone to lamellar bone), remove damaged bone and to ensure a correct calcium homeostasis. (9, 16)

This process, shown is figure 4, is carried out by osteoclasts and osteoblasts, responsible for resorbing (or destroy) old bone and form new bone respectively, in four sequential phases: activation, resorption, reversal and formation. (4)

Activation phase

The first step of bone remodeling process starts with an initiating remodeling signal which can be any external mechanical strength on bone that causes damages to the bone or internal factors, such as hormones, that leads to bone cells response. (2)

In this phase, mononuclear monocyte-macrophage osteoclast precursors are recruited from the circulation and activated. Endosteum, which contains the lining cells on its surface, is stimulated and multiple mononuclear cells form multinucleated pre-osteoclasts by fusion. (4)

Resorption phase

During resorption phase, osteoclasts secrete hydrogen ions into the resorbing compartment in order to locally decrease the pH in the compartment and dissolve the inorganic matrix (HA crystals). To digest the organic matrix, these cells also secrete TRAP, cathepsin K, matrix metalloproteinase 9, and gelatinase. (4)

Osteoclast activity during this phase produces irregular cavities at the trabecular bone surface named Howship's lacunae or cylindrical Haversian canals in cortical bone. (3)

Reversal phase

The reversal phase takes place when the osteoclasts have completed the previous phase. The events that occur in this phase are still unclear but it is presumed that additional collagen degradation, deposition of proteoglycans and release of growth factors to start next phase may occur. (13)

Formation phase

Finally, the cavity created by resorption is filled in with osteoblasts. These cells are responsible for deposition of a mineralizable matrix, after having differentiated from their mesenchymal precursors. When this task is complete, osteoblasts acquire a flattened shape

and then line on the bone surface, becoming osteocytes when trapped in bone or else proceed to apoptosis. (13)

Normal bone remodeling, with the correct balance between osteoclastic and osteogenic functions, is a crucial process to maintain a constant bone mass. (16) It is more active in trabecular bone than in cortical bone because the first one has much larger surface area/ volume ratio. (12)

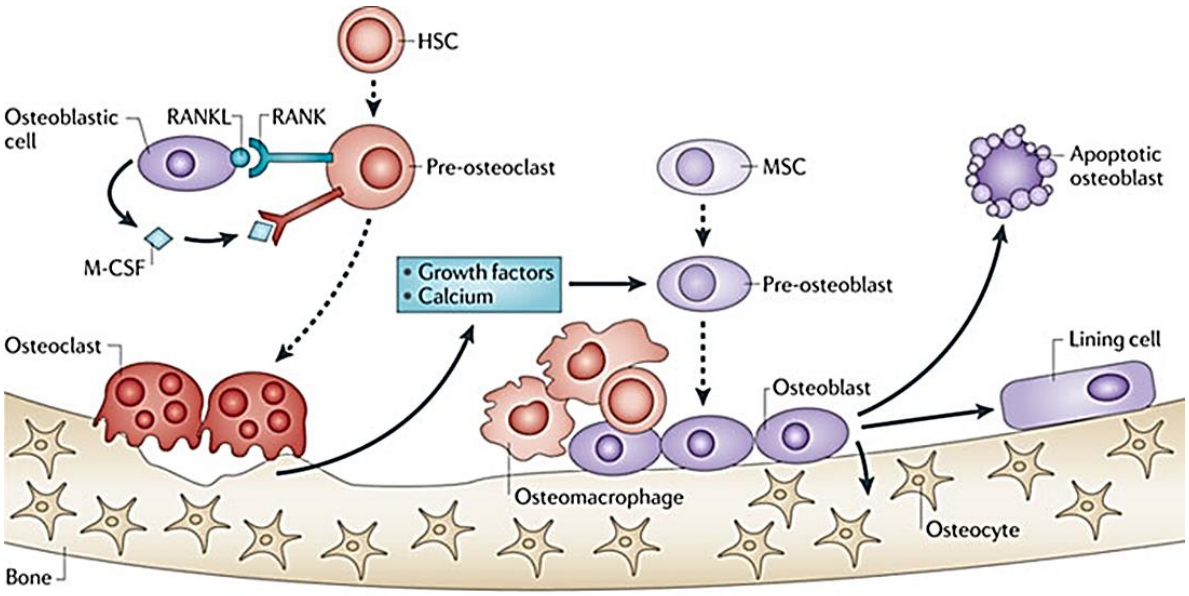


Figure 4. Bone remodeling process. (17)

1.5. Osteomyelitis

Osteomyelitis is a bone infection characterized by severe local inflammation, usually with pus formation, accompanied by bone destruction. This kind of infection can be caused by many species of organisms, with *Staphylococcus aureus* being the most commonly isolated organism.

Osteomyelitis is normally caused by a combination of local trauma and an acute infection in any other part of the body. Then, it can remain localized or it can spread through other parts of the bone such as bone marrow, cortex, periosteum and the surrounding soft tissue. (18) This disease can also be caused by bacteremia, surgery or orthopedic implants and it occurs more frequently in patients with diabetes mellitus, vascular insufficiency, or immunosuppression. Many other clinical conditions can make it easier for the infection to occur, such as neuropathy, intravenous drug use, open fractures, hemodialysis, sickle cell disease, dental infections, urinary tract infections and catheter-related bloodstream infection. (9)

The patients normally experience symptoms like fever, irritability, fatigue, nausea, tenderness and swelling around the affected bone and lost range of motion. These symptoms are very similar in the different cases of osteomyelitis. (9, 19)

Its management depends on many factors such as the organism involved, the specific affected bone, the type (acute or chronic), the spectrum of the disease (extensive or localized), vascular supply, nerve function and others. So it must be individualized. (20)
According to Lew & Waldvogel, osteomyelitis can be classified as acute or chronic, hematogenous or contiguous, and with or without vascular compromise. (21)

Acute Osteomyelitis

Acute osteomyelitis is usually a blood-borne disease that affects mostly children and it takes over days or weeks to evolve. (18, 21) The main symptoms are focal bone pain accompanied by fever or malaise. (22)
Normally, a patient with acute osteomyelitis experiences sudden pain with tenderness, heat, swelling, and restricted movement of the affected area. Tachycardia, sudden fever, nausea, and malaise are some of the systemic symptoms associated to this condition. (18)

Chronic Osteomyelitis

Chronic osteomyelitis is a rare long-standing infection defined by clinical signs that persist for months or even years, with development of necrotic bone. It is a relapse of an acute osteomyelitis in the same area and generally accompanied by fever. (18, 21)
Usually, chronic and acute osteomyelitis share the same clinical features, except that chronic osteomyelitis can persist for years. (18) It is characterized by necrotic bone (called sequestrum), new bone formation and the presence of characteristic cells of an infection/inflammation such as leukocytes, lymphocytes, and histiocytes. (9)

Hematogenous Osteomyelitis

Hematogenous osteomyelitis is characterized by the direct seeding of the bone from bacteria present in the bloodstream. (23)
This type of infection affects predominantly children and elderly because the metaphyseal regions of the long bones are much vascularized and more vulnerable to minor trauma. (24)
In adults, it is more commonly caused by a secondary infection where the causing bacteria migrates by entering bloodstream and reaching the distal bone and marrow sites. Sometimes it is caused by the reactivation of an old focus developed previously. The most common

clinical features are fever, lethargy, tenderness and decrease of mobility of the injured area. (23)

Contiguous Osteomyelitis

Contiguous osteomyelitis normally appears after a trauma and nosocomial contamination acquired during surgical procedures. It can be considered as a secondary osteomyelitis once the infection originates from adjacent sources. (23, 25)

1.5.1. Epidemiology

Due to an increase of cases of trauma, namely the ones caused by motor vehicles accidents, and orthopedic surgical procedures, there has been a high incidence of osteomyelitis caused by direct inoculation or contiguous focus of infection.

In numbers, there are 3 to 25 % of cases of the infection that are secondary to open fractures; 15% of patients with foot ulcers caused by diabetes mellitus develop osteomyelitis and 36 % of the patients with diabetes tent to have recurrent infection. Vertebral osteomyelitis occurs in 2 % to 4 % of the patients and, in these cases, men are more frequently affected. (9)

1.5.2. Mechanisms of disease

The affected area normally shows purulent inflammation where the bacteria are embedded.

The destruction of bone trabeculae and bone matrix is stimulated by the inflammatory factors and leucocytes derived from all the inflammation process. This inflammation process is also responsible for the vascular channels compression, causing ischemia and consequent necrosis of the adjacent tissue. Due to this event, the antibiotics and inflammatory cells are not able to reach the affected area and the treatment of osteomyelitis may fail.

Apparently, there is an increase of the osteoclast activity which causes a reactive hyperemia. The hyperemia is responsible for bone loss and localized osteoporosis. Once the growth factors and cytokines hormones are involved at the regulation of the bone cells (osteoclasts and osteoblasts) proliferation and activity, when their local concentration are altered, they also influence bone cells development and death. (21)

1.5.3. Pathogenesis: *Staphylococcus aureus*

Among the wide range of pathogenic microorganisms that can cause osteomyelitis, *Staphylococcus aureus* has proved to be the most virulent and it is the one implicated in most cases. (20, 21) Despite the considerable amount of effective antibiotics for this type of microorganisms, *Staphylococcus aureus* presents a good versatility in escaping the treatment, becoming responsible for nosocomial and community-based infections, and also the major cause of morbidity and mortality. (20)

S. aureus, shown in figure 5, is a gram-positive and non-motile coccus bacteria that grows by aerobic respiration or fermentation with diameters of 0.5-1.5μm and arranged like cluster of grapes. (26, 27) The colonies present a gold pigmentation and positive results for coagulase tests. (28) The cell wall of this bacteria is composed by a thick layer of peptidoglycan and teichoic acids, with adhesins and exotoxins attached. (26)

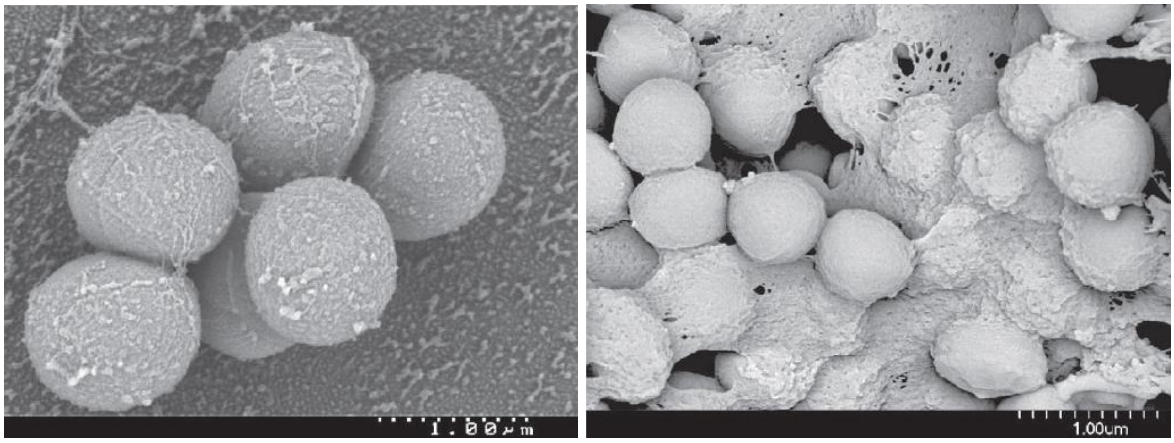


Figure 5. SEM images of *Staphylococcus aureus* morphology (left) and staphylococcal biofilm covering a layer of bacteria. (26)

In case of gram-positive bacteria, peptidoglycan constitutes over 50 percent of the cell wall's weight. It is a polymer formed by a long polysaccharide chain composed by two sugar derivated subunits - N-acetylglucosamine (NAG) and N-acetylmuramic acid (29) residues - alternated and linked by 1,4-β-linkages and many other amino acids (figure 6). Therefore, the peptidoglycan chains are cross-linked by tetrapeptide chains bound to NAM and by a pentaglycine bridge specific for *S. aureus*. (27, 28) Three of the amino acids that compose this polymer (D-glutamic acid, D-alanine, and meso-diaminopimelic acid) cannot be found in proteins and their main function is to protect the bacteria against degradation by peptidases. Peptidases can only recognize the L-amino acid form. (27)

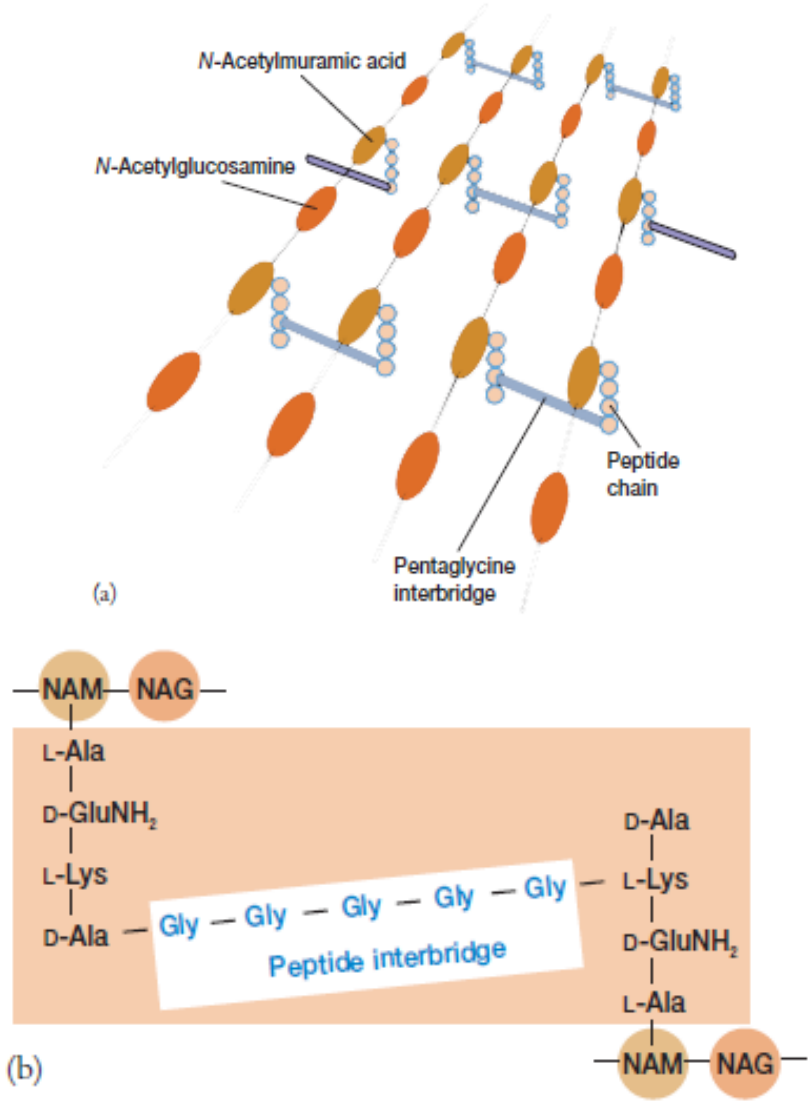


Figure 6. (A) Schematic diagram of peptidoglycan structure. (B) *Staphylococcus aureus* peptidoglycan cross-links:. NAM - N-acetylmuramic acid; NAG – N-acetylglucosamine; Gly – Glycine. Adapted from (27)

Besides the main component of the gram-positive cell wall being a peptidoglycan, a fair amount of teichoic acids covalently bound to the polymer is also found. The teichoic acids are polymers of glycerol phosphate or ribitol phosphate attached by phosphate groups. When the teichoic acid is connected to the peptidoglycan by a covalent bond to plasma membrane lipids, they are called lipoteichoic acids. (27, 28) Figure 7 shows the arrangement of these components.

