UNIVERSITA' POLITECNICA DELLE MARCHE

FACOLTA' DI INGEGNERIA

Department of Industrial Engineering and Mathematical Sciences

Master Degree in Biomedical Engineering

BONE SUBSTITUTE MATERIALS DELIVERING ANTIBIOTICS

Advisor:

Prof. Lorenzo Scalise

Co-Advisor:

Dr. Paolo Fattori

Author:

Jessica Di Monte

ACADEMIC YEAR **2019** / **2020**

Index

1 Introduction

1.1 Bone Regenerative Medicine

Human skeleton bones have the exceptional restorative capacity, shared probably with only the liver, to heal and to regenerate themselves after lesion, restoring shapes, sizes and strengths essentially equal to their pre-lesion form [1].

The bone tissue is involved in several biological functions in the body e.g., structural support, organs protection, production site of blood cells and storage of minerals [2].

Structurally, the bone is characterized by a hard-outer layer, called cortical bone, usually dense and strong, and by an inner-spongy core, called cancellous or trabecular bone, that is porous and softer than the external part; moreover, bones incorporate different cell types, as well as proteinaceous matrix (osteoid), and matrix-deposited inorganic minerals [3].

Bone is a very metabolically active tissue, highly vascularized and dynamic. Both cortical and cancellous bone grow, adapt and turnover by means of two fundamentally distinct mechanisms termed modelling and remodelling; during the former process, bones not fully developed at birth continue to be formed out of cartilage and connective tissue into strong, lamellar bone, until the ossification of the “growth plates”, at the end of the adolescence, stops the growth of the bones. During adulthood, the remodelling mechanism causes the bone - which ages losing strength, elasticity and minerals - to be constantly removed and replaced, obtaining the repairing of microscopic

and macroscopic damaged tissue, together with the mobilization of calcium during the self-regulating process of calcium homeostasis [4].

Unfortunately, spontaneous bone healing, even with internal fixation technologies that facilitate the osteosynthesis, is not always effective due to infection, poor vascularity, malnutrition and substantial bone or soft tissue loss [1].

Bone nonunion, due to its high morbidity and disability rate, is nowadays one of the most serious consequences of fractures, bone infections, tumours or revision arthroplasty [5].

Several factors affecting bones, muscles, and joints (e.g., aging, lifestyle, physical activity family history or drug usage) are responsible for causing bone-related diseases like osteoarthritis, rheumatoid arthritis, fibromyalgia and bone fractures. Moreover, the spontaneous, natural bone repairing could occur too slowly or in an inadequate way; hence, an alternative bone grafting substitute strategy is indispensable to solve these complications [6].

Over two million bone grafting procedures are performed every year [7], being the second most frequent organ transplantation in the world, immediately after blood transfusion [8].

Oncologic surgery, traumatology, maxillofacial surgery and orthopaedics, with revision prosthetic and spine surgery, are the main fields for bone substitutes application, due to their high rate and variety of pathological bone defects [9,10].

For centuries, the intention of substituting missing bone tissues has characterized human inventiveness and the first attempts of orthopaedic and dental procedures have been traced back to Pre-Columbian and Egyptian civilizations [11]. During the 17th century, the first succeeded bone grafting, consisting of the transplantation of a piece of bone from a dog's skull into a cranial defect of a soldier, was performed by the surgeon Job Van Meekeren, while two centuries later, both autogenic and allogenic successful grafts were reported [12]. Non osseous materials, like wood or marble, were employed during the same period, but the first positive result was recorded only in 1892, when Dreesman used plaster of Paris (calcium sulfate) as bone substitute [13].

Bone grafts are fundamentally bone-like materials, obtained from living donor, post mortem donors, or synthetically engineered, with the aim of replacing, healing, strengthening or improving bone function in disease or injury [6].

In the past, bone grafts succeeded application was measured essentially by the graft's ability to withstand the mechanical stresses that surrounded it, while today bone grafting is considered as a whole biological structure, capable of being incorporated, revascularized, and eventually assume the desired shape, while withstanding mechanical and shear stress (extrinsic and intrinsic) during its long-term healing process [14].

The ideal bone substitute material should exhibit several important properties [15,16], including:

- *Biocompatibility* — The graft should not cause an immune response against the implanted tissue.
- *Durability* — The graft should maintain shape and volume over time, avoiding losing of structural properties.
- *Vascularity and Angiogenesis* — A multiple apertures porous structure (at least 100 μm diameter) is an indispensable requirement to meet high blood demands in bone tissue, sustaining the proper transport of nutrients and oxygen for cells survival. Moreover, it has been seen that polymeric, ceramic, or composite materials are preferred for these purposes over metallic graft since they tend to not fuse completely and dissociate after insertion.
- *Bio-absorbability* — Bone substitutes should be absorbed and provide the space for new bone generation. Yet, the duration of availability mainly depends upon the site of surgery, since for spinal fusion the graft should be degraded approximately after 9 months while in skull or maxillofacial bone replacement, the required time is around 3–6 months.
- *Cost Efficiency and Availability* — The bone substitute should be reasonably priced to purchase and use.

Bone substitutes must reinforce bone healing by several properties of action [1], involving:

- *Osteogenesis* — The process in which living cells from the host or graft donor allow the synthesis of new bone at the graft site.

- *Osteoinduction* — The process by which exogenous growth factors - e.g., bone morphogenetic proteins (BMP) -2, -4, -7, platelet-derived growth factor (PDGF), angiogenic factors, etc. - regulate the differentiation of recruited mesenchymal stem cells into chondroblast and osteoblast which, in turn, form new bone.
- *Osteoconduction* — The process by which a degradable support or scaffold passively allow ingrowth of host vasculature, cells and new bone tissue. Among the most frequently used osteoconductive bone substitutes there are calcium phosphate ceramics such as hydroxyapatite, calcium sulfate and bioactive glass-ceramics.

Hence, the term bone graft substitute can define any implanted material that, alone or combined with other materials, provides a bone healing response by means of osteogenic, osteoinductive or osteoconductive processes [17]. In fact, it has been seen that an effective therapeutic concept for the treatment of impaired fracture healing, by means of bone substitutes, is the ‘‘diamond concept’’, based on five equally important factors: osteogenicity (mesenchymal stem cells), osteoconduction (scaffolds), osteoinduction (growth factors), mechanical stability and vascularity [18].

1.1.1 Bone Substitute Materials Classification

Bone substitutes can be classified, according to their source, in four main categories that are *autografts, allografts, xenografts* (derived from biological products) and *alloplasts* (derived from synthetic products) [1,19-21].

Autograft (autologous or autogenous bone graft), still considered the ‘‘gold standard’’ in bone substitution, involves bone obtained from the same individual receiving the graft [22]. In this case, the bone substitute can be harvested from non-essential bones such as the iliac crest or from the fibula, the chin, the ribs, the mandible or parts of the skull.

Ideally, autograft is the best option since it possesses all the properties that are essential for bone formation, including osteoconduction, osteoinduction, growth factors and osteogenic cells, with no immune response or infective risk.

The disadvantages of autografts reside in the inherent morbidity, since the required surgical donor site can lead to possible post-operative pain and complications [23]; moreover, possible blood loss or hematomas, infection, fracture, neurovascular injury, as well as cosmetic deformity and longer operative time, can represent significant drawbacks.

It should also be emphasized that autograft availability, especially in paediatric patients and in the elderly, could represent an insurmountable limit [24].

Autografts include:

- *Cancellous Bone Graft* — It is highly osteogenic and capable of encouraging at the recipient site both revascularization and incorporation, due to its large trabecular surface area [25].
- *Cortical Bone Graft* — It has both limited osteogenic and osteoinductive properties due to the lack of progenitor cells and poor

revascularization power owing to the highly dense and organized structure that confers, on the other hand, an excellent mechanical support [25].

Allograft bone substitutes are obtained from either living donors (discarded bones from total hip replacement surgery, usually from elderly patients) or non-living donors and represent a convenient alternative to autografts since, in this case, the extraction of human tissue spares any donor site morbidity and reduces surgical times [26]. Allografts are osteoconductive, poorly osteoinductive - depending on the processing, growth factors may still be present - and essentially not osteogenic, due to the absence of viable cells [27].

Furthermore, allografts require sterilization, usually via gamma irradiation, to eradicate any bacterial, fungal and viral transmission, causing adversely effects such as the impairment of the bone's mechanical properties and the deactivation of proteins normally found in healthy bone, diminishing the bone's structural integrity [25].

Allografts can be cancellous allograft, cortical allograft or demineralized bone matrix (DBM), which is a highly-processed bone substitute consisting of collagens, non-collagenous proteins and growth factors, that bestow it with greater osteoinductive and osteoconductive properties [1].

Xenograft are bone substitutes obtained from a species other than human - e.g., bovine bone, porcine bone or corals - that are freeze dried or demineralized and deproteinized before their application [21].

Coral based xenografts, mainly calcium carbonate, can be industrially transformed into hydroxyapatite through a hydrothermal process, leading to a non-resorbable xenograft able to match the natural human bone, actually composed by hydroxyapatite along with calcium phosphate and carbonate; alternatively, the coralline material can remain in its calcium carbonate state allowing for a better resorption by the natural bone [28].

Xenografts, which have shown better results in dentistry than in orthopaedics, have the advantages to be easily available and osteoconductive, with good mechanical properties and low costs [21].

Alloplasts are biocompatible, inorganic and synthetic bone substitutes, representing a broad class of osteoconductive materials with different chemical compositions, structures and physical properties. An ideal synthetic bone substitute should be resorbable in the long term, providing a solid framework for new bone formation, with a microporous configuration permitting biological fixation and able to act as vehicle for other materials such as antibiotics, growth factors and steroids [29].

Common examples of alloplasts include:

- *Polymethylmethacrylate (PMMA) Polymers* — They are biocompatible, poorly bioactive and nondegradable microporous bone cements, formed by methylmethacrylate liquid and PMMA powder in which an aqueous biodegradable carboxymethylcellulose gel is dispersed to create pores in the cement allowing osteoconduction and a better fixation of the prostheses [30].

