Magda Sofia Catroga Ferreira

Licenciada em Bioquímica

Antimicrobial Effect of Polymeric Biomaterials for Bone Infection Treatment

Dissertação para obtenção do Grau de Mestre em Bioquímica

Orientador: Ana Bettencourt, Professora Doutora, Faculdade de Farmácia, Universidade de Lisboa
Co-orientador: Luísa Jordão, Professora Doutora, Instituto Nacional de Saúde Dr. Ricardo Jorge

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“Antimicrobial Effect of Polymeric Biomaterials for Bone Infection Treatment”

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Abstract

Bone infection, mainly caused by *Staphylococcus aureus,* is a public health concern. Treatment is challenging due to multi-resistant strains, and *S. aureus* ability to adhere and form biofilm on bone and implant surfaces, as well as to invade and persist in osteoblast cells.

The present work consisted in the preparation and evaluation of novel acrylic polymeric systems that provide local and controlled antibiotic delivery for the treatment of bone infection, namely levofloxacin-loaded acrylic bone cement (BC), and vancomycin or daptomycin-loaded acrylic microparticles (MP).

Properties of both delivery systems with high impact on clinical performance were tested. Namely, contact angle and surface energy were determined in BC matrices and encapsulation efficiency in MP formulations. Release studies of levofloxacin-loaded BC matrices were also conducted. Also, the anti-biofilm activity of these systems was evaluated against *S. aureus* strains. Furthermore, BC and MP formulations were tested concerning the antibacterial intracellular activity using a human osteoblast infection model.

Overall, both BC formulations' surface characteristics and MP encapsulation efficiency were in agreement with previously published data. The release studies of levofloxacin from BC matrices showed that the drug release is size- and incubation medium-dependent. All BC matrices loaded with levofloxacin concentrations of 1.5 % or higher exhibited anti-biofilm activity against all *S. aureus* tested strains. For BC matrices and Vancomycin-loaded MP, a decrease of viable intracellular bacteria was observed. For Daptomycin-loaded MP, no viable intracellular bacteria were detected.

In conclusion, this work has shown that the BC formulations with drug concentration of 1.5 % or 2.5 % and daptomycin-loaded MP show potential to be used in the context of bone infection treatment.

Keywords: Bone infection; bone cement; microparticles; levofloxacin; *Staphylococcus aureus*; intracellular infection.

Resumo

A infeção óssea, causada principalmente por *Staphylococcus aureus*, é um grave problema de saúde pública. O seu tratamento é difícil devido a estirpes multi-resistentes, à sua habilidade de aderir e formar biofilmes em osso e implantes, bem como à sua capacidade de invadir e persistir em osteoblastos.

Este trabalho consiste na preparação e avaliação de novos sistemas poliméricos acrílicos, que promovem a libertação local e controlada de antibióticos para o tratamento de infeções ósseas, nomeadamente cimento ósseo (BC) carregado com levofloxacina, bem como vancomicina ou daptomicina encapsulada em micropartículas (MP).

Foram testadas propriedades com elevado impacto no desempenho clínico de ambos os sistemas, nomeadamente ângulos de contacto e energia de superfície das matrizes de BC, assim como a eficiência de encapsulação das formulações de MP. Foram também realizados estudos *in vitro* de libertação de levofloxacina das matrizes de BC e da sua atividade anti-biofilme contra estirpes de *S. aureus*. Usando um modelo de infeção de osteoblastos humanos, ambos os sistemas foram avaliados quanto à sua atividade antibacteriana intracelular.

Tanto as características de superfície das matrizes de BC como a eficiência de encapsulação de MP estão em concordância com estudos realizados anteriormente. Os estudos de libertação de levofloxacina a partir de diferentes formulações de BC revelaram que esta é dependente do tamanho das placas e do meio de incubação. As formulações de BC com concentração de fármaco igual a 1.5 e 2.5 % demonstraram atividade anti-biofilme. Todas as formulações testadas reduziram o número de bactérias intracelulares viáveis, sendo mais eficazes as MP com daptomicina.

Este trabalho conclui que as formulações de BC com concentração de fármaco igual a 1.5 e 2.5 % e as MP carregadas com daptomicina demonstram potencial para utilização no tratamento de infeções ósseas.

Palavras-chave: Infeção óssea; cimento ósseo; micropartículas; levofloxacina; *Staphylococcus aureus*; infeção intracelular.