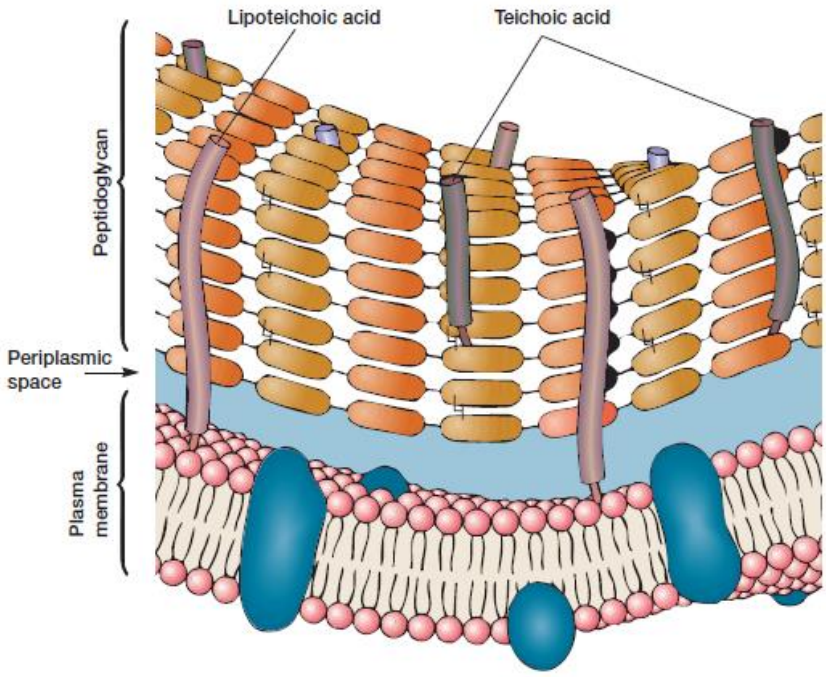


Figure 7. A gram-positive cell wall components. (27)

The real function of the teichoic acids remains unclear but they seem to extend the surface of the peptidoglycan and maintain the cell wall structure. They also seem to increase the negative charge of the cell wall, once they are negatively charged. (27)

Staphylococcus aureus uses bacterial adhesins expressed on its surface (microbial surface components recognizing adhesive matrix molecules - MSCRAMM) to adhere to extracellular matrix proteins (such as fibrinogen, fibronectin, collagen, vitronectin, laminin, thrombospondin, bone sialoprotein, elastin, or von Willebrand factor) on host tissues. (30) This is an important factor in the early colonization of host tissues and implanted biomaterials because when *S. aureus* adheres to a surface by using the MSCRAMM, host cells cannot get rid of it. (21, 26, 31)

S. aureus uses some factors, like protein A, toxins and capsular polysaccharides, to escape host defense mechanisms. It also uses exotoxins and degrading components of extracellular matrix (hydrolases) to invade and to penetrate the tissue (figure 8). All of the factors cited above show the ability of this bacteria to invade mammalian cells, colonize tissues and even persist after bacteremia.

This bacteria can also survive within the epithelial or endothelial cells by promoting its endocytic uptake. This ability to survive inside the cells can explain the persistence of bone infection. (21) The biofilms that this bacteria forms increases the difficulty of the treatment with antimicrobial agents because biofilms are able to slow down the penetration of the

antimicrobial agents into the bacteria, protecting them from phagocytosis and antibiotics action. (21, 26)

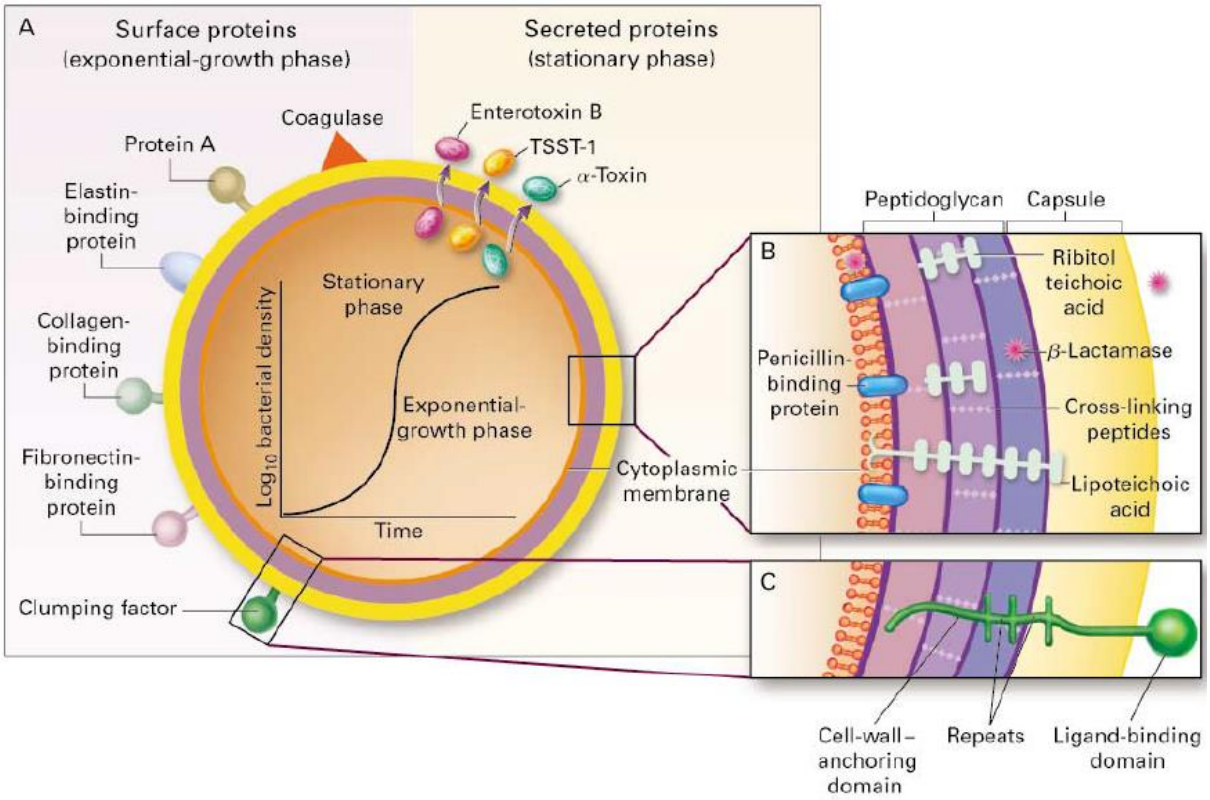


Figure 8. *S. aureus* structure and secreted proteins which are important virulence factors. (A) Surface and secreted proteins; (B, C) Cross-section of the cell envelope. (30)

Staphylococcus aureus is a native colonizer of skin and nasopharynx but it can underline damaged tissue causing disease. The major problem is when the bacteria acquire resistance which is supported by the hospital environment, becoming hard to eradicate. (26)

1.5.4. Current Treatment

In all cases of osteomyelitis should be considered a combined antimicrobial and surgical approach even though, in case of hematogenous osteomyelitis, the infection can be eradicate only by antibiotic treatment. The antimicrobial therapy requires observation and, if the infection persist after one week, surgery must be considered.

A treatment including the administration of semisynthetic penicillin or vancomycin is the currently recommended treatment for osteomyelitis caused by *Staphylococcus aureus* although this treatment has some complications like a long stay at the hospital, which has high costs, and adverse events due to the use of intravenous catheters.

Surgery is absolutely necessary in cases where sequestra has already been formed, to remove the dead bone and surrounding soft tissue which act as foreign material. This procedure is delicate as the inadequate removal procedure of the infected parts is the major cause of recurrence in chronic osteomyelitis. (21) Even with appropriate medical and surgical treatment, about 20 % to 30 % of the patients has recurrence within 2 years. (9)
This infection may have some complications including abscess formation, sepsis, bone deformity, limited range of movement, and motor and sensory deficits. (9)

1.6. Vancomycin

Vancomycin is one of the most clinically important glycopeptide antibiotics because it is often considered the last line of defense against resistant pathogens, such as *Staphylococcus aureus*, that have developed resistance to penicillin, methicillin and other β-lactam antibiotics. (32)

1.6.1. Structure

This antibiotic is a subclass of linear sugar from the vancomycin-ristocetin family, formed by peptides containing seven amino acids. (33) Vancomycin's carboxyl terminal is composed by a β-hydroxylated tyrosine, two phydroxyphenylglycine, a β-hydroxyty-rosine and a m,m`-dihydroxyphenylglycine, and it also has aliphatic amino acids (generally leucine and asparagine), as shown in figure 9. (33)

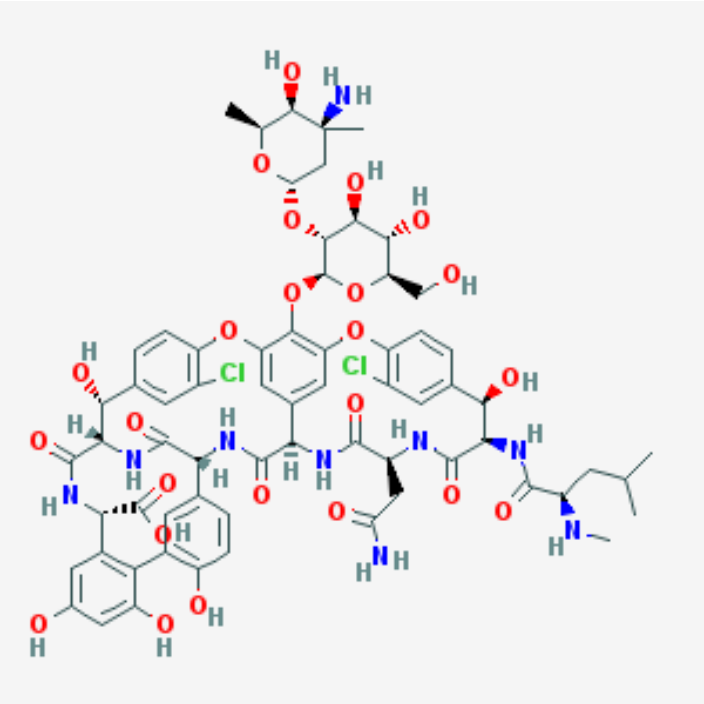


Figure 9. Vancomycin structure (34)

1.6.2. Mode of action

Some studies have shown that vancomycin's mode of action includes the inhibition of peptidoglycan synthesis and, therefore, the bacteria cell wall synthesis. (33, 35)

This antibiotic interacts with C-terminal D-Ala–D-Ala peptides of the lipid II, avoiding grow of the cell wall precursor by interfering with transglycosylase activity and possibly transpeptidase activity. Transglycosylase and transpeptidase activity are essential for synthesis of new cell wall and if these two are inhibited, a weak point in the new cell wall is created and bacteria become susceptible to lysis. (32, 36) Vancomycin also alters the permeability of the cell membrane and RNA synthesis. (33)

1.6.3. Physicochemical properties

Compared to other antibiotics, vancomycin has shown many advantages concerning physicochemical properties and cytotoxicity.

Vancomycin presents an isoelectric point (pI) about 8.3 (37) and it becomes cationically charged at physiological pH. The importance of being cationic lies in the interaction by charge-charge and hydrophobic interactions between the antibiotic and the membrane

bilayer of the bacteria (38) , i.e., being cationic improves the targeting of the antibiotic to the negatively charged bacterial cell membranes. (39)

This antibiotic is considered an acidic antibiotic with a standardized low minimum inhibitory concentration (MIC) of 1µg/mL for *S. aureus* (40, 41) and is also considered one of the least cytotoxic antibiotics, an ideal point to contemplate when choosing antibiotics for bone application. (40, 41) Vancomycin has been documented as an effective antibiotic against Staphylococcus strains. (42)

1.7. Tissue regeneration: a new approach

Nanohydroxyapatite (NanoHA) scaffolds, intended as a porous device, have been widely used as bone substitute to reduce the size of the hole created by necrosis. A scaffold can be permanent or temporary and offers mechanical support to adjacent tissue until the affected tissue naturally regenerates and remodels itself. Moreover, the characteristics of the material can be taken as advantageous to maximize tissue growth by adding some specific signaling molecules or even cells. (43)

From bone infections perspective, having a local drug delivery system with a controlled and sustained release of antibiotic that could remain between the MIC and MTC values and, once the drug releasing is over, could still stimulate new bone formation and repair bone defects would be ideal for a successfully treatment. (42)

Studies have shown that nanohydroxyapatite composites may be osteoconductive material and could stimulate bone regeneration. So, they have been indicated as an adequate bone substitute in the normal physiological environment. (44)

Additionally, the referred biomaterial reveals an excellent release profile which is characterized by an initial rapid release of antibiotic and a long term sustained release to control infection over a longer period, according to some experiments. (42)

Based on these studies, a biomaterial with the properties mentioned above which mimic bone composition was developed in order to treat bone infection and stimulate bone regeneration. Frequently, materials like hydroxyapatite and collagen are used for this purpose.

1.7.1. Hydroxyapatite

Hydroxyapatite (HA, $Ca_{10}(PO_4)_6(OH)_2$) is a bioactive ceramic very similar in chemical composition and crystalline structure to natural HA crystals found in mineralized bone and

teeth (45, 46) which has been widely and successfully used for biomedical applications, including bone remodeling and matrices for drug release control. (47-49)

This calcium phosphate-derived material has become increasingly used as a good candidate for orthopedic implants, artificial bones and scaffolds for tissue engineering owing to its positive biological response, the ability to adsorb various ions and organic molecules on its surface and also because of its interaction and strong affinity to host hard tissue. (45, 50)

HA molecule features a hexagonal shape, as shown in figure 10, with a stoichiometric Ca/P ratio of 1.67 (47) , very similar to the Ca/P ratio found in natural bone. It is considered the most stable calcium phosphate ceramic compound in terms of thermodynamic properties when exposed to pH, temperature and composition of physiological fluid. (47)

Patients with osteomyelitis usually presents bone defects with irregular shapes (21) and it has been reported that one of the best way for of HA to be used in these cases are granules, besides this material can be molded into many shapes for clinical use such as powders, granules, porous blocks and films. (51)

1.7.2. Nanohydroxyapatite

HA possesses an excellent bioactivity and biocompatibility however it reveals to have poor mechanical strength. (48, 52, 53)

NanoHA (figure 10) is a new material that has taken advantage over HA and has been extensively studied in order to improve HA characteristics and therefore its performance as biomaterial for bone remodeling. NanoHA revealed a promising candidate to mimic bone tissue because of its nanometer dimensions compared to HA, which is more similar to the hierarchical structure of natural bone, and because of the large surface to volume ratio and higher surface reactivity it presents. (51)

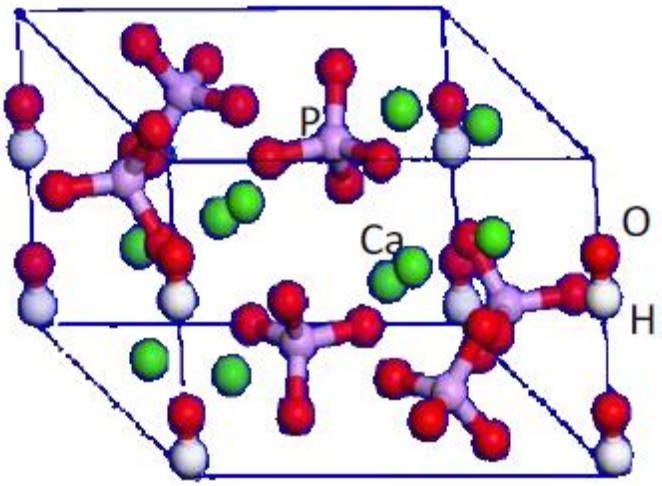


Figure 10. Crystal structure of nanohydroxyapatite (5)