- *Hydroxyapatite (HA)* — Synthetic hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$, can be found in porous or dense formats, as granules or blocks [31]. Several are the advantages of HA as bone substitute, such as the close resembling to the mineral content of native bone, the lower immunoreaction due to the excellent biocompatibility, the gradual post-operative adsorption concomitantly with osteoconductive bone growth and the optimal ability of carrying and releasing bioactive molecules. The main clinical disadvantage of hydroxyapatite is the tendency of the particles to not stay in place in a bleeding site [32].
- *Calcium Phosphate Cement (CPC)* — The white powder of calcium phosphate is mixed with a liquid and forms a workable paste which can be shaped during surgery to fit the contours of bone loss. The cement hardens, with an isothermic reaction at physiologic pH, within 20 min, forming nanocrystalline hydroxyapatite without any tissue damage during the setting reaction and allowing osteoconduction [21].
- *Tricalcium Phosphate (TCP)* — It is a porous calcium phosphate compound, bioabsorbable and biocompatible, existing in either α or β-crystalline forms and used as bone void fillers in orthopaedic and dental applications. Thanks to the reduced particle size and the interconnected sponge like microporosity, TCP is able of improving osteoconduction as well as promoting proper resorption within the process of remodelling [33].
- *Calcium Sulfate* — It is the most rapidly absorbed synthetic bone substitute available, with resorption typically occurring in one to three months, at a rate that could be faster than the actual bone

generation. It is present on the market in various forms, including injectable viscous fluids, that harden in vivo, for the percutaneous filling of bone voids [34].

- *Bioactive Glasses* — They are biocompatible, osteoconductive and widely employed for filling bone defects (alone or in combination with cancellous autografts and allografts); bioglasses are composed mainly of silica, sodium oxide, calcium oxide and phosphates. The behaviour of these alloplasts depends on their composition, the surrounding pH, the temperature and their porosity, which provides an osteoconductive scaffold for the new bone [33].

1.2 Bone Infection – Osteomyelitis

Surgical site infections, representing approximately 20% of the overall nosocomial infections in orthopaedic wards, are unfortunate and troublesome complications after surgeries, usually leading to increased morbidity, mortality, length of hospital stay, and health care costs [35].

Postoperative bone infection is actually the most difficult complication after the operative treatment in orthopaedics and traumatology. The duration of treatment, the recidivism, various number of operations, socio-psychological consequences for the patient and his surroundings, as well as the fear of potential amputation throw a special light on this problem. Highly active modern way of life and numerous daily activities are creating the possibilities for injuries which require surgical treatment which could lead to various complications, such as infections [36].

Osteomyelitis is the inflammatory process accompanied by bone destruction and caused by an infecting microorganism that gains access to the bone; spreading locally from a contiguous contaminated source of infection - that can be a trauma, a bone surgery or a joint replacement - osteomyelitis can be limited to a single portion of the bone or can involve several regions, such as marrow, cortex, periosteum, and the surrounding soft tissue [37].

Acute osteomyelitis evolves over several days or weeks, as opposed to chronic osteomyelitis, which is somewhat arbitrarily defined as long-standing infection that evolves over months or even years, characterised by the persistence of microorganisms, low-grade inflammation, and the presence of dead bone (sequestrum) and fistulous tracts [38].

Various inflammatory factors, and leucocytes themselves, contribute to tissue necrosis and the destruction of bone trabeculae and bone matrix. Vascular channels are compressed and obliterated by the inflammatory process, and the resulting ischaemia also contributes to bone necrosis. Segments of bone devoid of blood supply can become separated to form sequestra and can continue to harbour bacteria despite antibiotic treatment. Antibiotics and inflammatory cells cannot reach this avascular area, so medical treatment of osteomyelitis fails. At the infarction edge, there is reactive hyperaemia that is associated with increased osteoclastic activity. This activity, in turn, produces bone loss and localised osteoporosis. Meanwhile, bone apposition occurs, in some cases exuberantly, causing periosteal apposition and new bone formation [37].

Besides common high-risk factors (e.g., extreme ages, overweight, malnutrition, diabetes, smoke or immunodepression), for every specific patient there are several elements that influence the nascent stage and the development of osteomyelitis such as virulence and bacteria type, type of bone-break, operation room, operation staff, operative technique, size and duration of the operation [39,40].

On the other hand, various factors can decrease the possibility of infection e.g., early operation, radical debridement of the injury, stabilization of the fracture, early reconstruction of the soft tissue coverlet, in-time use of appropriate antibiotics prophylaxis and good post-operative care [41].

The positioning of biomaterials within the host tissues, in cases of implantation of synthetic media or arthroplasty, is able to favor the onset and maintenance of infections even for rather modest bacterial loads. In fact, it has been shown that 10 bacterial cells are enough to cause a deep infection in prosthetic surgery [42,43]. The reason for this peculiarity in the pathogenesis of prosthetic infections has been clarified thanks to the studies conducted by Anthony Gristina et al. since 1984. Following the implantation of orthopedic or vascular prostheses, what has been defined with a suggestive term "race for the surface" (competition for the surface) between macromolecules, bacteria and tissue cells is established. If healthy tissue colonizes this surface, the bacteria are confronted with living, well-integrated cells. But if bacteria win this race, they are able to proliferate in a particular environment (biofilm) on the surface of the implants, under a glycoprotein envelope created by themselves (slime), which protects them

from the aggression of the host's immune system and even from the antibacterial agents used against them. Infectious agents are therefore 50 to 5,000 times more resistant to antibiotics than the same microorganisms in planktonic form [44].

Many lab tests, conventional radiograph methods, CT, MRI, scintigraphy and biopsy of the seat can be used to detect bone infection with different stage of sensitivity and specificity [41].

In haematological analysis, indices of bone bacterial infection are the increase of erythrocyte sedimentation rate, as well as the higher production of globulins and osteoblast-released alkaline phosphatase, together with the decrease of serum albumin [45].

Bacteriological examination is a special kind of lab work with main aim to find out what bacteria is the cause of bone infection, analysing blood, pus from the abscess bone cavities, fistulae cavity, sequester and materials from the surface of the injury. In the same time, it is possible to determine the sensitivity of the bacteria to antibiotics (antibiogram), establishing the most suitable therapy for the patient [46].

Pathohistological examination can result in minimizing differential diagnostic dilemma, especially in separating specific from unspecific bone infections. Malignant processes in the bones are often difficult to differentiate from ordinary osteomyelitis, so the pathologic examination is the best diagnostic method.

Radiographic examination can make visible bone morphological changes provoked by bacteria. The first morphological changes visible on the x-ray (e.g., bone condensation, periosteal reaction, osteolysis and sequestra) can be seen after 15 to 21 days since the beginning of the infection, because it takes some time to affect the periosteum and for osteoblasts to start forming new bone around the area of necrosis. After being cured from bone infection, the bone remains permanently deformed in the structure [47].

Both Computer Tomography (CT) and Magnetic Resonance Imaging (MRI) have excellent resolution power and can reveal the destruction of medulla as well as periosteal reaction, cortical destruction, articular damage, and soft-tissue involvement, even when conventional radiographs are normal [48].

In orthopaedic surgery, *Staphylococcus aureus* and *Staphylococcus epidermidis* are the most common causative organisms of musculoskeletal infections [37,49].

Staphylococcus aureus (*S. aureus*), by far the most involved pathogen in osteomyelitis, is a member of the Micrococcaceae family. On microscopical examination, the organisms appear as gram-positive cocci, distinguished from other staphylococcal species on the basis of the gold pigmentation of colonies and positive results of coagulase, mannitolfermentation, and deoxyribonuclease tests. In humans, *S. aureus* can be present in the upper respiratory tract, gut mucosa, and skin as a member of the normal microbiota [50]. It expresses numerous surface proteins that mediate adherence to components of bone matrix and collagen. These bacterial cell

surface receptors are known as adhesins or MSCRAMMs (microbial surface components recognizing adhesive matrix molecules). Moreover, *S. aureus* is able to form biofilms on foreign materials that act as sanctuary sites, where it is relatively protected from antimicrobial agents and the host immune response. Finally, *S. aureus* can invade osteoblasts and form small-colony variants (SCVs) in the intracellular compartment, where they are able to survive in a metabolically inactive state while preserving the integrity of the host cell [51].

Staphylococcus epidermidis (*S. epidermidis*), gram-positive bacterium, is the most frequently isolated member of the group of coagulase-negative staphylococci. Together with more rarely found coagulase-negative staphylococci, *S. epidermidis* colonizes the skin and mucous membranes of the human body and represents the major part of the normal bacterial flora of this habitat. Whereas *S. epidermidis* has been regarded for a long time as relatively innocuous, it has now generally been accepted as a pathogen and cause of nosocomial infections. In contrast to *S. aureus*, *S. epidermidis* does not usually cause pyogenic infections in non-compromised patients, due to its distinctly reduced arsenal of toxins. Generally, *S. epidermidis* infections, complicated by the formation of antibiotic-resistant biofilms, are mainly caused by foreign bodies, such as indwelling catheters and any implanted devices, thanks to the pathogen's ability to adhere to surfaces [52].

1.2.1 Prevention and Treatment of Osteomyelitis

The increased awareness of the high levels of mortality and morbidity associated with postoperative infections, and the difficulties related to their

treatment, especially in orthopaedic surgery, have led to improve prevention over the years. This is based on four principles: patient preparation, surgical technique, perioperative antibiotic prophylaxis and postoperative wound management. The main purpose of antibiotic prophylaxis is to reduce the incidence of surgical site infections, without altering the patient's immune response and with the least number of side effects, using protocols supported by evidence or clinical efficacy. Perioperative antibiotic prophylaxis does not represent an attempt to sterilize tissues, but pursues the aim of reducing bacterial growth secondary to intraoperative contamination to a level that can be contained by the host's immune system, minimizing the effects on endogenous bacterial flora. Orthopaedic surgery, and in particular prosthetic joint replacement interventions and osteosynthesis of unexposed fractures, represents a type of “clean” surgery, that should not require an operative antibiotic prophylaxis [44].