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List of abbreviations

Ala – Alanine
ALABC – Antibiotic-loaded Acrylic Bone Cement
ATCC - American Type Culture Collection
ATP – Adenosine triphosphate
a.u. – Absorvance units
BC – Bone Cement
Bis-GMA/TEGDMA – 2,2'-bis-[4-(methacryloxypropoxy)-phenyl]-propane/Tri(ethyleneglycol) dimethacrylate
BPO – Benzoyl peroxide
CFU – Colony Forming Unit
CLSI – Clinical & Laboratory Standards Institute
CPC – Calcium Phosphate Cement
CRP – C-reactive Protein
CT – Computed Tomography
Dapto – Daptomycin
DBM – Demineralized Bone Matrix
DCM – Dichloromethane
DL – Drug Loading
DMPT – N, N-Dimethyl para-toluidine
DMpt – Dimethyl para-toluidine
DNA – Deoxyribonucleic Acid
DNS – Dinitrosalicylic Acid
Eap – Extracellular Adhesion Protein
ECM – Extracellular Polymeric Matrix
EE – Encapsulation efficiency
ESR - Erythrocyte Sedimentation Rate
EUD – Eudragit RL 100
FDG-PET – 18-fluoro-D-deoxyglucose Positron Emission Tomography
FnBPA – Fibronectin Binding Protein A
FnBPB – Fibronectin Binding Protein B
GlcNAc – *N*-acetylglucosamine Acid
HA – Hydroxyapatite
HAI – Healthcare Associated Disease
HPLC – High Performance Liquid Chromatography
Lac – Lactose
Lev – Levofloxacin
MBC – Minimum Bactericidal Concentration
MBIC – Minimum Biofilm Inhibitory Concentration
MIC – Minimum Inhibitory Concentration

MIC_{90} – Minimum Inhibitory Concentration necessary to inhibit bacteria growth in 90 %

MH – Müeller-Hinton

MMA – Methyl methacrylate

MOI – Multiplicity of Infection

MP – Microparticles

MRI – Magnetic Resonance Image

MRSA – Methicillin-resistance *Staphylococcus aureus*

MSCRAMM – Microbial Surface Components Recognizing Adhesive Matrix Molecules

MSSA - Methicillin-susceptible *Staphylococcus aureus*

MurNAc – *N*-acetylmuramic Acid

Nano-HA-PHBV/PEG-GM – Nano-hydroxyapatite/poly (3-hydroxybutyrate-hydroxyvalerate)-polymethyl lene glycol gentamicin drug delivery system

OD – Optical density

PBS – Phosphate Buffered Saline

Pls – Plasmin-sensitive Protein

PMMA – Poly(methyl methacrylate)

PVA – Poly(vinyl alcohol)

RPMI – Roswell Park Memorial Institute medium

Sccmec I – Staphylococcal Chromosomal Cassette type I

SCV – Small Colony Variants

SEM – Scanning Electron Microscopy

SD – Standard Deviation

TRAIL – TNF-Related Apoptosis-inducing Ligand

UDP – Uridine Diphosphate

UV – Ultraviolet

Vanco – Vancomycin

Vanco-AB-FG – Vancomycin-Alginate Beads-Fibrin Gel

Objective and thesis structure

The present thesis' main objective is the evaluation of antibacterial activity of antibiotic-loaded polymeric biomaterial systems, namely bone cement and microparticles, against the main pathogenic organism causing bone infections (*Staphylococcus aureus*).

Specific aims were:

- Production of polymeric systems with incorporation of respective drugs, levofloxacin in bone cement formulations and daptomycin or vancomycin in microparticles;
- Brief characterization of drug delivery systems;
- Release studies of drug and lactose (release modulator) from bone cement matrices for method optimization;
- Assessment of antibacterial activity, namely anti-biofilm efficacy of bone cement matrices;
- Evaluation of the antibacterial intracellular activity of bone cement formulations and microparticles.

This work is organized in four chapters and annexes including: Introduction, Materials and Methods, Results and Discussion, and Conclusion and Future Work.