Furthermore, NanoHA is even more similar to bone showing peculiar properties when compared to microsized HA (54) regarding protein adsorption and bone cells adhesion, which can be controlled by varying pore and grain sizes as well as wettability. (55, 56) It has been reported that these properties confer to the material the ability to enhance osteoblastic functions. (55)

According to some studies, nanoHA also reveals a good potential as Drug Delivery System, (42) so it can be used to treat chronic osteomyelitis by loading the material with antibiotic, namely vancomycin, due to its excellent mechanical and biological properties. (42, 57)

1.7.3. Collagen

Collagen is a natural polymer and the main structural element of the extra cellular matrix (ECM) in many tissues, playing an important role on its integrity. (53, 58, 59) It is synthetized by fibroblasts, (60) osteoblasts, chondrocytes and other cells. (60-62)

There are over 20 different types of collagen. The main types of collagen identified in bone, cartilage, tendon, skin and muscle are type I, type II, type III, and type V, being type I collagen the most abundant in bone tissues where it serves as support to the nanosized hydroxyapatite crystals,(58) with its nanofibrous structure containing fiber bundles in a diameter range between 50 to 500 nm. (59)

Regarding collagen structure, collagen molecule is formed by three polypeptide chains (α-chains) wound around each other, creating a triple-helical rod-like structure named tropocollagen. (60, 62)

About 1000 amino acid residues compose each α-chain and it is believed that the high content of glycine affects a helix formation because of the uncommon amino acid sequence of the chains, following the formula $(Gly\text{-}X\text{-}Y)_n$, where X is a proline and Y is a 4-hydroxyproline. So, collagen is considered a polypeptide of tripeptide units where every third amino acid is a glycine. (62) The three α-chains are linked together by hydrogen bonds between adjacent -CO and -NH groups, and also by covalent bonds, as seen in figure 11. (60)

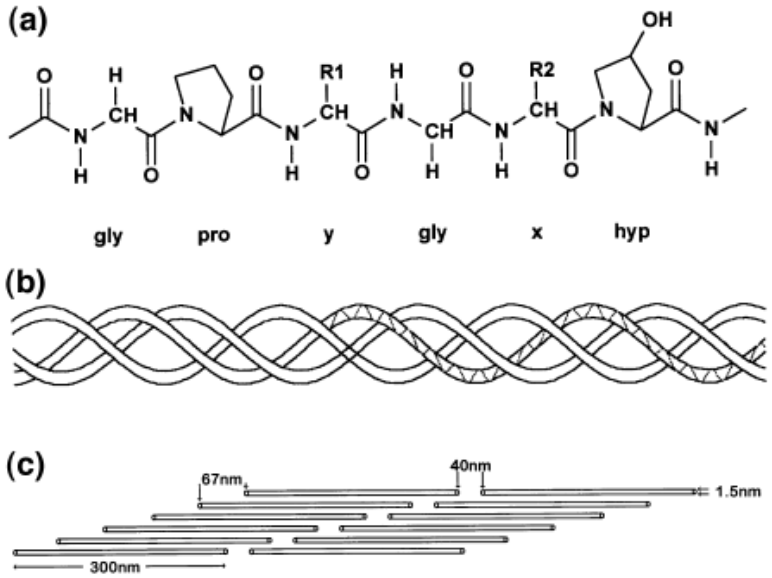


Figure 11. Chemical structure of collagen type I. (a) Primary amino-acid sequence. (b) Secondary left handed helix and tertiary right handed triple-helix structure. (c) Staggered quaternary structure (63)

Collagen has been more and more used in tissue engineering because of its good biodegradability and weak antigenicity compared to other natural polymers (53, 64) and it also permits good cell attachment. (43) Moreover, depending on the desired mechanical properties for bone, fibril orientation of collagen can be changed by the degree of collagen crosslinking, which means that the polymer can be perfectly adapted to the purpose. (58)

For many other reasons, such as good bioreabsorbability, non-toxicity, biocompatibility, compatibility with synthetic polymers, etc, (60) this biological polymer added to a ceramic structure can offer many advantages when applied in bone regeneration. (65)

Studies have shown that collagen may be a good choice as a component of a drug delivery system because it revealed a sustainable and adequate release of antibiotics. (60, 63)

1.7.4. Combined hydroxyapatite and collagen material

Natural bone composition and structure are some of the characteristics that must be taken in account when considering bone substitute material. (66)

Creating a material similar to actual bone in composition, nanostructure and biological response is the main goal of bone substitutes (67) and composites combining hydroxyapatite and type I collagen material have shown great potential as biomaterial for bone substitution applications, once they closely mimic bone composition. (53, 66)

Separately, these two material enhance osteoblast differentiation (68) but studies have proved that when combined their properties are improved and tend to accelerate osteogenesis. (53) Additionally, the few reported cases concerning the mechanical properties of synthetic HA/collagen material and the toughness characteristics that it presents rend it a very promising material as bone substitute. (66)

Some studies revealed that nanoHA/collagen material can be biodegraded by macrophages and the material presents good conditions which promotes bone cell attachment and proliferation. (66) Therefore, nanoHA/collagen material is considered a promising system for bone remodeling (69) because of its similarity to real bone, bioactivity and biodegrability. (66)

1.7.5. Heparin: Improving the combined hydroxyapatite and collagen material properties

As referred above, creating a Drug Delivery System (DDS) with a controlled and sustained release of antibiotic would be ideal for the treatment of osteomyelitis. Associating heparin on biomaterials could be a good choice as heparin improves the performance of the biomaterial as drug carrier because of its advantageous biological activities helping to control the antibiotic release kinetics. (70)

Heparin is a linear polysaccharide synthesized from a common precursor proteoglycan and produced in mast cells. (71)

When it was first discovered, heparin was known as a blood coagulation inhibitor, i.e, with anticoagulant activity. But over time, heparin showed useful when used in delivery systems for different biomedical applications. This polysaccharide consists on repeating disaccharides units, mainly uronic acid and glucosamine residues (figure 12). (70, 71)

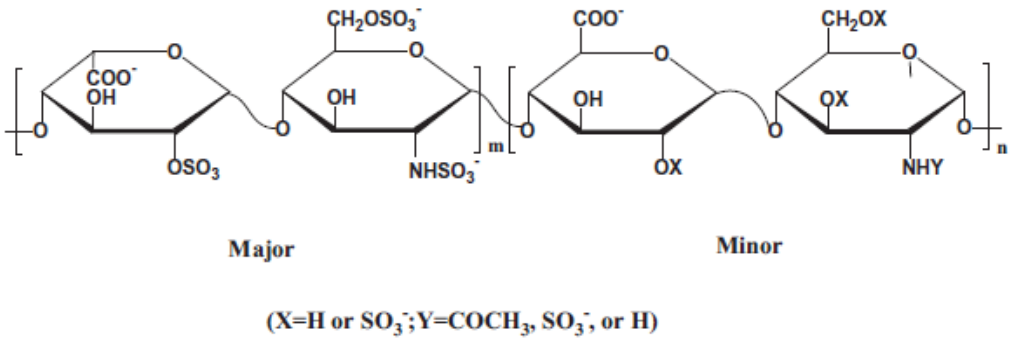


Figure 12. Heparin major and minor disaccharide sequences (70)

Heparin (figure 13) has sulfate and carboxylate groups that give a high negative charge to the molecule. The extremely negative charge allows electrostatic interactions between heparin and many proteins which is a good reason to include heparin in the design of scaffolds for tissue regeneration and DDS with drug controlled release. (70, 72)

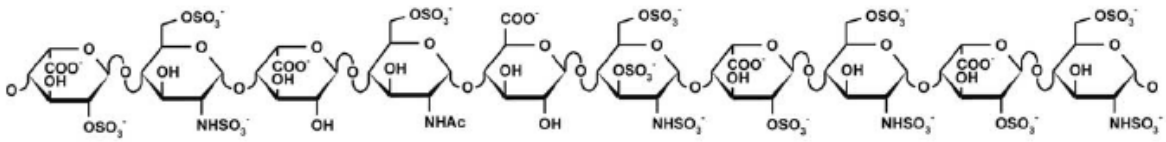


Figure 13. Current conception of Heparin structure. (73)

Studies have shown that heparin can be successfully conjugated to collagen during the crosslinking and thereby creating a composite that can be used both as bone regenerating tissue scaffold and as drug delivery system with a continuous release of molecules of interest, (72) namely vancomycin. The reported affinity of the delivery systems to specific small molecule and protein-based drugs used in biomedical applications is based on non-covalent interaction to immobilize them in the scaffold and protect their biological activity and control the diffusion from the scaffold. (71)

Chapter 2: Materials and Methods

2.1. NanoHA granules

A nanophased hydroxyapatite (nanoHA) slurry was impregnated to polyurethane (PU) sponges (Reticel Belgium) to produce scaffolds.

To obtain the optimal consistence of the slurry, nanoHA powder (nanoXIM Hap202, Fluidinova S.A., Portugal), ultra-pure water and dispersive agent Dolapix CE64 (Zschimmer & Schwarz, Germany), were used in the ratio of 5 g : 4.4 mL : 200 µL respectively.

After the impregnation, the sponges were let in the oven at 37 °C for 30 minutes before being heat-treated in a sintering furnace (Thermolab). Afterwards, the scaffolds were let in the furnace until they were completely cooled.

The sintered scaffolds were smashed and passed through sieves to obtain granules in a range size of 1.18 to 1.70 mm. (51)

2.2. Collagen addition to the nanoHA granules

Type I collagen (Bovine Achilles tendon, Sigma-Aldrich) was dissolved overnight in HCl (0.01 M, pH =2) at 4 °C to prepare a 0.5 % (w/v) collagen solution, by using an Ultra Turrax (T25 D, IKA®) at 10000 rpm. The solution was homogenized during 3 hours on ice.

The 0.5 % collagen solution was diluted to a concentration of 0.05 % before it was applied to each granule. The nanoHA granules were distributed on petri dishes and collagen solution was added. Then, the granules were dried for 48 hours at room temperature (RT, 25 °C), in a vacuum oven (Binder, Germany) to allow collagen to penetrate the granules.

2.3. Collagen crosslinking

Since collagen-based matrices are not very durable without crosslinking, this procedure is carried on to stabilize and provide good mechanical properties to the material. (74)

Therefore, collagen crosslinking was performed by using 57.5 mg of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC, Aldrich) and 20.8 mg of N-hydroxysuccinimide (NHS, Fluka) dissolved on 25 mL of 2-mofpholinoethane sulfonic acid (MES buffer, 0.05 M, pH=5.4, Sigma).

The samples were covered with the EDC/NHS crosslinking solution during 2 hours at 4 °C. After the crosslinking reaction, the solution was removed and samples were washed 3 times with MES buffer. The samples were dried in a vacuum oven overnight.
From several types of crosslinking procedures, the EDC/NHS crosslinking was chosen because of its non-cytotoxicity at low dosages. (74)

2.4. Collagen crosslinking with heparin immobilization

Heparin immobilization was done at the same time as the collagen crosslinking was being performed. Therefore, heparin (Heparin sodium salt from porcine mucosa, Sigma) was dissolved in MES buffer (0.05 %, pH=5.4, Sigma) along with the EDC and NHS, in a ratio of 188.3 mL MES : 0.433 g EDC : 0.157 g NHS : 3.766 g Heparin. The granules were distributed over a 24 wells plate, where the solution was added and left for 2 hours at 4 °C. Once the reaction was completed, the crosslinking solution was removed and a phosphate buffer saline (PBS, pH=7.4) was added to the granules for 2 more hours to stop the heparin immobilization.
Afterwards, the PBS was removed and a process of several washes was started with 2 M NaCl (during 4 hours), 4 M NaCl (6 hours) and ultra-pure water (8 hours). Then, the granules were dried overnight in a vacuum oven at RT.

2.5. Loading nanoHA/collagen granules with vancomycin

In order to adsorb vancomycin onto the nanoHA/collagen/Heparin granules, a 25 mg/mL (pH=4) concentrated solution of vancomycin was prepared and 1 mL of this solution was added to Eppendorf tubes with 20 mg of granules each. Then, the Eppendorf tubes containing the granules immersed in vancomycin solution were put in the orbital shaker (KS 4000 IC, IKA®) at 37 °C and 120 rpm for 24 hours.

2.1. Vancomycin release profile from heparinized nanoHA/collagen granules

20 mg of nanoHA/collagen/Heparin/Vancomycin granules were transferred to polystyrene 96-wells plate and 100 µL of fresh PBS (pH=7.4) were added in order to assess the vancomycin release profile. Granules were incubated in similar conditions of the performed cell culture, described in section *"Osteoclast culture"*. As negative control, granules with no added antibiotic were tested under the same experimental conditions.

Every 24 hours, 20 % of the content of the Eppendorf was collected for analysis and replaced by fresh PBS (Experiment A). In a parallel assay, in exactly the same conditions, all the content of the Eppendorf was withdrawn for analysis three times a week and replaced by fresh PBS (Experiment B).

Experiment A, where 20 % of the PBS was replaced by fresh one every 24 hours, was first designed to simulate the normal extracellular fluid renewal of body. Since this was not what would happen in cell cultures, Experiment B was designed to simulate the culture medium changing, ensuring that the vancomycin release profile was similar in both cases, although Experiment B does not correspond to actual extracellular fluid renewal. Experiment C was performed in similar conditions as experiment A and B but using a ratio of 20 mg/mL with full PBS replacement three times a week.

Before the determination of vancomycin concentration on the supernatant, samples were centrifuged for 5 minutes at 14000 rpm to avoid the presence of particles in suspension. Vancomycin concentration was determined by using NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific) at 280 nm.

To stablish a standard curve of vancomycin (figure 14), 5, 10, 15, 20, 25, 30, 40 and 50µg/mL of vancomycin solution samples were measured and the regression equation was calculated. Concentration and optical values are indicated in the X and Y axis, respectively, showing a linear correlation between these two values.

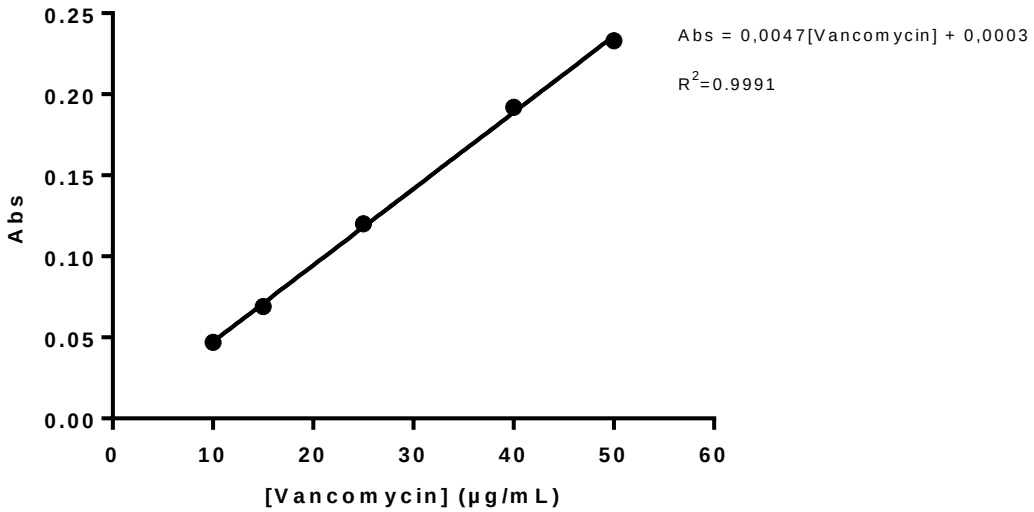


Figure 14. Standard curve of vancomycin

2.2. Bioactivity assay with *S. aureus*

Staphylococcus aureus (S. aureus) ATCC 25923 was first inoculated in a Nutrient Agar (NA, Liofilchem, Italy) and then a bacterial suspension on Tryptic Soy Broth (TSB, Difco, USA) was created. The bacterial suspension was adjusted to approximately 1.5 x 10^8 colony-forming units per milliliter (CFU/mL).