The ideal antimicrobial agent, to be used in prophylaxis, should have excellent in vitro activity against the most common agents of infection, penetrate effectively into tissues, have a relatively long serum half-life (in order to ensure adequate antimicrobial action for duration of the intervention) and be relatively non-toxic [53].

Prophylaxis target identification is of great importance. As said before, the main agents of infection in orthopaedic surgery are *Staphylococcus epidermidis* and *Staphylococcus aureus*, therefore, perioperative prophylaxis, in this case, is essentially an antistaphylococcal prophylaxis. The most used drugs in orthopedics are cefazolin, cefuroxime and

cefamandole (cephalosporins of I and II generation), particularly active on many gram-positive bacteria and fairly active against aerobic gram-negative bacteria [54].

An alternative is represented by glycopeptides (vancomycin or teicoplanin) or clindamycin, indicated in patients with hypersensitivity to cephalosporins. Prophylaxis with glycopeptides is also recommended if a prevalence of methicillin-resistant staphylococci is ascertained within the hospital [55].

A correct prophylaxis protocol must take into account the pharmacokinetic characteristics of the drug used, its spread in the host tissues and the type and duration of surgery performed. The dosage of the chosen antibiotic, whatever it is, must be adapted in order to ensure the maintenance of serum and tissue levels of antibiotic capable of inhibiting microbial contamination for the entire duration of the surgery [44].

1.2.2 Use of Rifampicin in Staphylococcal Osteomyelitis

Several studies have shown that rifampicin can been enrolled for the treatment of staphylococcal osteomyelitis in patients with orthopaedic-device-related infections, killing inactive sessile bacteria within biofilm, including methicillin-resistant species. Highly bioavailable and associated with few adverse effects, it should be only used in combination with another antistaphylococcal agent, such as vancomycin, trimethoprim-sulfamethoxazole, or fluroquinolones, in order to avoid emergence of rifampicin resistance [37,56].

Rifampicin (or rifampin) ($C_{43}H_{58}N_4O_{12}$) is a semisynthetic derivative of the rifamycins, a class of antibiotics that are fermentation products of *Nocardia mediterranei.* Rifampicin produces the antimicrobial activity by inhibition of DNA dependent RNA polymerase (RNAP) either by sterically blocking the path of the elongating RNA at the 5′ end or by decreasing the affinity of the RNAP for short RNA transcripts. It specifically inhibits the microbial RNAP and has no action on the mammalian enzyme, thereby decreasing the number of adverse effects it can cause in humans.

Rifampicin is a highly lipid-soluble drug and is available in oral or intravenous formulations. When given orally, it is rapidly absorbed and distributed throughout the body. It is excreted equally in bile and urine and has a half-life of 2.5 hours. Rifampicin is generally a well-tolerated drug but is associated with both dose-dependent and dose-independent adverse effects. The former include orange discoloration of body fluids like tears, sweat, saliva, urine, and gastrointestinal symptoms like nausea, anorexia, and diarrhea; the latter adverse effects include hypersensitive reactions like urticaria, flu-like symptoms, thrombocytopenia, haemolysis, and renal failure. These hypersensitivity reactions are common when used intermittently or for a prolonged duration [57].

1.3 Bone Antibiotics Carriers

Systemic antibiotic prophylaxis is mandated for certain surgical procedures, according to the contamination risk assessment of the surgeon, the regular practice in the hospital in question, and numerous guidelines published on the topic [58]. One of the problems related to systemic delivery of antibiotics

is that insufficient concentrations are reached at vascular compromised locations, such as a fracture site or other compromised tissues. In fractures, the bony structure may be affected and the local vascularity may be disturbed making it impossible to achieve appropriate local antibiotic concentrations via systemic delivery via the bloodstream. Increasing the dose of systemically delivered antibiotics is not a suitable approach since high concentrations of antibiotics over an extended period might cause systemic toxicity problems [59].

Local delivery of antibiotics maximizes target tissue concentration and minimizes systemic toxicity risks. Any drug delivery device intended for prophylactic use should have a broad antibiotic spectrum incorporated, in order to prevent both Gram-positive (e.g., *S. aureus* and *S. epidermidis*) and Gram-negative (e.g., *Pseudomona aeruginosa* and *Escherichia coli*) bacteria from colonizing the surgical site [58].

One potential side effect of high local concentrations of antibiotics is the risk of cytotoxicity since elevated concentration of various antibiotic agents can have effect on osteoblast cell viability and activity. Thus, in the local delivery of antibiotics it is not only necessary to reach concentration levels above the minimal bactericidal concentration (MBC) in order to prevent bacterial resistance, but it is also important to keep peak concentration at a level which does not affect bone healing [60].

Depending on the material used for biosynthesis of the carrier, local drug delivery systems are divided into *nonbiodegradable* and *biodegradable* and the release of the antibacterial agent is governed by the rate of dissolution

of the drug in its own matrix and by its penetration through the pores of the carrier. For highly soluble agents, the amount of released drug depends on the surface area of the carrier and on the initial concentration of the drug in the prepared system. For relatively insoluble agents, the rate of drug release depends on the porosity of the matrix and on the dissolution of the drug in the matrix [61,62].

1.3.1 Nonbiodegradable Carriers

Nonbiodegradable delivery systems are represented by PMMA carriers. They exist in 2 forms: antibiotic-impregnated spacer cement applied in arthroplasties and antibiotic-impregnated PMMA bead chains for musculoskeletal infections. The success of these carriers is related to the fact that PMMA does not usually trigger any immune response from the host, and the form of a bead confers a wide surface area, allowing rapid release of the antibiotic [63].

PMMA beads are found in both commercial and non-commercial preparations. Commercially available beads have a consistent diameter of 7mm and are available in strands of 10 or 30. Non-commercial preparations, prepared by surgeons themselves, have the disadvantages of a lack of thorough mixing of the antibiotic into the beads and a lack of uniform size of bead, resulting in lower antibiotic availability. Selection of the type of antibiotic is based on its stability at the high temperatures (up to 100°C) at which polymerisation occurs. The aminoglycosides are heat stable and are thus widely used in these preparations [64]. The use of rifampicin in combination with PMMA is avoided since it interferes with the

polymerization of the bone cement; however, is suitable for loading of degradable carriers such as hydroxyapatite [65]. PMMA serves both the antibiotic release and the induction of a biological active membrane on the surface of the PMMA spacer that stimulates bone healing during the bone reconstruction procedure [66]. Ultimately, the PMMA implant has to be removed since it is not biodegradable, and the implant itself can create bacterial resistance if the antibiotic concentration drops over the course of time, and the PMMA implant acts as a foreign body [67].

1.3.2 Biodegradable Carries

Biodegradable carriers could provide high local bactericidal concentrations in tissue for the prolonged time needed to completely eradicate the infection and the possibility to match the rate of implant biodegradability according to the type of infection treated. Biodegradation also makes surgical removal of the implant unnecessary. The implant can also be used initially to obliterate the dead space and, eventually, to guide its repair. Furthermore, secondary release of the antibiotic may occur during the degradation phase of the carrier, which could increase antibacterial efficacy compared to non-biodegradable carriers. The most commonly used biodegradable carrier systems are collagen sponges, lactic acid polymers and alloplastic bone substitutes such as calcium sulphate and hydroxyapatite [68].

Collagen has been studied widely as carrier system for antibiotics due to its biocompatibility, low costs, and availability. Commercially available products are mainly gentamicin-loaded collagen fleeces based on collagen from bovine or equine skin or soft tendon, with a relatively fast deliver of the

antibiotic over the first days, usually characterized by a burst release directly after implantation in the body [69]. The elution of the antibiotic takes place by its diffusion from the sponge upon implantation and principally during the degradation of collagen by the collagenases of macrophages, usually completed within 8 weeks [70]. Its ability to release drugs can be modified by changing the porosity of the matrix or by treating it with chemicals. It can also attract and stimulate the proliferation of osteoblasts, thereby promoting mineralisation and the production of collagenous callus tissue, which aids the formation of new bone [71].

Polymerised lactic acid carriers, biodegradable polymers from glycolide and lactide, can be used in the controlled delivery of pharmaceuticals especially with antibiotics such as ampicillin, gentamicin, polymyxin B and Chloramphenicol. Polylactide/polyglycolide was selected to act as a carrier because it undergoes a gradual degradation in a controlled manner and dissolves at physiological pH and removal is thus not necessary in patients who have osteomyelitis; in fact, lactic acid polymers are broken down into their monomeric forms and are then metabolised into either oxalic acid or carbon dioxide and water. Moreover, the kinetics of the release of the antibiotic can be modified by the selection of copolymers of varying monomeric composition, polymer crystallinity and molecular weight as well as by alteration of the geometry of the implant [68].

1.3.2.1 Alloplastic Bone Substitutes as Antibiotics Carries

Alloplastic biodegradable bone substitutes, employed as drugs carriers, combine the use of a local antibiotic delivery system, to eradicate infection,

with the possibility of treating bony defects and injury impaired fractures with a gradual post-operative adsorptive graft, supporting new bone formation via osteoconduction, in particular after debridement of an infected bone [72].

Calcium sulphate exists in several different hydrate forms, depending on how much water is incorporated into its crystal lattice. It is effective at delivering high levels of local antibiotic because it dissolves relatively quickly. Conversely, it is unable to provide any significant long-term mechanical support or act as a scaffold for tissue regeneration; moreover, as it dissolves, calcium sulphate produces an acidic microenvironment responsible for local inflammatory processes at the site of implantation [73].