Chapter 1 – Introduction

In Introduction, a brief clinical context of the bacterial bone infection is presented. It contains a sum of microorganisms that are usually responsible for bone infections and a description of the most common pathogen causing this disease, particularly the mechanisms and the problems with its biofilm assembly ability and its capacity to invade and persist within osteoblast cells. The challenge of the conventional antibiotic treatment and the characteristics of antibiotics used in this study are also detailed. Novel local drug delivery systems as a new therapeutic strategy including a list of some systems that have been developed, and a description of antibiotic-loaded acrylic bone cement and antibiotic-loaded polymeric microparticles. Finally, the assays that should be performed to characterize and evaluate the antibacterial activity of novel systems for applications in the treatment of bone infection. As such, for bone cement systems, surface and release studies and microbiological assays were performed and, for polymeric microparticles, encapsulation efficiency was determined. Also, for both systems, intracellular assays were conducted.

Chapter 2 – Materials and Methods

The first part of this chapter details the preparation of both systems, and the surface and release studies experiments for bone cement specimens. Furthermore, the experimental procedure for encapsulation efficiency of the antibiotic-loaded microparticles is also presented. The second part describes the microbiological assays conducted in bone cement formulations, and the intracellular survival assays for

both antibiotic-loaded polymeric systems. The reagents, equipment and experimental protocols used in this work are detailed in this chapter.

Chapter 3 – Results and Discussion

In this chapter, all results obtained from the experiments performed throughout this study and subsequent discussion are presented. It concerns the production of bone cement and microparticles formulations, their characterization, drug and lactose release studies, as well as the effects of bone cements matrices on microbiological activity and the antibacterial intracellular performance of all formulations.

Chapter 4 – Conclusion and Future Work

The last chapter of this thesis contains the main conclusions drawn from this work and possible alterations and improvements that can be made to the methodologies used in the present work. Moreover, the potential formulations that may be chosen for more advanced segments of studies for medical application are discriminated.

Chapter 1. Introduction

1.1. Bone infection

Infection is defined as a homeostatic imbalance between the host tissue and the presence of microorganisms [1]. Infections occur when pathogens (bacteria or fungi) successfully invade the host and begin to reproduce [2]. A major concern in terms of medical care is the infection involving bone, due to this being a significant cause of morbidity and mortality. Furthermore, they can result in prolonged hospital stays, long courses of systemic antibiotics and frequently will require a new surgical intervention [3].

Bone infection is associated with a variety of occurrences, mainly from complications following surgery and device implantation, leading to orthopedic implant failure and resulting in diseases such as osteomyelitis, and septic arthritis. Therefore, this infection can be acquired after bone surgery, or joint replacement, or even in consequence of a trauma. Consequently, people that suffer from immunosuppressive disorders face a higher risk of infection. Furthermore, the evidence of bacterial resistance is on the rise, and complications associated with infections are therefore expected to increase in the general population [1], [4]–[6]. This type of infection leads to necrosis and destruction of the bone. This pathology can affect all ages and involve any bone, and it can be limited to a single proportion of the bone or can involve several regions, such as marrow, cortex, periosteum, and the surrounding soft tissue, becoming a chronic disease and causing persistent morbidity [4], [7], [8].

Nowadays, with the emergence of modern standards in the control of sterility within the operating room environment and adequate protocols of peri-operative antibiotic prophylaxis, there is a decrease of the incidence of infections associated with orthopedic implants. Nevertheless, this infection still represents one of the most serious and devastating complications which may involve prosthetic devices [9]. Surgical implant procedures have become valid and extremely common procedures to restore the function of affected joints, fractured bone segments and impaired limbs. The enormous population of patients with orthopedic implants estimates only about 0.5-5 % of risk of infection. However, it has to be considered very relevant due to its serious consequences [9]. Thus, exposure to invasive medical devices is one of the most important risk factors. These devices predispose to infection by damaging or invading epithelial or mucosal barriers and by supporting growth of microorganisms, thus functioning as a reservoir [10].

The diagnostic and therapeutic of bacterial bone infections are a challenge to all clinicians [11]. The clinical diagnostic is made by different and complementary approaches, as laboratory tests, microbiological analysis of specimens and imaging modalities [3], [12]. Normally, main clinical symptoms are persisting local pain, erythema, oedema, warmth, tenderness, necrosis of wound edges, large post-operative haematoma and abrupt onset of high fever [12].