20 mg of nanoHA/collagen/heparin granules, previously loaded with vancomycin, were put in a glass tube with 1 mL bacterial suspension. TSB medium without bacteria and bacterial suspension without vancomycin were used as controls.

The glass tubes were incubated in water bath gently shaken for 24 hours at 37 °C. After incubation, absorbance was measured at 640 nm using a microplate reader (Spectramax M2e, Molecular Devices, Inc.).

Samples were drawn every 24 hours and the absorbance values obtained from each one were converted to a total number of bacteria/mL based on a calibration curve. TSB medium was completely removed and replaced by fresh one.

2.3. Osteoclast culture

Human peripheral blood mononuclear precursor cells (PBMNC) used in the osteoclast culture were isolated from blood of healthy donors, kindly provided by Instituto Português do Sangue (IPO, Porto).

The blood was diluted with PBS (2 parts of PBS: 1 part blood) and then applied over Ficoll-Paque (Sigma-Aldrich) to separate blood into its components. The mixture was centrifuged (400 x g, 10 minutes and room temperature). The cell layer on top of the Ficoll-Paque was collected, re-suspended in 45 mL of PBS and centrifuged once more under the same condition. Afterwards, supernatant was removed and cells were counted, re-suspended in culture medium and used immediately. (75)

Cells were cultured on tissue culture polystyrene (TCPS) 96-wells plate with alpha Minimum Essential Medium (α-MEM, Gibco), supplemented with 30 % of serum from the donor, 10 % of M-CSF and RANKL growth factors, 1 % of penicillin-streptomycin (Gibco), fungizone (Gibco), sodium heparin (Sigma-Aldrich) and L-Glutamine (Sigma-Aldrich).

20 mg of nanoHA/collagen/heparin granules, previously loaded with vancomycin, were placed in a TCPS 96-wells. NanoHA, nanoHA/collagen and nanoHA/collagen/Heparin (without vancomycin) granules were used as control. Samples were pre-incubated with α-MEM for 24 hours, at 37 °C and 120 rpm. Culture medium was changed three times a week.

2.4. Osteoblast culture

MG63, a human osteoblast-like cell line derived from human osteosarcoma, were cultured on TCPS 96-wells plate with α-MEM (Gibco), supplemented with 1 % of penicillin-streptomycin (Gibco), fungizone (Gibco) and ascorbic acid (Sigma-Aldrich) and 10 % of fetal bovine serum (FBS, Invitrogen). Cells were incubated in a humidified atmosphere of 95 % air and 5 % CO_2.

20 mg of nanoHA/collagen/heparin granules, previously loaded with vancomycin, were placed in a TCPS 96-wells. NanoHA, nanoHA/collagen and nanoHA/collagen/Heparin (without vancomycin) granules were used as control. Samples were pre-incubated with -MEM for 24 hours, at 37 °C and 120 rpm. Culture medium was changed three times a week.

2.5. Tartrate-resistant acid phosphatase activity

Tartrate-resistant acid phosphatase (TRAP) is an osteoclast enzyme marker that is involved in bone metabolism.

Osteoclasts cultured on granules were washed with PBS and incubated 15 minutes with 0.1 % Triton X-100, a cell lysis buffer. Afterwards, 80 µL of p-nitrophenyl phosphatase (p-NPP) substrate dissolved in 0.225 M sodium acetate, 0.3375 M KCl, 0.1 % Tx-100, 22.5 mM Sodium Tartrate, 0.225 mM iron chloride (pH = 5.8) were added and incubated at 37 °C for 1 hour. NaCl 5 M solution was added to stop the reaction and the absorbance was measured at 400 nm, using a SynergyMix, BioTek plate reader and Gen5 1.09 Data Analysis Software. The results of this test were normalized to total DNA content and expressed in nanomoles of p-nitrophenol produced per minute per microgram of protein (nmol min^{-1} µg $protein^{-1}$).

This test was performed at 7, 14 and 21 days of culture.

2.6. Alkaline phosphate activity

To measure the alkaline phosphatase activity (ALP), cell cultured on granules were washed with PBS and incubated 15 minutes with 0.1 % Triton X-100. Then, 80 µL of p-nitrophenyl phosphatase (p-NPP) substrate dissolved in PBS were added and incubated at 37 °C for 1 hour. NaCl 5 M was added to stop the reaction and the absorbance was measured at 400 nm using a SynergyMix, BioTek plate reader and Gen5 1.09 Data Analysis Software. The results of this test were normalized to total protein content and expressed in nanomoles of

p-nitrophenol produced per minute per microgram of protein (nmol min^{-1} μg $protein^{-1}$). Total protein content was measured as described in the following section.
ALP activity assay was performed at 7, 14 and 21 days of culture.

2.7. DNA and Protein content

Cell proliferation was measured by DNA/Protein content of the cells on the material using PicoGreen® - dsDNA Quantitation Reagent, an ultra-sensitive fluorescent nucleic acid stain for quantitating double-stranded DNA (dsDNA).
For that purpose, cells were washed twice with PBS and incubated for 15 minutes, at room temperature, with 100 μL 0.1 % Triton X-100. After incubation, 10 μL was transferred to a black TCPS 96-well plate with 90 μL of 1xTAE (Tris-acetate-EDTA) Buffer and 100 μL of PicoGreen® Reagent previously diluted in TAE Buffer were added to each well for 10 minutes.
This procedure had to be protected from light because PicoGreen® Reagent is susceptible to photodegradation. Fluorescence was determined on a SynergyMix, BioTek plate reader and Gen5 1.09 Data Analysis Software, with excitation at 485 nm and emission at 528 nm. This test was performed at 7, 14 and 21 days of culture.

2.8. Cell proliferation/viability

Resazurin test

Nontoxic Alamar blue dye (Resazurin) test was performed to measure the MG63 viability. Viable cells with active metabolism are able to reduce Resazurin to resofin, a fluorescent form of Alamar Blue and the amount of the reduced form produce is proportional to the number of viable cells. 100 μL of 10 % (v/v) Alamar blue dye solution in culture medium was added to each well and incubated at 37 °C in a humidified atmosphere of 95 % air and 5 % CO2. After 3 hours of incubation, the solution was transferred to a black TCPS 96-well plate and fluorescence was monitored at 530 nm excitation wavelength and 590 nm emission wavelength in a SynergyMix, BioTek plate reader and Gen5 1.09 Data Analysis Software plate reader. This test was performed at 1, 7, 14 and 21 days of culture. (76)

2.9. Cell morphology

Scanning Electron Microscopy (SEM)

Granules from 7, 14 and 21 days of culture were washed with pre-heated PBS and then fixed with a 1.5 % glutaraldehyde (v/v) solution in 0.14 M sodium cacodylate buffer at room temperature for 20 minutes. The granules were then washed twice with PBS and dehydrated with a growing ethanol (99%)/water gradient, maintaining them in 50, 60, 70, 80, 90 and 99% for 10 minutes each. 100 µL of hexamethyldisilazane (Sigma) was added to each well and the samples were dried overnight in the hood.

The samples were mounted onto aluminum supports using Araldite^{TM}, sputter coated with palladium-gold and then observed on SEM, using a High Resolution Scanning Electron Microscope with X-Ray Microanalysis with JEOL JSM 6301F/ Oxford INCA Energy 350 and Electron Backscattered Diffraction analysis with Quanta 400 FEG ESEM / EDAX Genesis X4M.

Confocal Laser Scanning Microscopy (CLSM)

Granules from 7, 14 and 21 days of culture were washed with pre-heated PBS and then fixed with a 3.7 % formaldehyde during 20 minutes. The samples were washed twice with PBS and 0.1 % Triton X-100 was added for 5 minutes to permeabilize cells. Afterwards, 1 % Bovine Serum Albumin (BSA) was added to the samples and incubated at 37 °C for 30 minutes.

Alexa Fluor 594 Phalloidin (1:4000, Molecular Probes A12379, Invitrogen) fluorescent dye in 1 % BSA was used to stain F-actin filaments for 30 minutes, at room temperature. Samples were washed with PBS and then, cell nuclei were stained with 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI, Sigma-Aldrich) solution at RT for 10 minutes in the dark. Samples were washed with PBS twice more and Vectashield Mounting Media was added to prevent photobleaching.

Granules were transferred to a proper support to obtain images with a Leica SP2 Confocal microscope, using a 10x water objective.

2.10. Statistical analysis

For each condition, three replicas were tested and data are expressed as the mean ± standard deviation. Statistical differences were tested by one-way analysis of variance (One-Way ANOVA), followed by the post hoc Tukey's test for multiple comparisons (SPSS 20.0 for windows, U.S.A). Difference was considered statistically significant for values of p 0.05.

GraphPad Software version 5.02 (GraphPad Software, Inc., U.S.A).was also used to perform data analysis.

Chapter 3: Results

3.1. Vancomycin release profile from heparinized nanoHA/collagen granules

Figure 15 shows the vancomycin release profile from heparinized nanoHA/collagen granules during 360 hours, with 20 % (experiment A) of PBS substitution everyday and total substitution of PBS three times a week (experiment B).

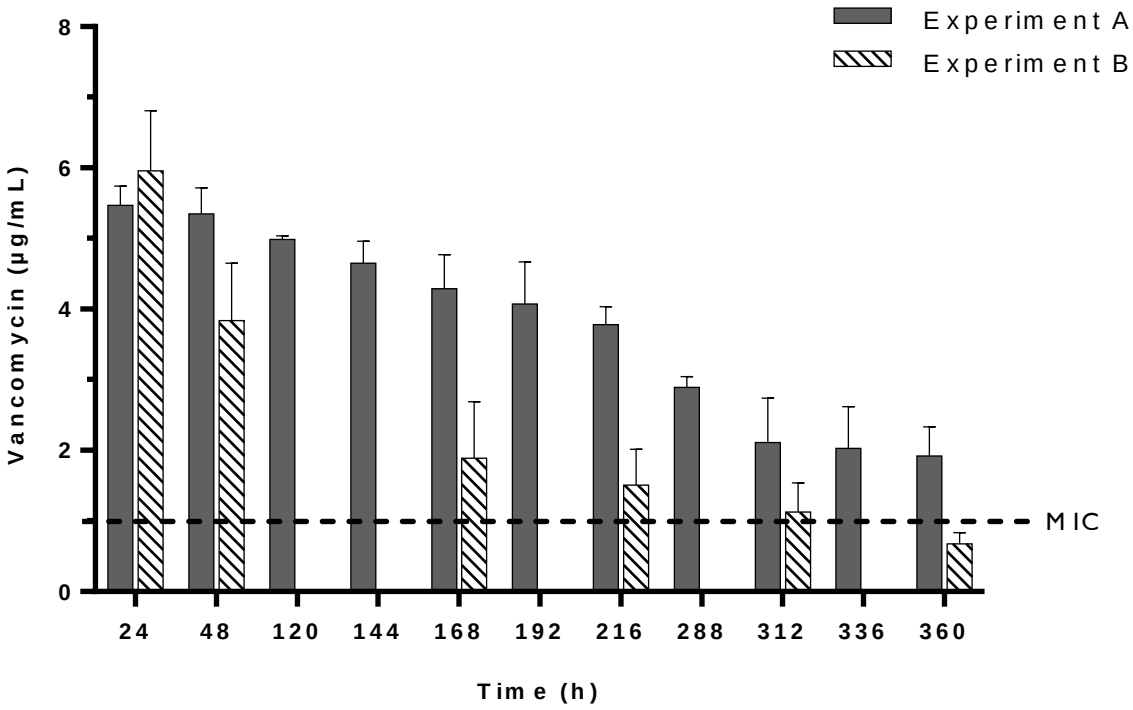


Figure 15. Vancomycin release profile from heparinized nanoHA/collagen granules over time. Experiment A (ratio= 200 mg/mL), where 20 % of PBS solution was substituted in each time point and Experiment B (ratio= 200 mg/mL), where complete substitution of PBS solution were performed in each time point.

Considering Experiment A, where 20 % of PBS was replaced every 24h and Experiment B, where all PBS was replaced by fresh PBS three times a week, the pattern of release is similar but in Experiment B, the concentration of vancomycin released at each time point is significantly lower than in Experiment A. For both experiments, vancomycin concentration remained above MIC for *S. aureus* (1µg/mL), at least until 312 hours, and below MTC (50

µg/mL) for *S. aureus*, although the mass of granules used in these two experiments was higher than the amount used in Experiment C.

Concerning Experiment C (figure 16), where all PBS was replaced by fresh one three times a week, the concentration of vancomycin released in each time point was similar compared to an experiment (77) where 20% of PBS was replaced every 24h, using the same amount of granules.

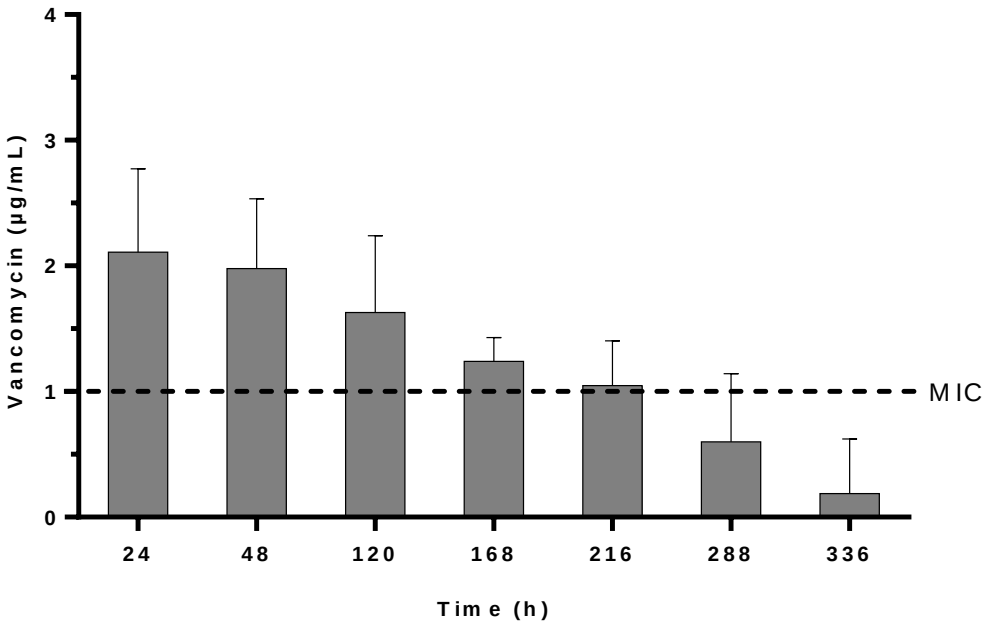


Figure 16. Vancomycin release profile from heparinized nanoHA/collagen granules over time. Experiment C (ratio = 20 mg/mL), where PBS was completely substituted by fresh one three times a week.

3.2. Bioactivity assay with *S. aureus*

Microbiology assays were performed with heparinized nanoHA/collagen granules, previously loaded with vancomycin in order to evaluate the amount of vancomycin being released and its capacity to inhibit *S. aureus* growth over time.

The results (figure 17) showed an inhibition of *S. aureus* growth in all 4 replicates up to 192 hours of antibiotic's release. From 312 hours of release, all replicates exhibited bacterial growth. Table 1 shows *S. aureus* growth inhibition by vancomycin being released from heparinized nanoHA/granules over time.