Calcium phosphate hydroxyapatite bone substitutes are similar in composition to bone mineral content and they are biocompatible, bioactive and osteoconductive and have the unique capability to be able to absorb several chemical (pharmacological) substances on their surface. Due to its porosity, hydroxyapatite is able to provide proper loading and long-term release of a broad spectrum of antibacterial agents, which is crucial for the antibacterial effectiveness of such a system. In contrast to hydroxyapatite granules or beads where pharmacological agents can only be absorbed on the surface, calcium phosphate cements can incorporate pharmacological agents throughout their entire structure [74]. On histological studies it has been observed that the bactericidal activity of the drug has not been affected by the incorporation in the ceramic device, that is gradually absorbed into the host bone with no recurrence of infection [68].

1.4 Analytical Methods

1.4.1 High Performance Liquid Chromatography (HPLC)

High-performance liquid chromatography (or high-pressure liquid chromatography, HPLC) is a specific type of column chromatography, widely used in biochemistry and analysis to separate, identify, and quantify pharmaceutical and biological samples.

During the HPLC separation, the sample components are distributed between a solid stationary phase, usually packed inside a stainless-steel column, and a liquid mobile phase; the characteristic time at which a specific analyte emerges from the column, called retention time, depends on the interactions that have been occurred among stationary phase, molecules being analyzed and mobile phase/solvent(s), as well as the operational pressure and temperature.

HPLC schematic instrumentation includes a degasser, sampler, pumps, and a detector.

For a correct analysis, some parameters are used as a standard according to nature and chemical properties of the analyzed compound. The most commonly used parameters are:

- *Internal Diameter (ID) of the Column* — It determines the quantity of analyte that can be loaded onto the column and also influences sensitivity. The use of low ID columns improves the sensitivity and decreases the solvent consumption.

- *Particle Size* — Traditionally, HPLC is performed with the stationary phase attached to the outside of small spherical silica particles (very small beads). Smaller particles generally provide more surface area and better separations.
- *Pore Size* — It affects the ability of the analyte molecules to penetrate inside the particle of the stationary phase and interact with its inner surface.
- *Pump Pressure* — Pump performance is measured on its ability to deliver metered amounts of mobile phase at constant (isocratic elution) or varying (gradient elution) composition, yielding a consistent and reproducible flow rate. Modern HPLC systems have been improved to work at much higher pressures, and therefore be able to use much smaller particle sizes in the columns (< 2 micrometres) [75,76].

In HPLC system, components are most often separated because of polarity differences, although other criteria, such as electrical charge or molecular size, can be utilized as well. Molecules with similar chromatographic polarity tend to be attracted to each other; those with dissimilar polarity exhibit much weaker attraction, if any, and may even repel one another. This becomes the basis for chromatographic separation modes based on polarity.

To design a chromatographic separation system, it is created a competition for the various compounds contained in the sample by choosing a mobile phase and a stationary phase with different polarities. Then, compounds in the sample that are similar in polarity to the stationary phase will be delayed

(higher retention time) because they are more strongly attracted to the particles. Compounds whose polarity is similar to that of the mobile phase will be preferentially attracted to it and move faster (lower retention time).

Normal phase chromatography is a type of HPLC with a very polar stationary phase (mainly pure silica) and a non-polar mobile phase (a non-aqueous solvent such as chloroform), whereas reverse phase chromatography has a non-polar stationary phase (modified silica substrate with long hydrophobic chains) and a polar mobile phase (mainly water, methanol or acetonitrile) [75].

After separation, the components can be quantified and identified.

There are several ways of detecting a substance when it has passed through the column. UV-visible absorption spectrophotometers are commonly used detectors in pharmaceutical analysis since many organic compounds absorb UV light of various wavelengths: the amount of light absorbed will depend on the amount of a particular compound that is passing through the beam at the time.

The output of the detector is recorded on a chromatogram, as a series of absorbance peaks function of time, resembling Gaussian profiles in the optimal separation case; each peak represents a compound in the mixture passing through the detector and absorbing UV light. The area under the peak is proportional to the amount of substance, which is passed through detector, and this area can be calculated automatically by the computer linked to the display [76].

1.5 Aim of the Work

Given the growing interest in biodegradable and osteoconductive synthetic bone substitutes, capable of regenerating bone and at the same time releasing antibiotics for local prophylaxis therapy aimed at eradicating possible osteomyelitic infections, and given the few studies in the literature describing a precise chromatographic technique for the in-vitro evaluation of rifampicin eluted from hydroxyapatite grafts, the purpose of the present thesis has been the development of a chromatographic method for the analysis of the release time of rifampicin loaded on bio-mimetic nano-structured hydroxyapatite bone substitutes in both porous chips and dense granules formats.

2 Materials and Methods

2.1 Loading of Rifampicin on Bone Substitutes

Two formats of synthetic hydroxyapatite bone substitutes, dense granules and porous chips (SpherHA®; Tiss'You srl, Domagnano, RSM), with a granulometry of 0.5-1 mm, have been employed. SpherHA® hydroxyapatite is a bio-mimetic calcium phosphate compound, remarkably similar to the human mineral bone matrix in composition, structure and size of nano-crystals (Ca / P ratio equal to 1.67). The highly porous and interconnected structure is optimally osteoconductive and completely degraded by osteoclastic activity, promoting cellular colonization, nutrients exchanges and rapid vascularization. Dense granules, with a micro-porosity of 2-3 μm, are compact and similar to human cortical bone with a slower remodelling time than porous chips, which have a macro-porosity of 200-500 μm resembling cancellous bone.

For this study all chemicals were of analytical grade and weighed using High-precision Balance Adventurer AX124 (Ohaus).

According to the recommended dosage based on in-vitro results provided by PerOssal® (OSARTIS GmbH), the antibiotic rifampicin (MW=822,95 g/mol, C16814500, Dr. Ehrenstorfer GmbH), solubilized in physiological solution 0.9%, has been loaded on SpherHA® dense granules, to initially evaluate the stability of the antibiotic at neutral pH.

The former stock of physiological solution 0.9% has been prepared by mixing Sodium Chloride (MW=58.44, S8776 Sigma-Aldrich) with bidistilled

water with a high degree of purity, produced by ELGA system (UK). Then, an amount of 5 mg of rifampicin has been dissolved in 1 ml of such solution, contained in a 1.5 ml Eppendorf tube, by 8 min of sonification at room temperature performed by the Ultrasonic Cleaner DK-80 (DK Sonic), followed by 5 min of shaking through the Incubating Cooling Thermal Shaker ISTHBLCTS (Ohaus), for three cycles.

Next, the solution of rifampicin has been loaded, by perfusion, in a syringe containing 0.5 cc (0.525 g) of SpherHA® dense granules. The hydroxyapatite loaded with the antibiotic has been place at the bottom of a 50 cc Falcon Tube, filled the former physiological solution, preventing the granules from going into suspension, simulating the in-vivo conditions after the implantation of the bone substitute in a bone, where the granules are compactly assembled and not dispersed into the blood or body fluids. The Falcon Tube has been positioned in the incubator (Incubator I, Memmert) at 35 °C, in order to keep the sample warm mimicking body conditions.

Unfortunately, rifampicin oxidizes easily in a neutral pH solution and is poorly detectable already after one day from its loading on the bone substitute.

For this reason, inspired by the study of Rajaram et al. [77], a preservative and antioxidant ascorbic acid (vitamin C) solution, with two different concentrations (2 mM and 10 mM) has been tested on dense granules to prevent the degradation of the rifampicin. Given the best performance of the higher concentration, the experiment with both types of bone substitutes has been accomplished with the 10 mM ascorbic acid solution.

In this second attempt, the physiological solution has been totally replaced and the amount of 5 mg of rifampicin has been solubilized in 1 ml of citrate buffer solution (pH 5.0) by means of the former technique composed by successive series of sonifications and shakings. The same 5 mg rifampicin solution has been prepared for both dense and porous bone substitutes loading.

The 10 mM ascorbic acid stock solution has been prepared by mixing L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate (MW=289.54 g/mol, A8960-5G Sigma-Aldrich) with buffer citrate solution, pH 5.0, as vitamin C is more stable at acidic pH .

Then, 5 mg rifampicin solution has been loaded on 0.5 cc (0.525 g) of SpherHA® dense granules, and the same quantity has been loaded on 0.5 cc (0.525 g) of SpherHA® porous chips, by placing and perfusing directly both hydroxyapatite materials at the bottom of two 100 ml Glass Jars. Both Jars have been filled with 100 ml of 10 mM ascorbic acid solution previously prepared (further millilitres extractions will not affect the ascorbic acid solution and the rifampicin release measurement), avoiding the suspension of SpherHA® particles, and placed in the incubator at 35 °C.

The experiment of rifampicin loading has been performed three times, for both dense granules and porous chips substitutes, in parallel (six Glass Jars in total).

2.2 Rifampicin Extraction and HPLC Analysis

2.2.1 Rifampicin Samples Extraction

Each of the three experiment has been conducted in the same manner. After the incubation of dense granules and porous chips loaded with rifampicin, every 24 h in the following 5 days, one sample of 3 ml from each Glass Jar has been extracted and filtered with 0.22 μm sterile syringe filters. After each extraction, 3 ml of buffer citrate solution (pH 5) have been added in the Glass Jars to avoid super concentrations of rifampicin.

All the daily extracted and filtered samples (thirty in total) have been stored in the dark at the temperature of 4°C in order to be analysed all at once.

The next step has been the samples preparation in order to perform the HPLC Analysis.

2.2.2 HPLC analysis

2.2.2.1 Liquid Chromatography

In order to perform HPLC analysis and evaluate the release time of rifampicin loaded on dense granules and porous chips bone substitutes and progressively eluted in the ascorbic acid citrate solution, the liquid chromatographer Flexar LC by Perkin Elmer (conventional LC 6000 PSI) [78] has been employed.