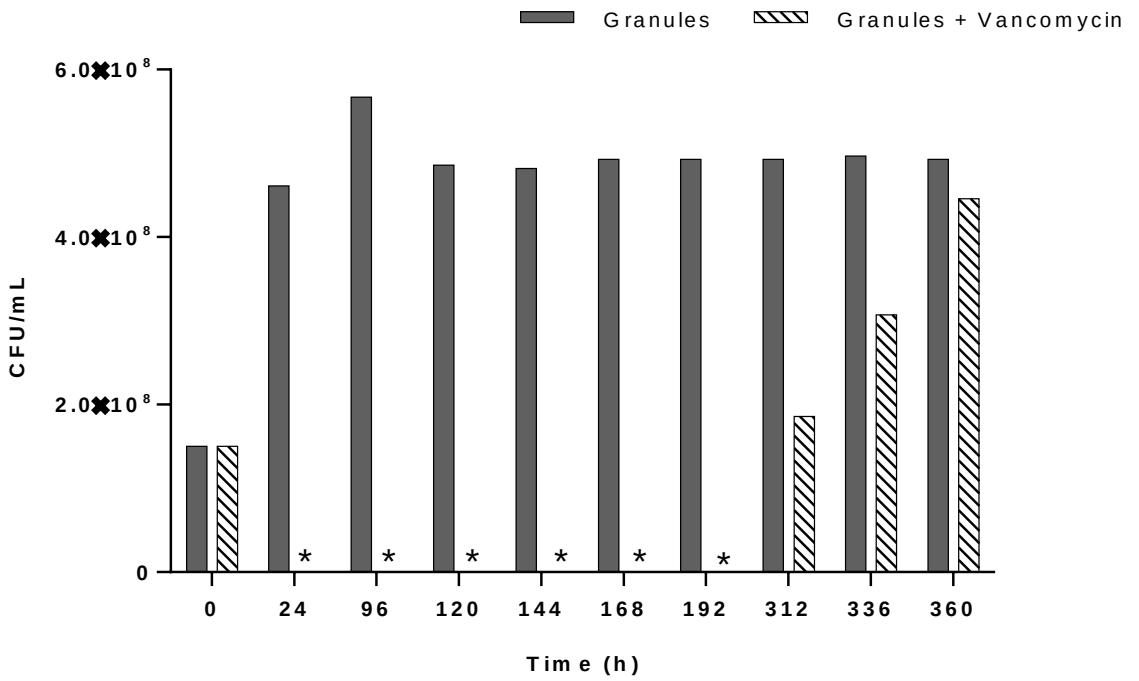


Figure 17. *S. aureus* growth in the presence of granules loaded with vancomycin compared to control (granules without vancomycin) versus time. Statistically significant differences were evident between replicates with and without vancomycin. * represents statistically significant differences when compared to *S. aureus* bioactivity at 0, 312, 336 and 360 hours. ($p<0.05$).

Table 1. *S. aureus* growth inhibition for each timepoint of released vancomycin. (-) No growth; (++) Growth in 2 replicates; (+++) Growth in 3 replicates; (++++) Growth in all replicates

Time (h)	24	48	72	96	120	144	168	192	216	240	264	288	312	336
S. aureus Growth	-	-	-	-	-	-	-	-	-	-	++	+++	++++	++++

3.3. Osteoclast culture

Tartrate-resistant Acid Phosphatase activity

Tartrate-resistant acid phosphatase activity (TRAP) was measured to determine the osteoclast differentiation. As observed in figure 18, TRAP activity increased in all materials combination, except for the condition with vancomycin.

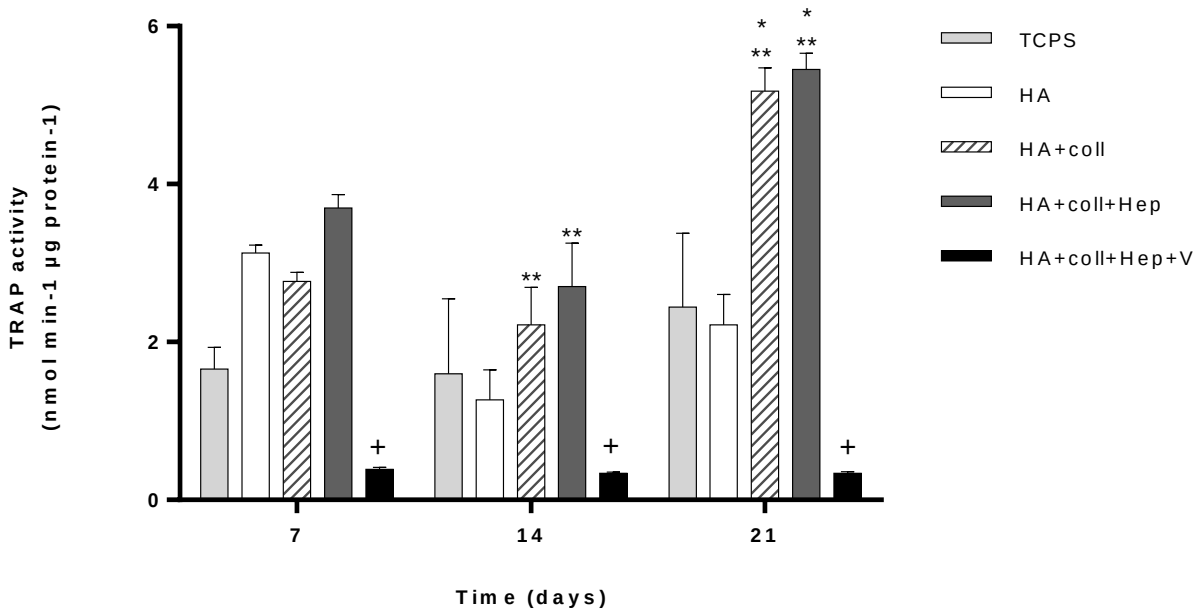


Figure 18. TRAP activity after 7, 14 and 21 days of osteoclasts cell culture with nanoHA, nanoHA/collagen, and nanoHA/collagen/Heparin granules (with and without vancomycin). TCPS was used as control and culture medium was changed once a week. * represents statistically significant differences when compared with the same material at 7 and 14 days of culture; ** represents statistically significant differences when compared to nanoHA granules at the same timepoint; + represents statistically significant differences when compared to granules without vancomycin and TCPS at the same timepoint. ($p<0.05$).

It is clear that vancomycin inhibits osteoclastogenesis at 7 days of culture and cells were not able to restore their function, even after 14 and 21 days of culture when the release of antibiotic is lower.

Comparing cell response to nanoHA/collagen and nanoHA/collagen/Heparin with TCPS (control), it is possible to see a significant osteoclast differentiation in the presence of the biomaterial after 21 days of culture. There were no statistical differences between osteoclasts cultured on nanoHA/collagen and nanoHA/collagen/Heparin.

As the effects of vancomycin showed to be devastating for osteoclast cells when medium was changed once a week, a comparative study was performed to test the leaching power by changing the medium culture three times a week. Osteoclast behavior was independent of culture medium since no statistically significant differences were observed between cultures (figure 19).

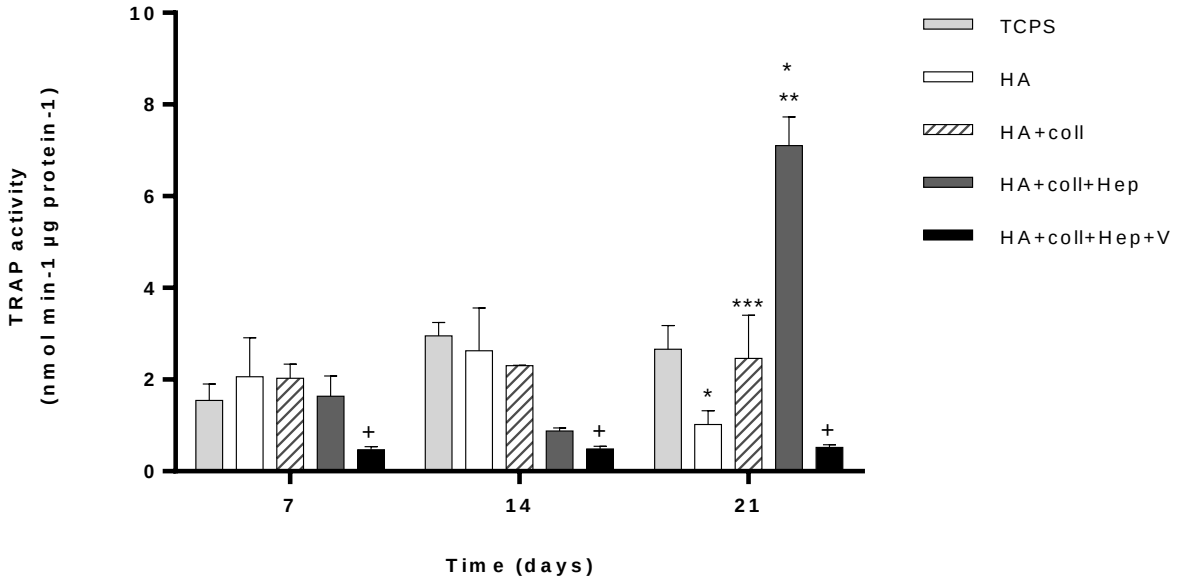


Figure 19. TRAP activity after 7, 14 and 21 days of osteoclast culture with nanoHA, nanoHA/collagen, and nanoHA/collagen/Heparin granules (with and without vancomycin). TCPS was used as control. Culture medium was changed 3 times a week. . * represents statistically significant differences when compared with the same material at 7 and 14 days of culture; ** represents statistically significant differences when compared with nanoHA/collagen granules at the same timepoint; *** represents statistically significant differences when compared to nanoHA granules at the same timepoint; + represents statistically significant differences when compared to granules without vancomycin and TCPS at the same timepoint. ($p<0.05$).

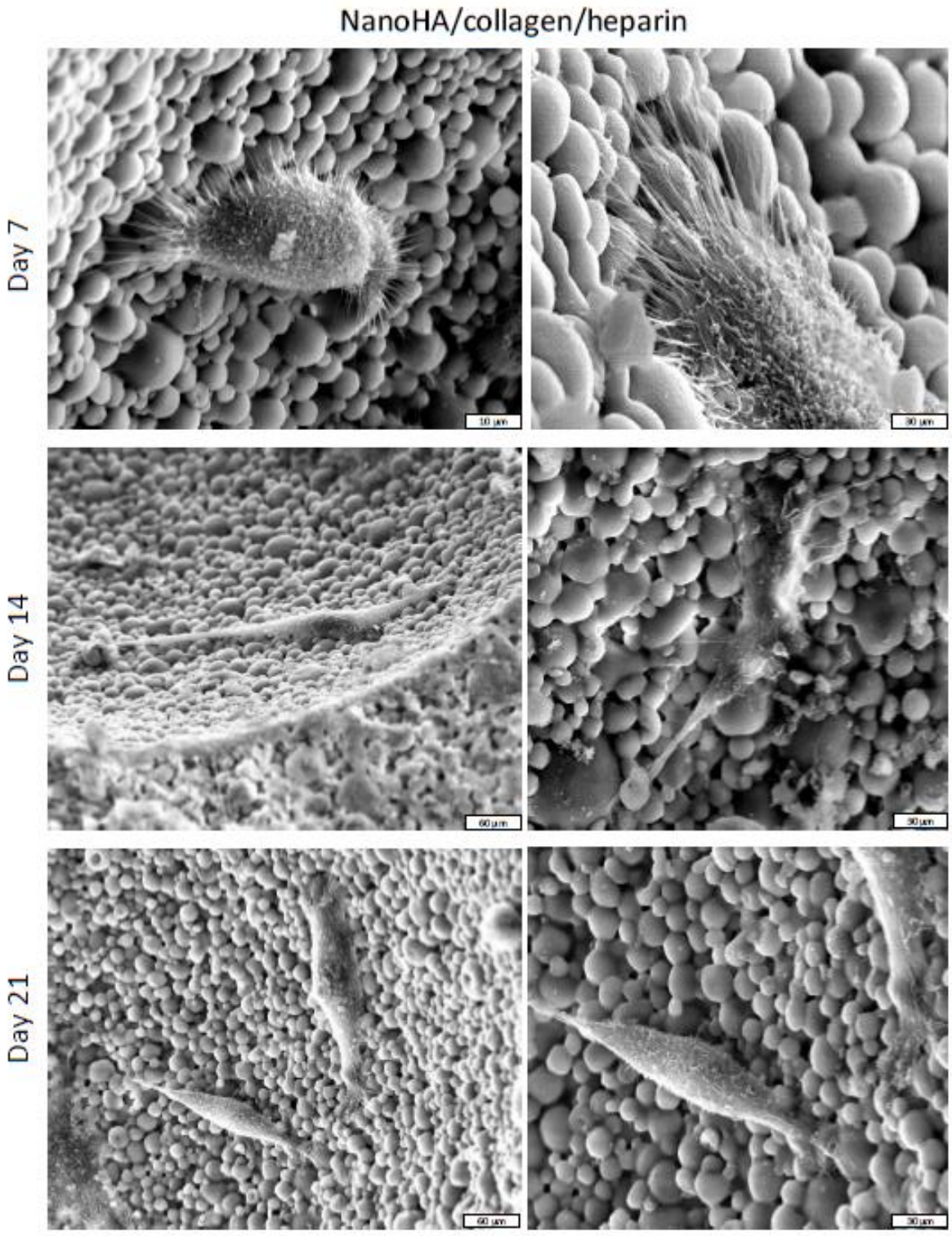
Cultures performed in the presence of heparinized nanoHA/collagen granules displayed significantly higher values of TRAP activity, when compared to cultures performed on other biomaterial combination at day 21. Cells cultured on heparinized material without vancomycin showed an increased cell differentiation from 14 to 21 days of culture.

Cell morphology

Osteoclast morphology on different materials combination, as previously described in "Materials and Methods", was possible to be assessed by SEM (Figure 21). But only results for nanoHA/collagen/Heparin (with and without vancomycin) were found relevant.

SEM images showed that cells adhered to the granules without vancomycin and were able to proliferate, acquiring elongated morphology at days 14 and 21. In the absence of vancomycin, there were no significant differences when comparing cells morphology on nanoHA/collagen/heparin and nanoHA or nanoHA/collagen granules, unlike the results shown for TRAP activity.

In the presence of vancomycin, round cells were observed until day 14 and at 21 days of culture. No cells were observed in any of the tested biomaterials.


NanoHA/collagen/heparin
Day 7
Day 14
Day 21

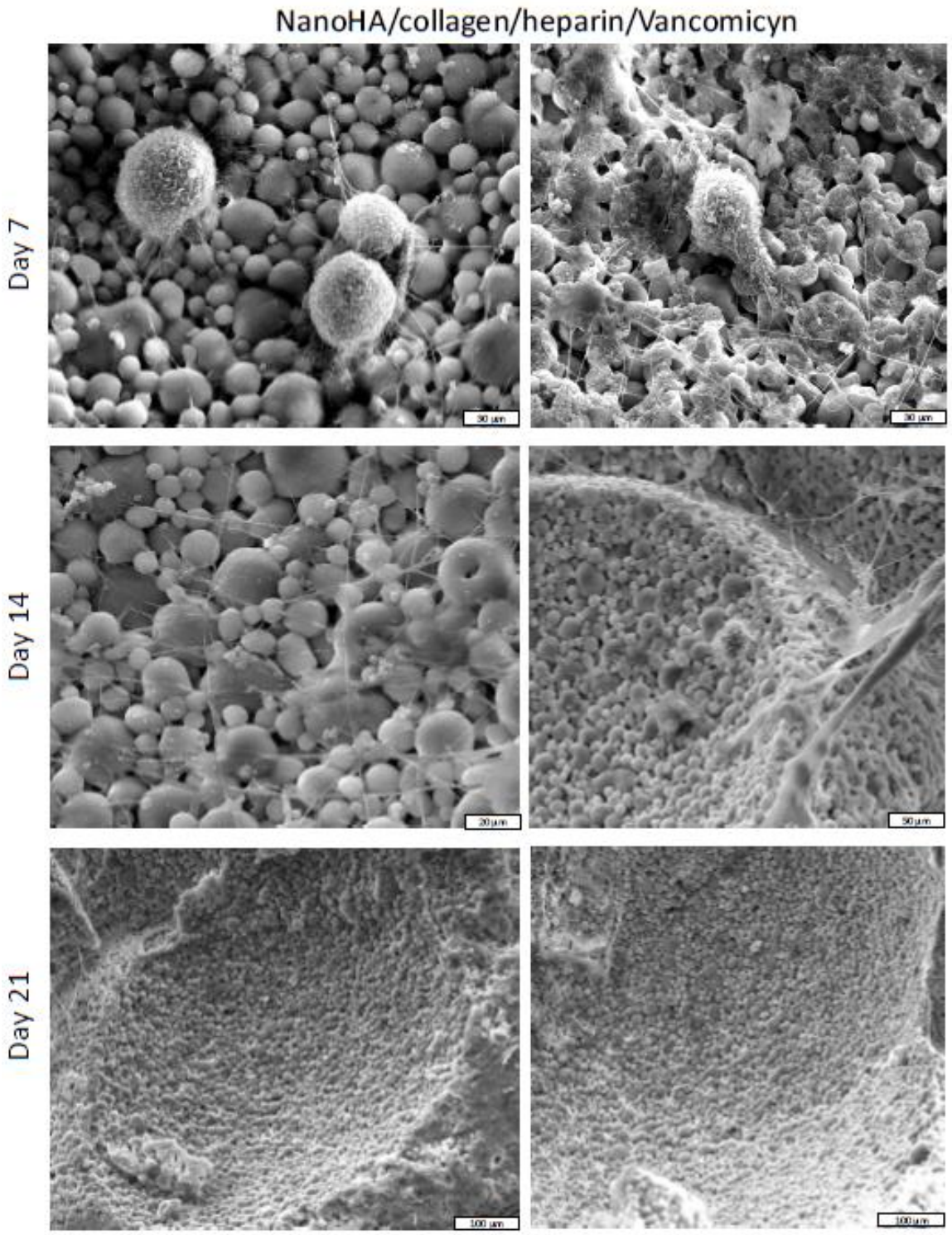


Figure 20. SEM images of osteoclasts on nanoHA/collagen/Heparin (with and without vancomycin)

3.4. Osteoblast culture

Resazurin test

Results of the Resazurin assay are represented in figure 21. These results show that metabolic activity of MG63 significantly decreased in the presence of vancomycin compared to TCPS. Vancomycin did not affect substantially cell proliferation in the first 24h but after 7 days of culture, metabolic activity was significantly lower.

At 21 days of culture, metabolic activity has increased in all tested materials when compared to the previous timepoint (14 days). No statistical differences were found between cells cultured with and without vancomycin, at 14 and 21 days of culture.

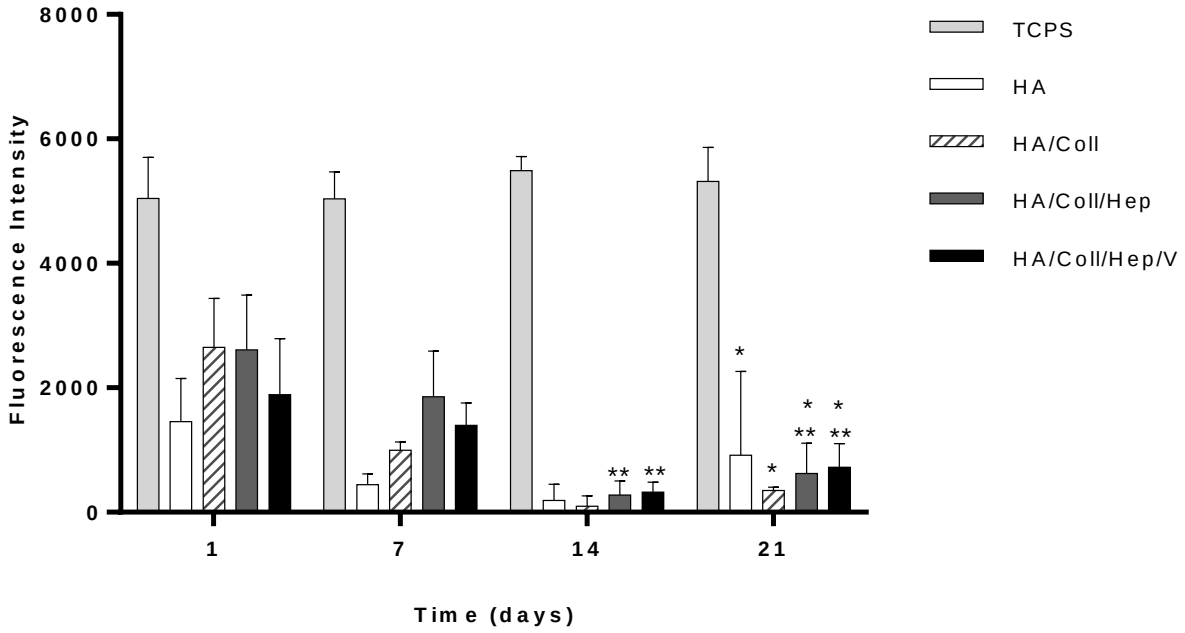


Figure 21. Proliferation of MG63 cells cultured on nanoHA, nanoHA/collagen, nanoHA/collagen/Heparin and nanoHA/collagen/Heparin/Vancomycin granules for 1, 7, 14 and 21 days, estimated by Resazurin assay. * represents statistically significant differences when compared with the same material at 14 days of culture; ** represents statistically significant differences when compared with nanoHA/collagen granules at the same timepoint, ($p<0.05$).

Alkaline Phosphate Activity (ALP)

Figure 22 shows that ALP activity diminished with culture time in all materials tested in the present study.

Excluding TCPS (control), the highest and more consistent values for ALP activity at 7, 14 and 21 days of culture were observed for nanoHA/collagen/heparin material. These values were significantly higher when compared to MG63 cultured on nanoHA and on nanoHA/collagen/heparin/vancomycin but showed no statistically differences when compared to MG63 cultured on nanoHA/collagen granules.

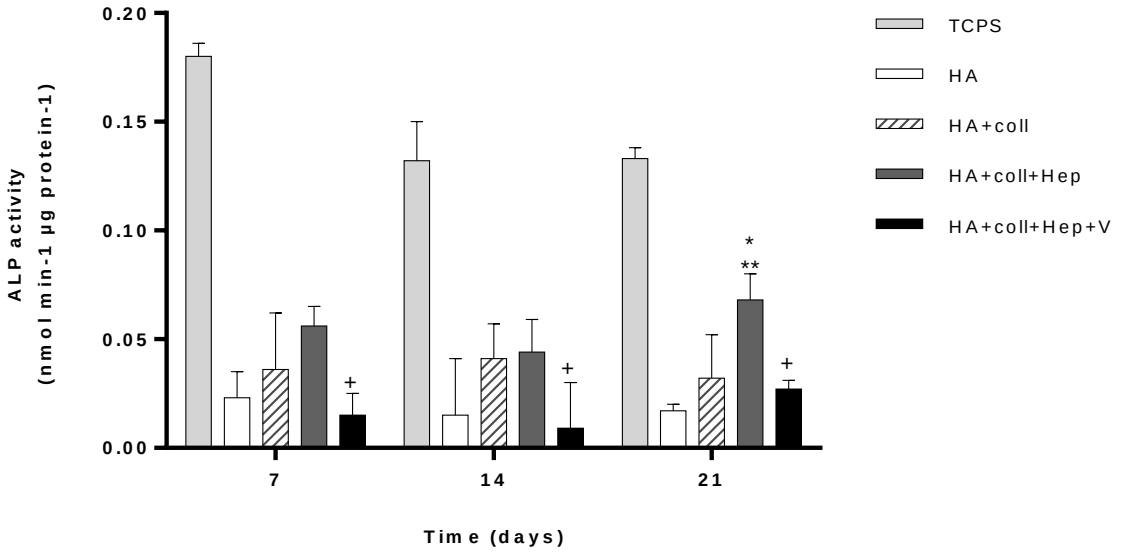


Figure 22. ALP activity of MG63 culture on nanoHA/collagen/Heparin granules (20 mg/L), with and without vancomycin after 7, 14 and 21 days of culture. . * represents statistically significant differences when compared with the same material at 7 and 14 days of culture; ** represents statistically significant differences when compared with nanoHA, nanoHA/collagen and nanoHA/collagen/heparin/vancomycin granules at the same timepoint; + represents statistically significant differences when compared to granules without vancomycin and TCPS at the same timepoint. ($p<0.05$).

MG63 cells were cultured under similar conditions but using a 100 mg/L ratio of granules instead of 200 mg/L. This preliminary study showed that the previously used ratio in osteoblast cultures might be inadequate for those cells growth (figure 23).

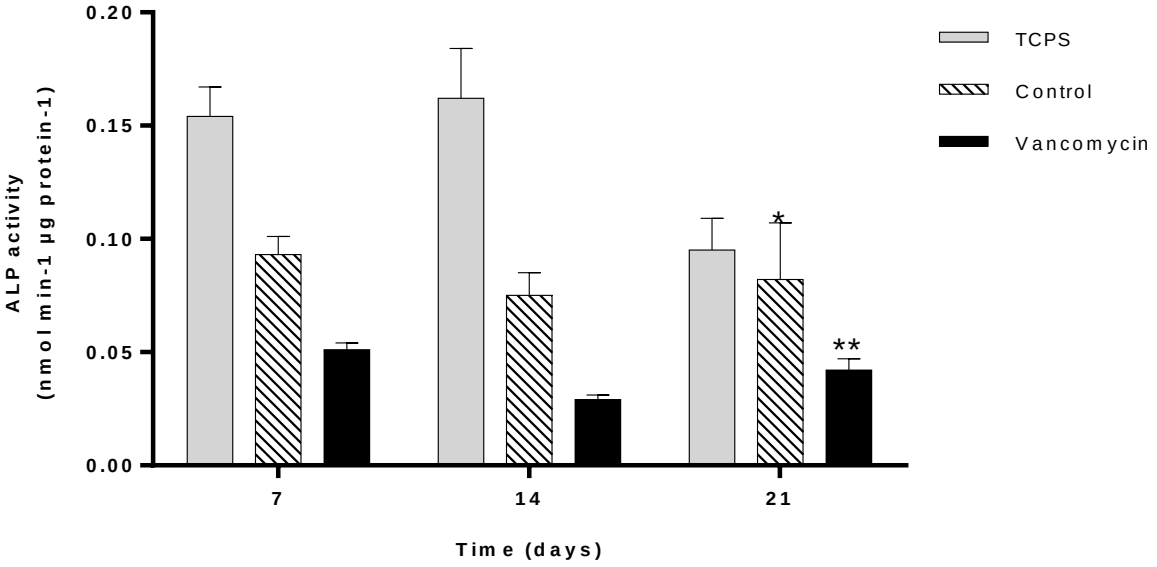


Figure 23. ALP activity of MG63 culture on nanoHA/collagen/Heparin granules (ratio = 100 mg/L), with and without vancomycin after 7, 14 and 21 days of culture. * represents statistically significant differences when compared with the same material at 7 and 14 days of culture; ** represents statistically significant differences when compared with the same material at 14 days of culture. ($p<0.05$).

Chapter 4: Discussion

Osteomyelitis is a bone infection usually accompanied by bone necrosis which is stimulated by an inflammation process. This process is responsible for the vascular channels compression, causing ischemia and resulting in necrosis of the adjacent tissue. As a consequence, the antibiotics administered by oral or intravenous route may not be able to reach the affected area and the treatment of osteomyelitis might fail. (18, 21) Hence, a local delivery of antibiotics from an implantable bone replacement material offers considerable advantages over these traditional methods. (41, 78)

It has been reported that a local and sustained release system of antibiotic in the treatment of this infection is advantageous as it maintains a localized release of the drug in adequate amounts and a more effective control of bacterial growth. (79) The nanoHA/collagen composites used in this work were specifically designed as a controlled drug delivery system to carry an antibiotic intended to locally treat bone infection. In this case the chosen antibiotic to treat osteomyelitis was vancomycin as glycopeptides are known to be very effective antibiotics against gram-positive bacteria, namely *S. aureus* and methicillin-resistant *Staphylococcus aureus* (MRSA). (32, 80)

Previously, these composites were studied and presented adequate dimensions (1.18-1.70 mm) to fill bone defects including those caused by infections in humans and to perform in vivo studies in rat models. (77) Granules with similar dimensions used for bone substitution were successfully used in clinical application and are already in the market. (81)

Strength and macroporosity, besides the micro and nanoporosity of these biomaterial were also ideal to be used in osteomyelitis cases,(77) inducing bone-like apatite formation (82) and enhancing initial cell attachment (83) . The granules were characterized by ATR-FTIR and micro-CT, showing that the material had adequate porosity (62.7 ± 1.5 %) for bone regeneration. The average mean pore size obtained (227.3 ± 7.2 μm) is adequate for osteogenesis. The granules also presented adequate porosity for osteoblasts invasion and mobility to the most inner regions of the granules (77) once osteoblast average size is 20 – 30 μm. (84)

Concerning collagen concentration used in the granules, 0.05 % was considered the most suitable among all concentrations tested. The inclusion of collagen provides a better interaction between the material and molecules, bacteria and cells as it happens in bone. (77) These biomaterials are also designed to promote osseointegration and osteoconductivity.(85) It is expected that nanoHA/collagen composites favor the

proliferation and differentiation processes of bone cells and accelerate the damaged tissue healing process. Therefore, the study of vancomycin influence continuously released from heparinized nanoHA/collagen composites on osteoblasts and osteoclasts cells is of great interest for bone tissue regeneration.

Considering the vancomycin release from granules, results showed that in experiment A the granules were able to release antibiotic in a sustainable way and enough to inhibit bacterial growth. The same was observed in experiment B and C. So, changing the medium (replacing 20 % of the PBS every 24 hours and replacing all the PBS three times a week) seems not to have influenced the release profile of vancomycin.

The results showed that even under extreme conditions, as observed in experiment B (total replacement of PBS), the controlled release profile of vancomycin was identical. So, based on results of this study, we can predict that under normal conditions the vancomycin release pattern from heparinized nanoHA/collagen granules should be similar.

Although the release profile seems to be adequate, *in vivo* conditions might be altered namely the pH found in the infection site. Therefore, it must be taken in account that this release profile of vancomycin can be completely altered once that the local pH in the vicinity of infection site is different from the pH of PBS used to perform this study. (86) Vancomycin is slowly released in PBS because it becomes less positive when in contact with the buffer solution (77) and therefore the links stablished between the biomaterial and the antibiotic become weaker, allowing the latter releasing. Initially, rapid release of antibiotic in local site of infection might be favorable in order to prevent bacterial resistance. (87) Consequently, an initial burst of vancomycin release may be advantageous as observed in this study.

Concerning the bioactivity studies of *S. aureus*, directly exposed to vancomycin released from heparinized nanoHA/collagen granules, the bacteria were not able to grow up to 192 hours of antibiotic release, as shown in figure 17, which means that the amount of vancomycin being released remained above MIC. (41) These results were expected since vancomycin release profile in PBS assay showed a controlled antibiotic release over time, presenting values considered adequate to inhibit bacterial growth.

A previous study (77) that used Broth microdilution method, showed that the released vancomycin was able to inhibit bacterial growth up to 216 hours and assumed that the dilution of released vancomycin concentration was responsible for the loss of capability to inhibit *S. aureus* growth.

In the clinical setting, a fluorescence polarization immunoassay (FPIA) is used to determine the concentration of vancomycin present in patient's serum. FPIA measures tracer binding directly by using competitive-binding assay principles. (88) The technique requires specific instrumentation to measure the polarized fluorescent light and the assay is generally more expensive. (89) Therefore, as it was impossible to measure vancomycin concentration directly in patients' serum with FPIA, the method used in the present study was a direct method to assess vancomycin release from the granules.