It is composed by five modular components:

- *Solvent Manager* — It removes dissolved gasses from HPLC solvents by a vacuum chamber in which the pressure is monitored by a microprocessor through an integrated absolute pressure sensor. Degassed mobile phase then enters the pump.
- *LC Autosampler* — It can provide different injection volumes and modes, with a pressure limit up to 6100 psi (428 bar).
- *Binary LC Pump* — It has a flow-rate range in the interval 0.01 - 10.0 ml/min for a pressure range that goes from 0 to 6100 psi.
- *LC Column Oven* — It is the column compartment, with temperature ranging from 5 to 90 (°C).
- *PDA Plus Detector* — It is an advance UV-VIS photo diode array detector (PDA) with a 190 - 790 nm wavelength range and a high acquisition rate (up to 200 HZ).

The speciation analysis is performed with the software system Chromera, able to support a post-run review analysis, displaying the final chromatogram after each sample run.

2.2.2.2 Protocol of Analysis

Samples previously stored have been prepared for the HPLC Analysis. From each of the thirty samples, 500 µl have been extracted and with the same amount of acetonitrile (MW=41.05 g/mol, 34851-2.5L Honeywell) and inserted in LC Clean Vials made of Type 1 Borosilicate glass – which conforms to all USP, JP and EP requirements.

In order to perform HPLC Analysis, it has been used a reverse phase chromatography with a non-polar stationary phase (modified silica substrate with octadecylsilane chains) and a polar mobile phase composed by two solvents (potassium dihydrogen phosphate and acetonitrile), inspired by Siddhartha et al. [79].

The 10 mM potassium dihydrogen phosphate solvent (pH 3.25), has been prepared by mixing Potassium Phosphate monobasic H_2KO_4P (MW= 136.09 g/mol, P5379-1KG Sigma-Aldrich), bidistilled water with a high degree of purity and Phosphoric acid (MW=98.0 g/mol, 695017-500ML Sigma-Aldrich) with a magnetic stirrer (Joan-SH-2, BIPEE); the solvent has been further filtered with 4-7 µm filter.

In order to measure pH solutions, the Bluetooth probe HALO HI13302 by Hanna Instruments has been used, transmitting the readings directly to an Apple or Android device.

The applied protocol has been the follow:

- *Chromatographic conditions*: isocratic, 60% A, 40% B, with A= 10 mM potassium dihydrogen phosphate solvent (pH 3.25) and B= acetonitrile.
- *Temperature*: 25.0 °C (± 1°C).
- *Flux and Runtime*: 1.2 ml/min for 15.0 min.
- *Absorbance*: wavelength of 335 nm with an analytical bandwidth of 5 nm.
- *Injection Volume*: 5 µl.

- *Column*: Agilent Eclipse Plus C18 5 µm, 4.6 x 150 mm.
- *Acquisition Sampling Rate*: 0.5 pts/sec.

The calibration of the instrument has been performed preparing a stock standard solution of rifampicin (50% v/v buffer citrate solution pH 5 in acetonitrile) such that the final concentration was approximately 0.2 mg/ml.

The calibration samples have been made with the stock standard diluted with a mixture of 1:1 v/v A and B (A+B), as follow:

- 5 µg/ml calibration sample (S0, 1:60) prepared with 25 µl of stock solution and 975 µl of A+B;
- 15 µg/ml calibration sample (S1, 1:20) prepared with 75 µl of stock solution and 925 µl of A+B;
- 30 µg/ml calibration sample (S2, 1:10) prepared with 150 µl of stock solution and 850 µl of A+B.

The calibration line has been created setting up a new measurement code with Chromera Software: three “Calibration Replace”, with samples S0, S1 and S2, have been inserted, processing the HPLC analysis two times for each sample. After 90 min of analysis, the calibration line ($y = ax + b$), between area under the peak (y) and rifampicin concentration (x), has been created with the Least Mean Squares Algorithm.

Then, the same protocol, but performing only one injection, has been applied to all the samples previously prepared, in order to analyse the rifampicin released from dense granules and porous chips. As follow, the “Average Area” of rifampicin peak (retention time between 8.6 and 9.8 min)

has been calculated by the software for each sample. The software, in order to compute the definite integral of the peak, applies the rectangular method: it defines the baseline of the peak and divide the interval between the onset point and the offset point of the peak into a number of small intervals with the same length, according to the sampling rate; the approximate area under the curve is then the sum of the areas of all the rectangles formed by subintervals, in which the height of each rectangle is the value of the function in the middle of the interval.

Once determined the area under the peak, it is possible to obtain the related rifampicin concentration [μg/ml] applying the following formula:

$$[rifampicin] = \frac{Area - b}{a}$$

where $Area$ is the "Average Area" [dimensionless], while a and b are respectively angular coefficient and y-axis intercept of the calibration line.

Each concentration has been multiplied by the volume of the ascorbic acid solution contained in the Glass Jar (100 ml) in order to obtain the total amount of rifampicin [μg] in solution, for each of the five days of measurement. Moreover, the daily rifampicin release [μg/day], as well as the final amount of rifampicin still present on both dense and porous bone substitutes five days after its loading, have been calculated.

The overall mean and related standard deviation of the released rifampicin quantity for day 1, day 2, day 3, day 4 and day 5, have been computed and

displayed in a comprehensive graph, for both dense granules and porous chips substitutes.

3 Results

3.1 Loading of Rifampicin on Bone Substitutes

Rifampicin chromatograms Time [min] – Absorbance [mAU], obtained from three different loading methods – physiological solution 0.9%, 2 mM ascorbic acid in citrate buffer and 10 mM ascorbic acid in citrate buffer, are shown in the following figures (Figure 1, Figure 2 and Figure 3).

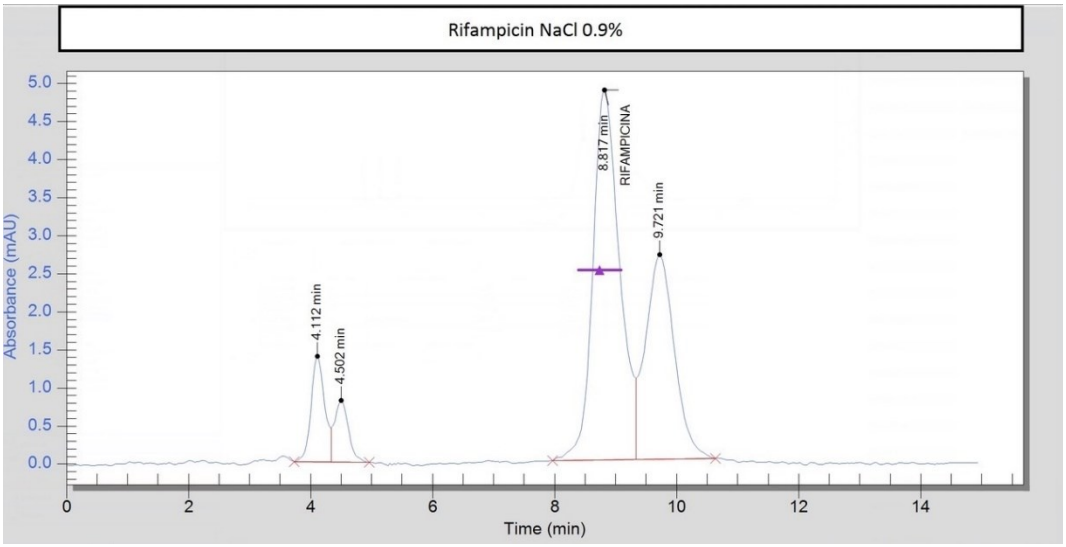

Figure 1.*Chromatogram of rifampicin in physiological solution 0.9%*

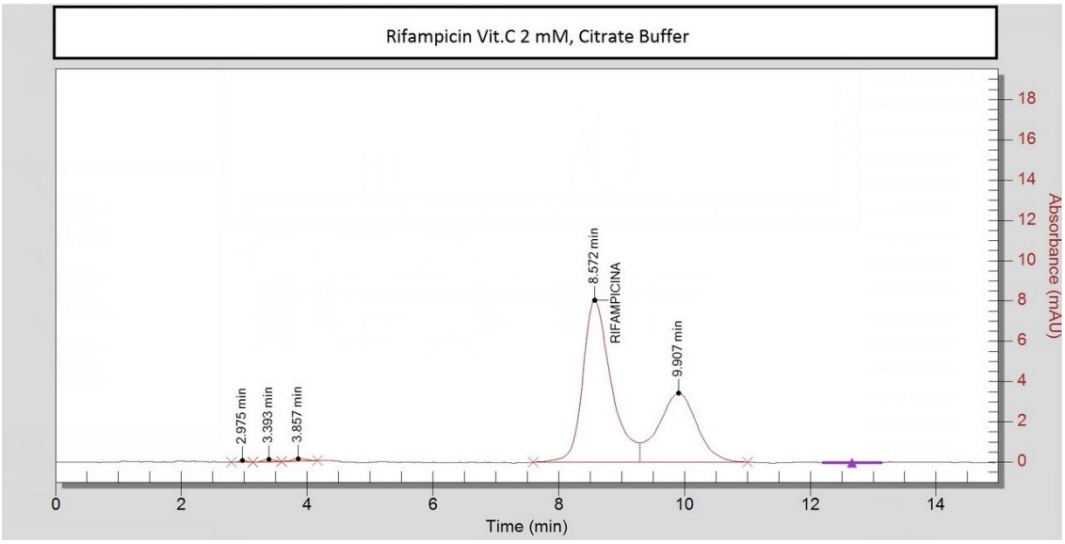

Figure 2. *Chromatogram of rifampicin with 2 mM ascorbic acid in citrate buffer.*