Once the antibiotic is being directly released on the bacteria, the direct method used allows a better simulation of the vicinity of the infection, avoiding the limitations of the Broth Microdilution method described above. The evaluation of bacterial bioactivity indicated that heparinized nanoHA/collagen granules loaded with vancomycin should be able to eradicate the bacteria after a shorter period of treatment than the one presently used in clinical applications by intravenous administration of antibiotic, which is usually 4 to 6 weeks. (24, 90, 91) In some cases, it can be even extended up to 6 months. (91)

By crossing data with the release profile of vancomycin from nanoHA/collagen in PBS assay (figure 24), the bacterial growth after 216 hours may be due to insufficient vancomycin release to inhibit *S. aureus* growth, i.e. the concentrations of vancomycin might be below *S. aureus* MIC (1 µg/mL), at this point.

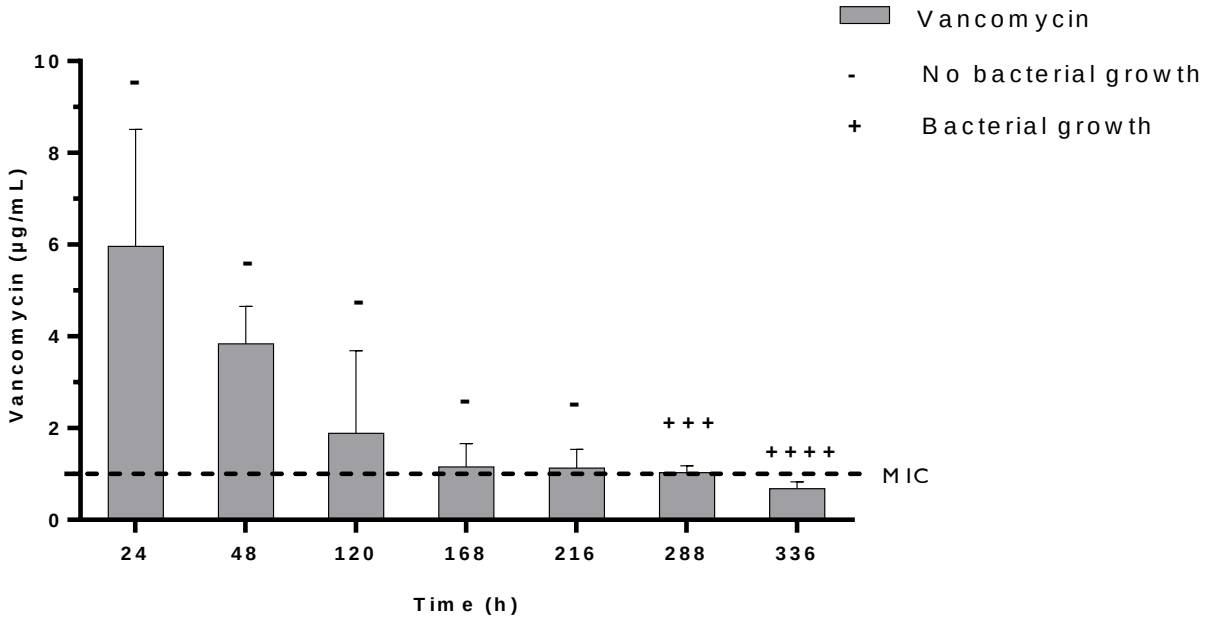


Figure 24. *S. aureus* growth inhibition for each timepoint of released vancomycin. (-) No growth; (+++) Growth in 3 replicates; (++++) Growth in all replicates.

Osteomyelitis is well known by the damage it causes in bone, presenting general vascular insufficiency which leads to bone necrosis. Several studies suggest that a successful management of the infection requires a combination of target antibiotic therapy and surgical interventions, which implicates bone debridement to remove dead tissue. (23, 29, 86, 90) It is important to take into account that the delivery system based on heparinized nanoHA/collagen granules loaded with vancomycin has as main purpose to eradicate the bacteria responsible for inducing osteomyelitis and, at the same time, to promote bone regeneration, by stimulating cells responsible for the bone remodeling process.

Osteoblast-like MG63 and human osteoclasts cells cultures were performed to assess cells behavior in the presence of the antibiotic. The results from both cultures showed a substantial decrease of cells' metabolic activity and differentiation over time when in contact with vancomycin.

Regarding osteoclasts culture, vancomycin did not reveal to be cytotoxic but instead a cytostatic effect on cells seems to occur. Cells' differentiation was low and presented a similar TRAP activity while cells cultured without vancomycin showed increased values over time.

Initial studies, where the culture medium was changed once a week (Figure18), showed a marked TRAP activity decrease in cells exposed to vancomycin, suggesting a low cell differentiation and cells exposed to vancomycin were unable to recover activity. However, osteoclasts cultured without vancomycin, namely nanoHA/collagen and heparinized nanoHA/collagen granules, increased significantly TRAP expression when compared to nanoHA granules, indicating a higher cell differentiation at 21 days of culture. Data suggest that collagen plays an important role in bone cells behavior, enhancing cell adhesion as also shown by other authors. (92) Studies suggested that collagen induces differentiation, (93) enhancing cells adhesion. (94) Since collagen is one of the main components of bone tissue, cells probably recognize the material as if it were real bone.

A new culture was performed under similar conditions but changing the culture medium three times a week to exclude the possibility of medium saturation by the vancomycin being released from the granules. In accordance to the release profile observed in figure 16, during the first days of culture, vancomycin released from granules was considerably higher.

The medium change does not seem to interfere in osteoclast differentiation since significant changes in TRAP activity were not observed.

Although the results did not significantly differ from the ones obtained with the first cultures (figure 18), cells also showed a significantly improved behavior in terms of differentiation when in contact with heparinized nanoHA/collagen granules.

Heparin seems to stimulate osteoclasts activity. It was reported by Atsushi *et al* that this glycosaminoglycan enhances bone resorption in rat osteoclasts in vitro (95) and Chowdhury *et al* also described an increase in osteoclast number in the presence of heparin. (96)
Concerning osteoclasts activity, it is known that bone resorption can be inhibited by physiological regulators such as Osteoprotegerin (OPG), Nitric Oxide, interleukins 4 and 18, Transforming Growth Factor beta (TGF-β), interferon gamma and systemic hormones (PTH, calcitonin and prostaglandins). (97, 98)
Some of these factors are secreted by osteoclasts, specially Nitric Oxide and TGF-β (99) (a potent inhibitor of osteoclastic bone resorption), (97) in response to external factors. In this case, high levels of vancomycin released from the granules associated to the small amount of medium used in the culture (100 µL) would be the external factor that stimulates the secretion of osteoclastic inhibitor factors.
In terms of MG63 osteoblast-like cells proliferation, it was possible to observe that metabolic activity decreased until day 14 in all tested materials and that heparinized nanoHA/collagen granules resulted in higher overall cell growth than nanoHA/collagen granules from 14 to 21 days of culture as nanoHA granules (figure 21).

As well as in osteoclasts culture, vancomycin showed a negative effect on MG63 differentiation (figure 22).
Although ALP activity was significantly lower in the presence of vancomycin when compared to MG63 cultured on nanoHA/collagen or heparinized nanoHA/collagen granules, ALP activity was considerable increased from 14 to 21 days of culture, meaning that cells might be able to restore their normal activity as vancomycin release from granules diminished over time. This result proved the efficacy of the nanoHA/collagen composites in enhancing the osteoblastic phenotype expression level and also osteogenic traits, as it has been already reported by Shiao-Wen Tsai *et al.* (100)
The comparison between the MG63 osteoblast-like cells culture using ratios of 100 mg/L and 200 mg/L granules showed that the 100 mg/L ratio performed better. More test must be carried out on the 200 mg/L as this ratio appears to be excessive for the available volume of culture medium inside the wells.
These findings are supported by a study (77) performed with of MC3T3-E1 cells on granules containing vancomycin and using a 100 mg/L ratio. C. Coelho (77) observed that metabolic activity significantly increased even after being exposed to the antibiotic.

The literature (101) also suggests that it is important to take into account these results that shows how even after being exposed to high levels of antibiotic, the remaining viable cells might be able to recover their osteogenic potential and proliferate. Rathbone *et al* studies revealed that very high concentrations of vancomycin (2000 µg/mL) were not enough to inhibit human osteoblasts cells normal activity.(101)

Regarding cell morphology, only preliminary assays were performed. Few or no cells attached to the material were observed by SEM and CLSM. This is probably a consequence of wrong preparation of the samples for microscopy observation.

Chapter 5: Concluding Remarks and Future work

5.1. Concluding remarks

In this study, a nanoHA/collagen composite was used as a Drug Delivery System for vancomycin and the effects of this antibiotic on bone cells behavior were evaluated.

Regarding the vancomycin release profile from heparinized nanoHA/collagen granules, it was observed that replacing 20 % of the PBS every 24 hours or replacing all the PBS three times a week did not affect vancomycin release profile, whose pattern was preserved.

This study has also shown that heparinized nanoHA/collagen granules are a suitable carrier for controlled release of vancomycin once that they were able to release antibiotic in a sustainable way and in enough amounts to inhibit *S. aureus* growth at least for 192 hours.

In fact, heparinized nanoHA/collagen granules were successfully used as drug carrier for a controlled release of vancomycin. This antibiotic seems to reveal a cytostatic effect on both osteoblasts and osteoclasts differentiation. Both cultures showed a substantial decrease of cells' metabolic activity and differentiation over time when in contact with vancomycin. NanoHA/collagen and heparinized nanoHA/collagen granules showed better properties for cell adhesion, proliferation and differentiation.

In osteoclasts culture, the change of medium did not seem to interfere with those cells' differentiation since significant changes in TRAP activity were not observed. But cells showed a significantly better behavior in terms of differentiation when in contact with heparinized nanoHA/collagen granules if culture medium was changed three times a week.

Concerning MG63 osteoblast-like cells culture, metabolic activity did not decrease in the initial 14 and 21 days after culture and, at this point, it was expected that cells would be able to recover their function and proliferate. Probably the 200 mg/L ratio used was not the ideal for cell growth.

As a conclusion, the heparinized nanoHA/collagen material proved to have a great potential to be used as DDS in clinical applications, namely in osteomyelitis treatment. The nanoHA/collagen granules used in this study have adequate dimensions, strength and macroporosity to be applied in those cases as they were designed to promote osseointegration and osteoconductivity. Although the cell cultures did not present the ideal conditions, bone cells were capable to adhere and proliferate on the material without vancomycin.

5.2. Future work

Concerning the MG63 osteoblast-like cells culture using a 100 mg/L ratio, the assay must be repeated in order to validate the preliminary results obtained. Therefore, further investigation are planned in order to establish the perfect culture conditions, namely concerning the amount of granules, as well as the techniques used in the experiment.

A co-culture system with osteoblasts and osteoclasts should be tried in order simulate a more likely bone environment and, therefore, assess the influence of the heparinized nanoHA/collagen material and vancomycin. Moreover, vancomycin release profile from the heparinized nanoHA/collagen granules must be tested in the infection environment once the conditions presented are quite different and may influence vancomycin's release.

In vivo studies on osteomyelitis rat models are already being performed to assess the possible effectiveness of this biomaterial in the treatment of osteomyelitis. It would be also interesting to explore vancomycin's bioactivity in other bacteria such as *Staphylococcus epidermidis* and MRSA and in VRSA, once that the occurrence of vancomycin resistant strains has been reported.

Additionally, fluorescence polarization immunoassay, which is commonly used in clinical practice, should be tried in order to assess vancomycin concentration directly from the patients' serum.

References

(1) SALGADO, A. J., COUTINHO, O. P. & REIS, R. L. 2004. Bone tissue engineering: State of the art and future trends. Macromolecular Bioscience 4, 743–765.

(2) RAGGATT, L. J. & PARTRIDGE, N. C. 2010. Cellular and molecular mechanisms of bone remodeling. The Journal of Biological Chemistry 285, 25103–25108.

(3) KINI, U. & NANDEESH, B. N. - **Physiology of bone formation, remodeling, and metabolism.** *In:* Radionuclide and hybrid bone imaging. ed.: FOGELMAN, I., GOPINATHGNANASEGARAN & WALL, H. v. d. (ed.)^(eds.),2012. 29-57.

(4) CLARKE, B. 2008. Normal bone anatomy and physiology. Clinical Journal of American Society of Nephrology 3, 131–139.

(5) WANG, X., *et al.* - **Fundamental biomechanics in bone tissue engineering**. Morgan &vClaypool Publishers, 2010.

(6) TATON, T. A. 2001. Boning up on biology. Nature, 412, 491-492.

(7) *Bones and skeletal tissues* [Online]. Available: http://classes.midlandstech.edu/carterp/Courses/bio210/chap06/lecture1.html [Accessed August 28th 2014].

(8) COWIN, S. C. & HEGEDUS, D. H. 1976. Bone remodeling i: Theory of adaptive elasticity. Jornal of Elasticity, 6, 313-326.

(9) *Osteomyelitis in adults* [Online]. Clinical Key Elsiever Available: https://www.clinicalkey.com/topics/orthopedic-surgery/osteomyelitis-in-adults.htmld [Accessed June 2nd 2014].

(10) ZIMMERMAN, M. & SNOW, B. - **Essentials of nutrition: A functional approach**. Flat World Education, Inc,. 2012.

(11) OSGOOD, E. E. & SEAMAN, A. J. 1944. The cellular composition of normal bone marrow as obtained by sternal puncture. American Physiological Society - Physiological Reviews 24, 46-69.

(12) HADJIDAKIS, D. J. & ANDROULAKIS, I. I. 2007. Bone remodeling. Annals of the New York Academy of Sciences, 1092, 385-396.

(13) RAISZ, L. G. 1999. Physiology and pathophysiology of bone remodeling. Clinical Chemistry 45, 1353-1358.

(14) HAYMAN, A. R., *et al.* 2000. Osteoclastic tartrate-resistant acid phosphatase (acp 5): Its localization to dendritic cells and diverse murine tissues. Journal of Hystochemistry and Cytochemistry 48, 219-227.

(15) JR., M. M. C. 2006. The new bone biology: Pathologic, molecular, and clinical correlates. American Journal of Medical Genetics, 140A 2646–2706.

(16) RUCCI, N. 2008. Molecular biology of bone remodelling. Clinical Cases in Mineral and Bone Metabolism, 5, 49-56.

(17) WEILBAECHER, K. N., GUISE, T. A. & MCCAULEY, L. K. 2011. Cancer to bone: A fatal attraction. Nature Reviews Cancer, 11, 411-425.

(18) HEALTH, W. K. - **Professional guide to diseases**.9th: Lippincott Williams & Wilkins, 2009.

(19) *Pain management health center: Osteomyelitis* [Online]. WebMD. Available: http://www.webmd.com/pain-management/osteomyeltis-treatment-diagnosis-symptoms [Accessed June 4th 2014].

(20) LONGO, D. L., *et al.* - **Harrison's principles of internal medicine**.18th: The McGraw-Hill 2012.

(21) LEW, D. P. & WALDVOGEL, F. A. 2004. Osteomyelitis. Lancet, 364:369-79.

(22) BAUTISTA, S. R., GHOLVE, P. & DORMANS, J. P. 2006. Pediatric musculoskeletal infections: Advances in diagnosis and management. Consultant for Pediatricians 360, 5.
(23) BRADY, R. A*., et al.* 2008. Osteomyelitis and the role of biofilms in chronic infection Federation of European Microbiological Societies, 52, 13-22.
(24) HATZENBUEHLER, J. & PULLING, T. J. 2011. Diagnosis and management of osteomyelitis. American Family Physician, 84, 1027-1033.
(25) *Osteomyelitis types* [Online]. Antimicrobial Therapy - The ultimate reference. Available: http://www.antimicrobe.org/new/printout/e12printout/e12type.htm [Accessed June 3rd 2014].
(26) HARRIS, L. G. & RICHARDS, R. G. 2006. Staphylococci and implant surfaces: A review. Injury, International Journal of the Care of the Injured, 3-14.
(27) PRESCOTT, L. M. - **Prescott, harley and klein's microbiology**.5th: The McGraw Hill Companies, 2002.
(28) LOWY, F. D. 1998. Staphylococcus aureus infections. The New England Journal Of Medicine 339, 520-532.
(29) EL-GHANNAM, A., AHMED, K. & OMRAN, M. 2004. Nanoporous delivery system to treat osteomyelitis and regenerate bone: Gentamicin release kinetics
and bactericidal effect. Journal of biomedical Materials Research. Part B, Applied Biomaterials, 73, 277-284.
(30) GORDON, R. J. & LOWY, F. D. 2008. Pathogenesis of methicillin-resistant staphylococcus aureus infection. Clinical Infectious Diseases, 46.
(31) FOSTER, T. J. & GEOGHEGAN, J. A. 2014. Adhesion, invasion and evasion: The many functions of the surface proteins of staphylococcus aureus. Nature Reviews - Microbiology, 12.
(32) SCHÄFER, M., SCHNEIDER, T. R. & SHELDRICK, G. M. 1996. Crystal structure of vancomycin. Current Biology, 4, 1509-1515.
(33) SATTUR, A. P*., et al.* 2000. Analytical techniques for vancomycin - a review Biotechnology and Bioprocess Engineering, 5, 153-158.
(34) *Vancomycin structure* [Online]. PubChem Compound - NCBI: National Center for Biotechnology Information. Available: http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=14969&loc=ec_rcs#itabs-2d [Accessed July 15th 2014].
(35) NAGARAJAN, R. 1991. Antibacterial activities and modes of action of vancomycin and related glycopeptides. Antimicrobial Agents And Chemotherapy, 35, 605-609.
(36) KIM, S. J*., et al.* 2002. Rotational-echo double resonance characterization of vancomycin binding sites in staphylococcus aureus. Biochemistry 41, 6967-6977.
(37) LAI, C.-Y*., et al.* 2003. A mesoporous silica nanosphere-based carrier system with chemically removable cds nanoparticle caps for stimuli-responsive controlled release of neurotransmitters and drug molecules. Journal of the American Chemical Society 125, 4451-4459.
(38) STRAUS, S. K. & HANCOCKB, R. E. W. 2006. Mode of action of the new antibiotic for gram-positive pathogens daptomycin: Comparison with cationic antimicrobial peptides and lipopeptides. Biochimica et Biophysica Acta (BBA) - Biomembranes, 1758, 1215-1223.
(39) YANG, S.-J*., et al.* 2012. The staphylococcus aureus two-component regulatory system, grars, senses and confers resistance to selected cationic antimicrobial peptides. Journal of Infection Immunity, 80, 74-81.
(40) ANDREWS, J. M. 2001. Determination of minimum inhibitory concentrations. Journal of Antimicrobial Chemotherapy, 48, 5-16.

(41) DION, A., *et al.* 2005. Vancomycin release behaviour from amorphous calcium polyphosphate matrices intended for osteomyelitis treatment. Biomaterials, 26, 7276–7285.
(42) JIANG, J.-L., *et al.* 2012. Vancomycin-loaded nano-hydroxyapatite pellets to treat mrsa-induced chronic osteomyelitis with bone defect in rabbits. Inflammation Research, 61, 207-215.
(43) WAHL, D. & CZERNUSZKA, J. 2006. Collagen-hydroxyapatite composites for hard tissue repair. European Cells and Materials, 11, 43-56.
(44) LIAO, J., *et al.* 2009. Development of nano-hydroxyapatite/polycarbonate composite for bone repair. Journal of Biomaterials Applications, 1-16.
(45) AHN, E. S., *et al.* 2001. Nanostructure processing of hydroxyapatite-based bioceramics. NanoLetters 1, 149-153.
(46) FERRAZ, M. P., *et al.* 2007. Nanohydroxyapatite microspheres as delivery system for antibiotics : Release kinetics, antimicrobial activity, and interaction with osteoblasts. Journal of Biomedical Materials Research, 81A, 994–1004, GUO, L., HUANG, M. & ZHANG, X. 2003. Effects of sintering temperature on structure of hydroxyapatite studied with rietveld method Journal of biomaterials Science: Materials in Medicine, 14, 817-822.
(47) KALITA, S. J., BHARDWAJ, A. & BHATT, H. A. 2007. Nanocrystalline calcium phosphate ceramics in biomedical engineering. Materials Science and Engineering 27, 441-449.
(48) WERNER, J., *et al.* 2002. Mechanical properties and in vitro cell compatibility of hydroxyapatite ceramics with graded pore structure. Biomaterials, 2002, 4285-4294.
(49) ZHU, W., *et al.* 2009. Experimental study of nano-ha artificial bone with different pore sizes for repairing the radial defect. International Orthopaedics, 33, 567–571.
(50) ELLIOTT, J. C. - Calcium phosphate biominerals. *In:* Phosphates: Geochemical, geobiological, and materials importance. ed. Reviews in Mineralogy and Geochemistry: (ed.)^(eds.),2002. 427-453, SANOSH, K. P., *et al.* 2009. Preparation and characterization of nano-hydroxyapatite powder using sol–gel technique. Bulletin of Materials Science, 32, 465–470.
(51) LARANJEIRA, M. S., FERNANDES, M. H. & MONTEIRO, F. J. 2010. Innovative macroporous granules of nanostructured-hydroxyapatite agglomerates: Bioactivity and osteoblast-like cell behaviour. Journal of Biomedical Materials Research, 95A, 891-900.
(52) RIMAN, R. E., *et al.* 2002. Solution synthesis of hydroxyapatite designer particulates. Solid State Ionics, 151, 393-402.
(53) WAHL, D. A. & CZERNUSZKA, J. T. 2006. Collagen-hydroxyapatite composites for hard tissue repair. Jounal of European Cells and Materials, 11, 43-56.
(54) WEI, G. & MA, P. X. 2004. Structure and properties of nano-hydroxyapatite/polymer composite scaffolds for bone tissue engineering. Biomaterials, 25, 4749-4757.
(55) FERRAZ, M. P., MONTEIRO, F. J. & MANUEL, C. M. 2004. Hydroxyapatite nanoparticles: A review of preparation methodologies. Journal of Applied Biomaterials & Biomechanics 2, 74-80.
(56) RIBEIRO, N., SOUSA, S. R. & MONTEIRO, F. J. 2010. Influence of crystallite size of nanophased hydroxyapatite on fibronectin and osteonectin adsorption and on mc3t3-e1 osteoblast adhesion and morphology Journal of Colloid and Interface Science, 351, 398-406.
(57) RAUSCHMANN, M. A., *et al.* 2005. Nanocrystalline hydroxyapatite and calcium sulphate as biodegradable composite carrier material for local delivery of antibiotics in bone infections. Biomaterials, 26, 2677-2684.
(58) CEN, L., *et al.* 2008. Collagen tissue engineering: Development of novel biomaterials and applications. Pediatric Research, 63.

(59) THOMAS, V., *et al.* 2007. Nanostructured biocomposite scaffolds based on collagen coelectrospun with nanohydroxyapatite. Biomacromolecules, 8, 631-637.
(60) LEE, C. H., SINGLA, A. & LEE, Y. 2001. Biomedical applications of collagen. International Journal of Pharmaceutics 221, 1–22.
(61) ROSSERT, J., EBERSPAECHER, H. & CROMBRUGGHE, B. d. 1995. Separate cis-acting DNA elements of the mouse pro-cd(i) collagen promoter direct expression of reporter genes to different type i collagen-producing cells in transgenic mice The Journal of Cell Biology, 129, 1421-1432.
(62) GRANT, M. E. 2007. From collagen chemistry towards cell therapy – a personal journey. International Journal of Experimental Pathology, 88, 203-214.
(63) FRIESS, W. 1998. Collagen – biomaterial for drug delivery. European Journal of Pharmaceutics and Biopharmaceutics, 45, 113-136.
(64) MAEDA, M., SANO, S. T. & FUJIOKA, K. 1999. Microstructure and release characteristics of the minipellet, a collagen-based drug delivery system for controlled release of protein drugs. Journal of Controlled Release, 62, 313-324.
(65) SCABBIA, A. & TROMBELLI, L. 2004. A comparative study on the use of a ha/collagen/chondroitin sulphate biomaterial (biostites) and a bovine-derived ha xenograft (bio-osss) in the treatment of deep intra-osseous defects. Journal of Clinical Periodontology, 31, 348-355.
(66) DU, C., *et al.* 1998. Tissue response to nano-hydroxyapatite/collagen composite implants in marrow cavity. Journal of Biomedical Materials Research, 42, 540-548.
(67) KIKUCHI, M., *et al.* 2001. Self-organization mechanism in a bone-like hydroxyapatite/collagen nanocomposite synthesized in vitro and its biological reaction in vivo. Biomaterials, 22, 1705-1711.
(68) XIE, J., BAUMANN, M. J. & MCCABE, L. R. 2004. Osteoblasts respond to hydroxyapatite surfaces with immediate changes in gene expression. Journal of Biomedical Materials Research - Part A, 71, 108-17.
(69) HUANG, Z., *et al.* 2009. A bone-like nano-hydroxyapatite/collagen loaded injectable scaffold. Biomedical Materials, 4, 1-7.
(70) LIANG, Y. & KIICK, K. L. 2014. Heparin-functionalized polymeric biomaterials in tissue engineering and drug delivery applications. Acta Biomaterialia, 10, 1588-1600.
(71) SAKIYAMA-ELBERT, S. E. 2014. Incorporation of heparin into biomaterials. Acta Biomaterialia, 10, 1581–1587.
(72) TEIXEIRA, S., *et al.* 2010. Heparinized hydroxyapatite/collagen three-dimensional scaffolds for tissue engineering. Journal of Materials Science: Materials in Medicine, 21, 2385–2392.
(73) PETITOU, M., CASU, B. & LINDAHL, U. 2003. 1976–1983, a critical period in the history of heparin: The discovery of the antithrombin binding site. Biochimie, 85, 83-89.
(74) VRANA, N. E., *et al.* 2007. Edc/nhs cross-linked collagen foams as scaffolds for artificial corneal stroma. Journal of Biomaterials Science, Polymer Edition, 1-19.
(75) SUSA, M., *et al.* 2004. Human primary osteoclasts: In vitro generation and applications as pharmacological and clinical assay. Journal of Translational Medicine, 2.
(76) *Alamarblue® assay* [Online]. Life Technologies. Available: http://tools.lifetechnologies.com/content/sfs/manuals/PI-DAL1025-1100_TI%20alamarBlue%20Rev%201.1.pdf [Accessed July 10th 2014].
(77) COELHO, C. C. 2013. *Heparinized nanohydroxyapatite/collagen granules for controlled release of vancomycin.* Master Degree in Biomedical Engeneering, Universidade do Porto.
(78) MANSOUR, H. M., *et al.* 2010. Materials for pharmaceutical dosage forms: Molecular pharmaceutics and controlled release drug delivery aspects. International Journal of Molecular Sciences, 11, 3298-3322.

(79) MARTINS, V. C. A., *et al.* 1998. The controlled release of antibiotic by hydroxyapatite: Anionic collagen composites. Artificial Organs, 22, 215-221.
(80) HIRAMATSU, K. 2001. Vancomycin-resistant *staphylococcus aureus*: A new model of antibiotic resistance. The Lancet. Infection Diseases, 1, 147-155, JACQUELINE, C., *et al.* 2010. Efficacy of the new cephalosporin ceftaroline in the treatment of experimental methicillin-resistant *staphylococcus aureus* acute osteomyelitis. Journal of Antimicrobial Chemotherapy, 65, 1749-1752.
(81) JAFARIAN, M., *et al.* 2008. Marrow-derived mesenchymal stem cells-directed bone regeneration in the dog mandible: A comparison between biphasic calcium phosphate and natural bone mineral. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontics, 105, e14-24.
(82) KARAGEORGIOU, V. & KAPLAN, D. 2005. Porosity of 3d biomaterial scaffolds and osteogenesis. Biomaterials, 26, 5474-5491.
(83) LEONG, M. F., *et al.* 2009. Effect of electrospun poly(d,l-lactide) fibrous scaffold with nanoporous surface on attachment of porcine esophageal epithelial cells and protein adsorption. Journal of Biomedical Materials Research. Part A 89, 1040-1048.
(84) PUCKETT, S., PARETA, R. & WEBSTER, T. J. 2008. Nano rough micron patterned titanium for directing osteoblast morphology and adhesion. International Journal of Nanomedicine, 3, 229-241.
(85) JR., A. B. N., *et al.* 2010. Influence of implant surfaces on osseointegration. Brazilian Dental Journal, 21, 471-481.
(86) FANG, R. C. & GALIANO, R. D. 2009. Adjunctive therapies in the treatment of osteomyelitis. Seminars in Plastic Surgery, 23.
(87) TANG, L., *et al.* 2009. Preparation, release profiles, and antibacterial properties of vancomycin-loaded poly(d,l-lactic) titanium alloy plates. Orthopedics, 32.
(88) JOLLEY, M. E., *et al.* 1981. Fluorescence polarization immunoassay. Iii. An automated system for therapeutic drug determination. Clinical Chemistry, 27, 1575-1579.
(89) SMITH, F. - **Handbook of forensic drug analysis**. Academic Press, 2004.
(90) FRAIMOW, H. S. 2009. Systemic antimicrobial therapy in osteomyelitis. Seminars in Plastic Surgery, 23, 90-99.
(91) ZIMMERLI, W. - **Osteomyelitis therapy – antibiotic therapy.** *In:* Osteomyelitis of the jaws. ed.: BALTENSPERGER, M. M. & EYRICH, G. K. H. (ed.)^(eds.),2009. 179-190.
(92) ZHU, X., *et al.* 2006. Characterization of nano hydroxyapatite/collagen surfaces and cellular behaviors. Journal of Biomedical Materials Research. Part A, 79, 114-127.
(93) KLEINMAN, H. K., KLEBE, R. J. & MARTIN, G. R. 1981. Role of collagenous matrices in the adhesion and growth of cells. Journal of Cell Biology 88, 473-485.
(94) ALBU, M. G., TITORENCU, I. & GHICA, M. V. - **Collagen-based drug delivery systems for tissue engineering.** *In:* Biomaterials applications for nanomedicine. ed.: PIGNATELLO, R. (ed.)^(eds.),2011
(95) IRIE, A., *et al.* 2007. Heparin enhances osteoclastic bone resorption by inhibiting osteoprotegerin activity. Bone, 41, 165-174.
(96) CHOWDHURY, M. H., HAMADA, C. & DEMPSTER, D. W. 1992. Effects of heparin on osteoclast activity. Journal of Bone and Mineral Research, 7, 771-777.
(97) ROODMAN, G. D. 1999. Cell biology of the osteoclast. Experimental Hematology, 27, 1229-1241.
(98) VAANANEN, K. 2005. Mechanism of osteoclast mediated bone resorption—rationale for the design of new therapeutics. Advanced Drug Delivery Reviews, 57, 959-971.
(99) OURSLER, M. J. 1994. Osteoclast synthesis and secretion and activation of latent transforming growth factor β. Journal of Bone and Mineral Research, 9, 443-452.
(100) TSAI, S.-W., HSU, F.-Y. & CHEN, P.-L. 2008. Beads of collagen–nanohydroxyapatite composites prepared by a biomimetic process and the effects of their surface texture on cellular behavior in mg63 osteoblast-like cells. Acta Biomaterialia, 4, 1332-1341.

(101) RATHBONE, C. R.*, et al.* 2011. Effect of various concentrations of antibiotics on osteogenic cell viability and activity. Journal of Orthopaedic Research, 29, 1070-1074.