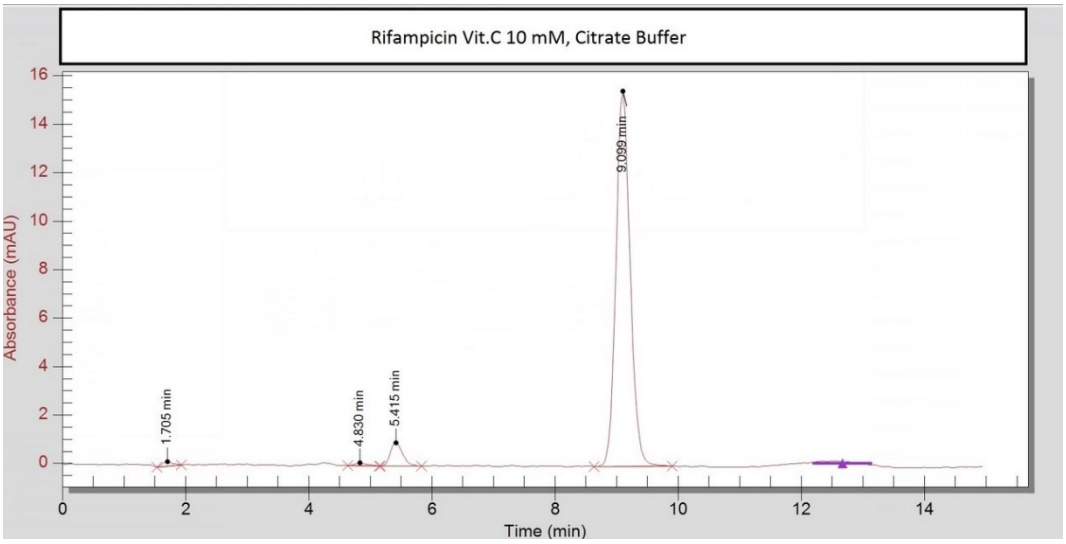

Figure 3. *Chromatogram of rifampicin with 10 mM ascorbic acid in citrate buffer.*

3.2 Rifampicin Extraction and HPLC Analysis

In Table 1 are reported the area [dimensionless] of the rifampicin peaks, computed by Chromera software, for each calibration sample.

Table 1. *Area [dimensionless] of rifampicin peaks, from both HPLC column injections, for each rifampicin sample during instrument calibration. (S* = 0 µg/ml, S0 = 5 µg/ml, S1 = 15 µg/ml, S2 = 30 µg/ml).*

Calibration sample	Injection	Area
S*	1:2	0
	2:2	0
S0	1:2	241371.66
	2:2	247592.24
S1	1:2	1010786.87
	2:2	1034824.96
S2	1:2	2469909.71
	2:2	2539606.09

The calibration line, created automatically by Chromera software thanks to the HPLC Analysis of calibration samples, is shown in Figure 4.

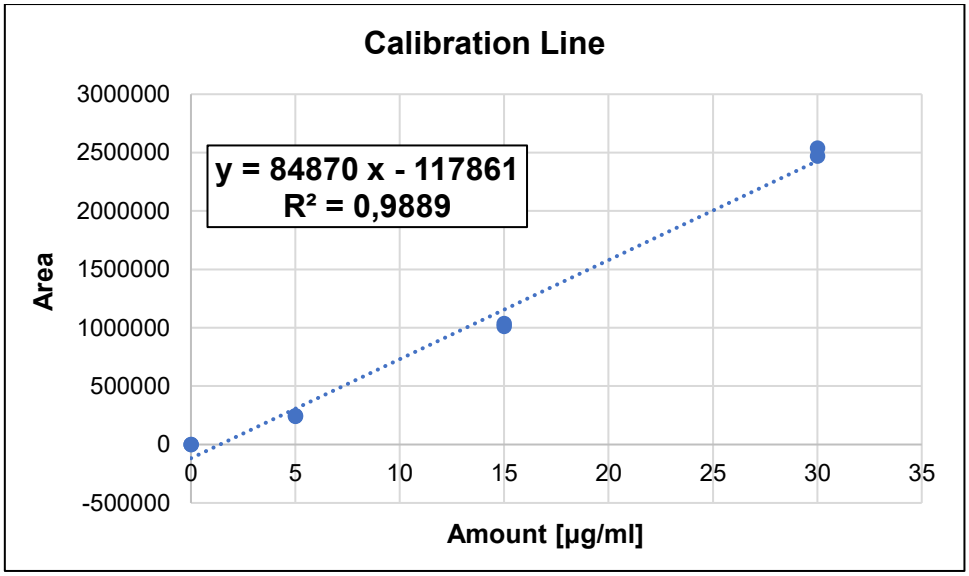

Figure 4. *Calibration Line used for HPLC Analysis.*

In Table 2 are reported, for the three trials, the area [dimensionless] of rifampicin peaks and the amounts of rifampicin present in the ascorbic acid solution [µg], for each of the five days of extraction, for both dense and porous bone substitutes.

Table 2. *Area [dimensionless] of rifampicin peaks and related amount in solution [µg], for each day of extraction from dense granules and porous chips, during first trial (T1), second trial (T2) and third trial (T3).*

		DENSE GRANULES		**POROUS CHIPS**	
		Area	**Amount of rifampicin [µg]**	**Area**	**Amount of rifampicin [µg]**
Day 1	**T1**	593973.56	838.7	428321.44	643.6
	T2	574932.81	816.3	452550.27	672.1
	T3	598526.67	844.1	408672.48	620.4
Day 2	**T1**	1085531.92	1417.9	487504.93	713.3
	T2	1059370.77	1387.1	633577.98	885.4
	T3	1099429.41	1434.3	494476.05	721.5
Day 3	**T1**	1358713.65	1739.8	722749.49	990.5
	T2	1324504.65	1699.5	736100.94	1006.2
	T3	1367279.13	1749.9	762580.38	1037.4
Day 4	**T1**	1529358.03	1940.9	1080404.67	1411.9
	T2	1492632.12	1897.6	972124.41	1284.3
	T3	1547033.79	1961.7	857380.17	1149.1
Day 5	**T1**	1635729.92	2066.2	1268646.33	1633.7
	T2	1595070.21	2018.3	1015068.63	1334.9
	T3	1655752.26	2089.8	939534.33	1245.9

The daily rifampicin release [μg/day], from both dense granules and porous chips, for each of the three trials, together with the overall mean and the related standard deviation for day 1, day 2, day 3, day 4 and day 5, are reported in Table 3 and displayed in Figure 5.

Table 3. *Daily rifampicin release [μg/day], during first trial (T1), second trial (T2) and third trial (T3), and overall mean and standard deviation (SD), for both dense granules and porous chips.*

	Rifampicin release [μg/day]							
	DENSE GRANULES				**POROUS CHIPS**			
	T1	**T2**	**T3**	**Mean [SD]**	**T1**	**T2**	**T3**	**Mean [SD]**
Day 1	838.7	816.3	844.4	833.0 [14.74]	643.6	672.1	620.4	645.4 [25.89]
Day 2	579.2	570.8	590.2	580.1 [9.73]	69.7	213.3	101.1	128.0 [75.49]
Day 3	321.9	312.4	315.6	316.6 [4.83]	277.2	120.8	315.9	238.0 [103.30]
Day 4	201.1	198.1	211.8	203.7 [7.20]	421.4	278.1	111.7	270.4 [154.99]
Day 5	125.3	120.7	128.1	124.7 [3.74]	221.8	50.6	96.8	123.1 [88.57]

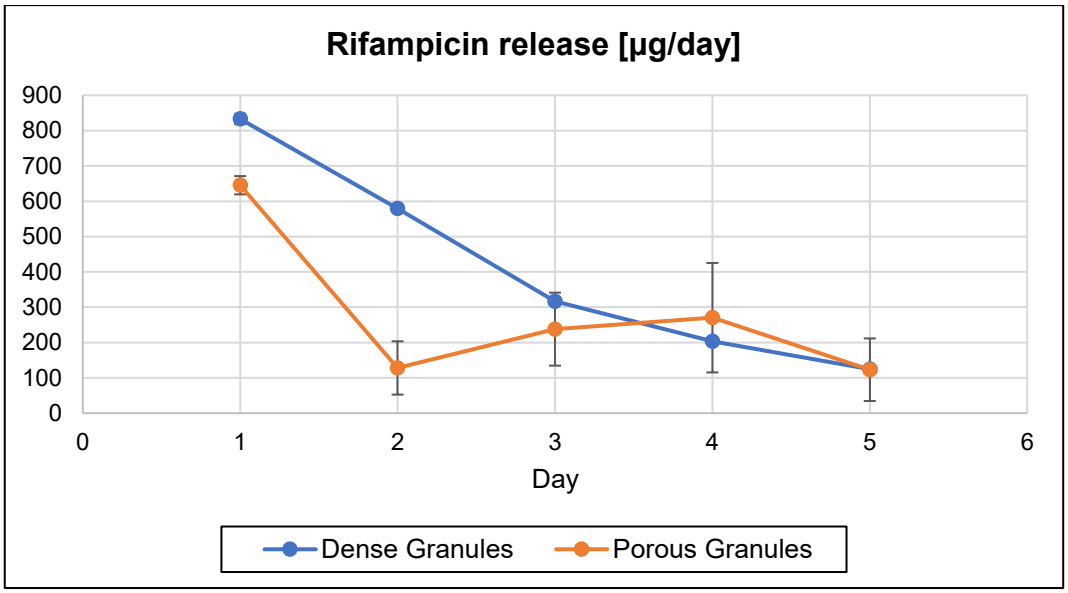

Figure 5. *Daily mean Rifampicin release and standard deviation [µg/day] for dense granules and porous chips.*

Finally, Table 4 shows the final amounts of rifampicin [µg] still present on both dense and porous bone substitutes, with respect to the initial rifampicin loading (5 mg), after five days from the placement in the ascorbic acid solution, for each trial, and the relative mean and standard deviation.

Table 4. *Rifampicin [µg] on bone substitute after five days, with respect to initial (5 mg) loading (%), during first trial (T1), second trial (T2) and third trial (T3), and overall mean and standard deviation (SD), for both dense granules and porous chips.*

	Rifampicin [µg] on bone substitute after five days, with respect to initial loading (%)	
	DENSE GRANULES	**POROUS CHIPS**
T1	2933.8 (58.67%)	3366.3 (67.33%)
T2	2981.7 (59.63%)	3665.1 (73.30%)
T3	2910.2 (58.20%)	3754.1 (75.08%)
Mean [SD]	2941.9 (58.84%) [36.43]	3595.2 (71.90%) [203.14]

4 Discussion

4.1 Loading of Rifampicin on Bone Substitutes

As it is possible to see in Figure 2 and 3 in Results, the use of ascorbic acid (solubilized in a buffer citrate solution, pH 5.0), for preventing the degradation of the antibiotic rifampicin, as indicated in the study of Rajaram et al.[77], led to profitable outcomes for the development of the chromatographic method; in fact, as can be seen in Figure 1, rifampicin oxides easily when placed in physiological solution and it is not perfectly detectable by the HPLC system already after one day from its loading on the bone substitute. Instead, by placing the bone substitute in a solution contained the antioxidant, the rate of oxidation has slowed down drastically (as can be seen on the left parts of the chromatograms in Figure 2 and 3, where the in-vitro degradation products of the antibiotic, detected by the HPLC, are significantly reduced); in particular, a better quantification of the rifampicin has been obtained by using a higher concentration of ascorbic acid (10 mM).

4.2 Rifampicin Extraction and HPLC Analysis

As shown by the calibration line (Figure 4), obtained with the values reported in Table 1, the concentrations of the analyte and the instrument response for each standard can be fit to a straight line; moreover, the value of the coefficient of determination (R^2 = 0.9889) claims that the calibration line accurately represents the instrument response. This result confirms the

efficiency of the chromatographic method developed by using an isocratic mobile phase, composed by potassium dihydrogen phosphate and acetonitrile [79], together with a non-polar stationary phase, to estimate the concentration of the analyte rifampicin in an unknown sample (Table 2).

By observing Table 3 and Figure 5, dense granules and porous chips are characterized by different rifampicin release times. There is a more coherent, homogeneous and gradual release of rifampicin from dense granules than from porous chips, in which the antibiotic is released with a more incongruent and uncontrolled trend, as confirmed by the daily standard deviation values, that are particularly high for porous bone substitutes.

A possible cause could be the macroporous structural conformation of porous chips, capable of retaining less effectively the rifampicin, with respect to the microporous structure of the dense granules. For this reason, even the slightest vibration or movement of either the sample or the solution surrounding the bone substitute can cause an uncontrolled release of rifampicin, affecting the actual release times. According to its homogeneous and controlled performance, a bone substitute made of dense granules could be more reliable as antibiotic carrier device.

Despite its peculiar and overall poor favourable behaviour, porous chips, as well as dense granules, are characterized by a high initial rifampicin release (day 1), probably due to the fact that the initial quantity of loaded antibiotic (5 mg) is particularly massive for the volume of bone (0.5 cc) used for the in-vitro experiment, and the majority of the drug doesn’t bind completely the

bone substitute. The advantageous aspect of this burst release of antibiotic from the carrier, soon after its loading, is the possibility to have high local bactericidal concentration in-vivo, capable of treating the first stages of bone infection onset.

Moreover, in the following days the tendency of releasing rifampicin is more gradual and delayed in time; in this case, the result is remarkably interesting since not all the loaded antibiotic is expelled at once but a consistent part, as shown in Table 4, is still present on the bone substitute after five days from the beginning of the trial. This behaviour could allow the use of the analyzed bone substitutes as reliable carriers for a local and prolonged antibiotic therapy, with no toxic consequences for the body, for prevention and treatment of osteomyelitis in orthopedics.

5 Concluding Remarks and Future Perspectives

The chromatographic method, developed in this work, has allowed to quantify correctly the release time of rifampicin loaded on bio-mimetic nano-structured hydroxyapatite bone substitutes, in both porous chips and dense granules formats; in particular the use of ascorbic acid as antioxidant enables to evaluate the release of rifampicin over a period of several days.

This study has shown that both bone substitutes - in particular dense granules, characterized by a more homogeneous and controlled antibiotic release - are suitable for the treatment of osteomyelitic infections.

The result obtained with this study is of great use in the application field of regenerative medicine.

The development of this effective chromatographic measurement method, capable of testing the in-vitro release times of antibiotics, provides fundamental information to biomedical companies, specialized in the production of synthetic bone substitutes, allowing them to combine the data relating to the functional and structural characteristics of their products with those relating to the performance of the biomaterials as carriers for antibiotic prophylaxis.

This study is therefore complementary to the use of those biomedical devices since it allows to verify which antibiotic is preferable to associate with a specific bone substitute used in orthopedics with the double role of

bone graft and effective carrier of antibiotics for the treatment and prevention of osteomyelitis.

In the future, it would be interesting and useful to analyse with a chromatographic method the in-vitro release times of other antibiotics commonly used in the treatment of osteomyelitis in orthopedics, such as glycopeptides (vancomycin) and aminoglycosides (tobramycin and gentamicin), loaded on biodegradable and osteoconductive bone substitutes.

References

[1] Roberts TT, Rosenbaum AJ. Bone grafts, bone substitutes and orthobiologics: the bridge between basic science and clinical advancements in fracture healing. *Organogenesis*. 2012;8(4):114-124.

[2] Kronenberg HM. Developmental regulation of the growth plate. Nature. 2003;423(6937):332-336.

[3] Kubota T, Michigami T, Sakaguchi N, Kokubu C, Suzuki A, Namba N, et al. Lrp6 hypomorphic mutation affects bone mass through bone resorption in mice and impairs interaction with Mesd. Journal of Bone and Mineral Research. 2008;23:1661-1671.

[4] Bartl R., Bartl C. (2017) Modelling and Remodelling of Bone. In: Bone Disorders. Springer, Cham.

[5] Leng Y, Yang F, Wang Q, Li ZH, Yuan BM, Peng CG, Ren GK, Wang ZH, Cui YT, Wang YB, Zhu LY, Liu H, Wu DK. Material-based therapy for bone nonunion. Materials and Design. 2019;183:108161.

[6] Dahiya, Ujjwal & Mishra, Sarita & Bano, Subia. (2019). Application of Bone Substitutes and Its Future Prospective in Regenerative Medicine.

[7] Greenwald AS, Boden SD, Goldberg VM, Khan Y, Laurencin CT, Rosier RN. Bone-graft substitutes: facts, fictions and applications. J Bone Joint Surg Am. 2001;83:98–103.

[8] Van Heest A, Swiontkowski M. Bone-graft substitutes. Lancet. 1999 Apr;353 Suppl 1:SI28-9.

[9] Bhatt RA, Rozental TD. Bone graft substitutes. Hand Clin. 2012 Nov;28(4):457-68.

[10] Deev RV, Drobyshev AY, Bozo IY, Isaev AA. Ordinary and Activated Bone Grafts: Applied Classification and the Main Features. Biomed Res Int. 2015;2015:365050.

[11] Urist MR, O'Conner BT and Burwell RG. *Bone graft, derivatives and substitutes*. Cambridge: Butterworth- Heinemann, 1994.

[12] Mainard D and Delagoutte J-P. Les substituts osseux. In: Mainard D, Merle M, Delagoutte J-P, et al. (eds) *Actualités en biomatériaux*. Paris Romilliat, 1990, pp. 128–176.

[13] Dreesman H. Über knochenplombierung. *Beitr Klin Chir* 1892; 9: 804–810.

[14] Balaji VR, Manikandan D, Ramsundar A. Bone grafts in periodontics. Matrix Sci Med 2020;4:57-63.

[15] Habibovic P, de Groot K. Osteoinductive biomaterials— Properties and relevance in bone repair. Journal of Tissue Engineering and Regenerative Medicine. 2007;1:25-32.

[16] Yi H, Ur Rehman F, Zhao C, Liu B, He N. Recent advances in nano scaffolds for bone repair. Bone Research. 2016;4:16050.

[17] Bauer TW, Muschler GF. Bone graft materials. An overview of the basic science. Clinical Orthopaedics and Related Research. 2000 Feb(371):10-27.

[18] Giannoudis PV, Einhorn TA, Schmidmaier G, Marsh D. The diamond concept – open questions. Injury. 2008;39(suppl 2):S5–S8.

[19] Fernandez de Grado G, Keller L, Idoux-Gillet Y, Wagner Q, Musset AM, Benkirane-Jessel N, Bornert F, Offner D. Bone substitutes: a review of their characteristics, clinical use, and perspectives for large bone defects management. J Tissue Eng. 2018 Jun 4;9:2041731418776819.
[20] Motilal R Jangid, PS Rakhewar, Abhishek Singh Nayyar, Anup Cholepatil, Pooja Chhabra. (2016). Bone Grafts and Bone Graft Substitutes in Periodontal Regeneration: A Review. Int. J. Curr. Res. Med. Sci. 2(8): 1-7.
[21] Campana V, Milano G, Pagano E, Barba M, Cicione C, Salonna G, Lattanzi W, Logroscino G. Bone substitutes in orthopaedic surgery: from basic science to clinical practice. J Mater Sci Mater Med. 2014 Oct;25(10):2445-61.
[22] Pape HC, Evans A, Kobbe P. Autologous bone graft: properties and techniques. J Orthop Trauma. 2010;24(Suppl 1):S36–40.
[23] Baumhauer J, Pinzur MS, Donahue R, Beasley W, Digiovanni C. Site selection and pain outcome after autologous bone graft harvest. Foot Ankle Int. 2014;35(2):104–7.
[24] Boden SD. Overview of the biology of lumbar spine fusion and principles for selecting a bone graft substitute. Spine. 2002;27(16 Suppl 1):S26–31.
[25] Khan SN, Cammisa FP Jr., Sandhu HS, Diwan AD, Girardi FP, Lane JM. The biology of bone grafting. J Am Acad Orthop Surg 2005; 13:77-86.
[26] Calvo R, Figueroa D, Dı´az-Ledezma C, Vaisman A, Figueroa F. Bone allografts and the functions of bone banks. Rev Med Chil. 2011;139(5):660–6.
[27] Bae DS, Waters PM. Free Vascularized Fibula Grafting: Principles, Techniques, and Applications in Pediatric Orthopaedics. Orthopaedic Journal at Harvard Medical School 2006; 8:86-9.
[28] Dumitrescu AL. Bone grafts and bone graft substitutes in periodontal therapy. In: Chemicals in surgical periodontal therapy. Berlin: Springer; 2011. p. 92.
[29] Ashman A. The use of synthetic bone materials in dentistry. Compendium 1992;13:1020, 1022, 1024-1026.
[30] Bruens ML, Pieterman H, de Wijn JR, Vaandrager JM. Porous polymethylmethacrylate as bone substitute in the craniofacial area. J Craniofac Surg. 2003 Jan;14(1):63-8.
[31] Kuo TC, Lee BS, Kang SH, Lin FH, Lin CP. Cytotoxicity of DP-bioglass paste used for treatment of dentin hypersensitivity. J Endod 2007;33:451-454.
[32] Oonishi H, Kushitani S, Yasukawa E, Iwaki H, Hench LL, Wilson J, et al Particulate bioglass compared with hydroxyapatite as a bone graft substitute. Clin Orthop 1997;334:316-325.
[33] Nandi, S. K., et al. "Orthopaedic applications of bone graft & graft substitutes: a review." *Indian Journal of Medical Reseach* 132.1 (2010): 15-30.
[34] Hak DJ. The use of osteoconductive bone graft substitutes in orthopaedic trauma. J Am Acad Orthop Surg 2007;15:525-36.

[35] Li GQ, Guo FF, Ou Y, Dong GW, Zhou W. Epidemiology and outcomes of surgical site infections following orthopedic surgery. Am J Infect Control. 2013 Dec;41(12):1268-71.
[36] Cheatle M D. The effect of chronic orthopedic infection on quality of life. Orthop Clin of North Am 1991; 22:539-46.
[37] Lew, Daniel P., and Francis A. Waldvogel. "Osteomyelitis." The Lancet 364.9431 (2004): 369-379.
[38] Jauregui LE, Senour CL. In: Jauregui LE, ed. Diagnosis and management of bone infections. New York: Marcel Dekker, 1995:37–108.
[39] Pšorn V. Value of preoperative preparation and postoperative care in prevention of the infections in orthopedic surgery. Acta Orthop Yugosl 1973; 1: 21-24.
[40] Milankov M. Treatment of unspecific bone infection with fluid drenaging. Offset print. Novi Sad, 1996.
[41] Soldatovi, G., Milenkovi, S., Mitkovi, M., Bumbairevi, M., Anelkovi, D., & Mili, D. (2004). Bone infections and antibiotics use in orthopaedics. Acta Fac. Med. Naiss, 21, 245-52.
[42] Charnley J, Eftekhar N. Postoperative infection in total prosthetic replacement arthroplasty of the hip joint. Br J Surg 1969;56:641-9.
[43] Lidwell OM. Air, antibiotics and sepsis in replacement joints. J Hosp Infect 1988;11(Suppl C):18-40.
[44] Tucci G, Amorese V, Venditti M. Profilassi antibiotica in chirurgia ortopedica:evidenze scientifiche e pratica clinica.GIOT2005;31:121–9.
[45] Baščarević Lj, Baljošević I. The role and the significance of immunoglobulin in infections of skeletal system. XVI ortopedsko-traumatološki dani Jugoslavije, Zbornik radova, 1986; 7-10.
[46] Mitchell C, Ehrlich G. M, Siffert S R. Comparative bacteriology of early and late orthopaedic infection. Clin. Orthop. 1973; 96:277-287.
[47] Weinstein D, Wang A, Chambers R, Stewart C. A, Motz H. A. Evaluation of magnetic resonance imaging in the diagnosis of osteomyelitis in diabetic foot infection. Foot Ankle. 1993; 14: 18-22.
[48] Santiago Restrepo C, Gimenez CR, McCarthy K. Imaging of osteomyelitis and musculoskeletal soft tissue infections: current concepts. Rheum Dis Clin North Am 2003; 29: 89–109.
[49] Rao, Nalini M.D.; Ziran, Bruce H. M.D.; Lipsky, Benjamin A. M.D. Treating Osteomyelitis: Antibiotics and Surgery, Plastic and Reconstructive Surgery: January 2011 - Volume 127 - Issue - p 177S-187S.
[50] Lowy FD. Staphylococcus aureus infections. N Engl J Med. 1998 Aug 20;339(8):520-32.
[51] Tong SY, Davis JS, Eichenberger E, Holland TL, Fowler VG Jr. Staphylococcus aureus infections: epidemiology, pathophysiology, clinical manifestations, and management. Clinical Microbiology Reviews. 2015 Jul;28(3):603-661.
[52] Vuong C, Otto M. Staphylococcus epidermidis infections. Microbes Infect. 2002 Apr;4(4):481-9.

[53] Nafziger DA, Saravolatz LD. Infection in implantable prosthetic devices. In: Wenzel RP, ed. Prevention and control of nosocomial infections. Third Edition. Baltimore, USA: Williams & Wilkins 1997, pp. 889-923.
[54] Brown AR, Vicca AF, Taylor GJS. A comparison of prophylactic antibiotic regimens against airborne orthopaedic wound contamination. J Hosp Infect 2001;48:117-21.
[55] Periti P, Mini E, Mosconi G. Antimicrobial prophylaxis in orthopaedic surgery: the role of teicoplanin. J Antimicrob Chemother 1998;41:329-40.
[56] Rao, Nalini, Bruce H. Ziran, and Benjamin A. Lipsky. "Treating osteomyelitis: antibiotics and surgery." Plastic and reconstructive surgery 127 (2011): 177S-187S.
[57] Beloor Suresh A, Wadhwa R: Rifampin. StatPearls. StatPearls Publishing, 2020, Treasure Island (FL).
[58] ter Boo, Gert-Jan A., et al. "Antimicrobial delivery systems for local infection prophylaxis in orthopedic-and trauma surgery." Biomaterials 52 (2015): 113-125.
[59] Ito K, Perren SM. Biology and biomechanics in bone healing. In: Rüedi TP, Buckley RE, Moran CG, editors. AO principles of fracture management. Davos Platz: AO Publishing; 2007. p. 9e31.
[60] Rathbone CR, Cross JD, Brown KV, Murray CK, Wenke JC. Effect of various concentrations of antibiotics on osteogenic cell viability and activity. J Orthop Res 2011; 29:1070-4.
[61] Rushton N. Applications of local antibiotic therapy. Eur J Surg 1997; Suppl. 578: 27-30.
[62] Allababidi S, Shah JC. Kinetics andmechanism of release from glyceryl monostearate-based implants: evaluation of release in a gel simulating in vivo implantation. J Pharm Sci 1998; 87 (6): 738-44.
[63] Henry SL, Galloway KP. Local antibacterial therapy for the management of orthopaedic infections. Pharmacokinetic considerations. Clin Pharmacokinet 1995; 29 (1): 36-45.
[64] Nelson CL, Griffin FM, Harrison BH, et al. In vitro elution characteristics of commercially and noncommercially prepared antibiotic PMMA beads. Clin Orthop 1992 Nov; 284:303-9.
[65] Rauschmann MA, Wichelhaus TA, Stirnal V, et al. Nanocrystalline hydroxyapatite and calcium sulfate as biodegradable composite carrier material for local delivery of antibiotics in bone infections. Biomaterials. 2005; 26:2677–2684.
[66] Masquelet AC, Fitoussi F, Begue T, et al. Reconstruction of the long bones by the induced membrane and spongy autograft [In French]. Ann Chir Plast Esthet. 2000;45:346–353.
[67] Neut D, van de Belt H, Stokroos I, van Horn JR, van der Mei HC, Busscher HJ. Biomaterial-associated infection of gentamicin-loaded PMMA beads in orthopaedic revision surgery. J Antimicrob Chemother. 2001;47(6):885–891.

[68] Nandi, Samit Kumar, et al. "Local antibiotic delivery systems for the treatment of osteomyelitis–A review." Materials Science and Engineering: C 29.8 (2009): 2478-2485.
[69] Alt, Volker & Franke, Jörg & Schnettler, Reinhard. (2015). Local Delivery of Antibiotics in the Surgical Treatment of Bone Infections. Techniques in Orthopaedics. 30. 230-235.
[70] Stemberger A, Grimm H, Bader F, et al. Local treatment of bone and soft tissue infections with the collagen-gentamicin sponge. Eur J Surg 1997; Suppl. 578: 17-26.
[71] El-Husseiny M, Patel S, MacFarlane RJ, Haddad FS. Biodegradable antibiotic delivery systems. J Bone Joint Surg Br.2011Feb;93(2):151-7.
[72] Hanssen AD. Local antibiotic delivery vehicles in the treatment of musculoskeletal infection. Clin Orthop Relat Res. 2005;437:91–96.
[73] Ferguson J, Diefenbeck M, McNally M. Ceramic Biocomposites as Biodegradable Antibiotic Carriers in the Treatment of Bone Infections. J Bone Jt Infect. 2017;2(1):38-51.
[74] van de Belt H, Neut D, Uges DR, Schenk W, van Horn JR, van der Mei HC, et al. Surface roughness, porosity and wettability of gentamicin-loaded bone cements and their antibiotic release. Biomaterials 2000;21(October (19)):1981–7.
[75] Malviya, Rishabha & Bansal, Vvipin & Pal, Om & Sharma, Pramod. (2010). High performance liquid chromatography: A short review. Journal of Global Pharma Technology. 2. 22-26.
[76] World Health Organization. The international Pharmacopoeia. 9 ed. Columbia University Press; 2019.
[77] Rajaram S, Vemuri VD, Natham R. Ascorbic acid improves stability and pharmacokinetics of rifampicin in the presence of isoniazid. J Pharm Biomed Anal. 2014 Nov;100:103-108.
[78] Perkin Elmer, Flexar LC Systems, 008523D_01, 2017.
[79] Siddhartha, T. SRIRAM, et al. "Development and validation of high performance liquid chromatographic method for the determination of rifampicin in human plasma." *Int J Pharm Pharm Sci* 4.5 (2012):362-7.