Diagnostic from laboratory tests can be made by blood tests, that will show raised inflammatory markers, as erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP), which are both elevated in almost all patients, and a modestly elevated white cell count (rarely >15,000/mm^3) [3], [12]. Microbial

analysis consists of the culture of pathogens. These assays, although helpful in the diagnostic of bone infection, entail several disadvantages that influence the results of the test. One such example is the administration of antibiotics before collection of samples, or the improper management of specimens that may alter the results and the growth of microbes [12]. For imaging modalities, such as radiographs, it is only possible to collect evidence of infection up to 10-14 days after onset, although there may be some observable soft-tissue changes before. Moreover, the presence of implants makes the interpretation of radiographs difficult, decreasing the sensitivity and specificity of diagnostic active infection. Several imaging techniques besides radiography are also required for diagnostic of bone infection, mainly when the site of infection is unknown, such as 18-fluoro-D-deoxyglucose positron emission tomography (FDG-PET) or magnetic resonance image (MRI). Nevertheless, these techniques also entail a number of factors that influence the results, such as post-operative scars or artefacts to residual abrasions of metallic implants, in the case of the MRI.

When it is known that it is bone infection, computed tomography (CT) is routinely used for pre-operative planning. This method demonstrates better the details of the cortical bone, cortical erosion or destruction, peri-osteal and endoesteal reaction, as well as the presence of sequestrum and intraosseous fistulas. This happens due to optical imaging of the bone structure, regarding mineralisation and extension of disease. However, in presence of metallic implants, the CT loses quality. It is also possible to carry out directly a bone biopsy. Still, many other techniques of diagnostic in bone infection can be applied, all of which with great difficulty in interpreting the results, which makes diagnostic a challenge in clinic context [3], [12].

When an infection is diagnosed, a number of therapeutic approaches may be adopted. The selection of the proper approach depends on host factors, such as age, baseline mobility, comorbidities, etc., virulence and antimicrobial susceptibility of the infecting organism, duration of infection, prosthesis factors, such as stability of implant, loss of bone stock and soft tissue because of previous surgeries or infections, and patient expectation [5]. The treatment of bone infections involves operative debridement and chemotherapy with antimicrobial agents. However, both the simple debridement procedures with retention of prosthesis and antibiotic therapy treatments are not always effective on infections that have already established. Often, prosthesis removal and replacement, when not even joint fusion, it is the only solution to definitively eradicate severe infections [8], [9]. Another inherent problem is that the therapy with antibiotic takes long periods of time. By intravenous route, the antibiotics are prescribed for three weeks, followed by more three weeks of oral antibiotics. Furthermore, to achieve effective therapeutic drug concentration in the bone, at the precise site of infection, there is a need of high parenteral dose of antibiotic. These facts can lead to systemic toxicity of the antibiotic. [8], [13].

These drastic interventions, as it is obvious, have serious implications in terms of attendant patient trauma, prolonged hospitalization as well as in terms of health and social costs [9], [14]. As a consequence, there has been a progressive increase of the attention focused on the epidemiology and the pathogenesis of these infections, mainly those associated to implant materials, in order to acquire knowledge and better control over this phenomenon. Many efforts have been made to investigate which

are the most relevant etiologic agents involved, the pathogenetic mechanisms leading to microbial adhesion, colonization of implant surfaces, and evasion of the host defences, the most important virulence factors, the nature and properties of microbial biofilms and the progressive alarming appearance of antibiotic resistant strains [9].

1.1.1. Aetiology

Bone infection is usually caused by bacteria but sometimes can also be caused by a fungus. The microorganisms that normally are responsible for bone infection are staphylococci, streptococci, Gram-negative bacilli enterococci and anaerobes. In Table 1.1 it is listed a series of organisms present in bone and joint infections [15]. The most common pathogens that cause these infections, mainly implant-associated infections, are *Staphylococcus aureus* and *Staphylococcus epidermidis*. *S. aureus* is generally responsible for metal-biomaterial, bone-joint, and soft-tissue infections. Whereas that, *S. epidermidis* is better known for causing polymer-associated implant infections [6], [13], [16], [17].

Table 1.1 – Summary of causative organisms.

Disease	Causative organisms
Osteomyelitis	*Staphylococcus aureus* (90 % adults, >50 % children); *Staphylococcus epidermidis;* *Streptococcus* groups A, B, C and G; Gram-negative bacilli (e.g. *Escherichia coli*, *salmonella*); *Polymicrobial* (5 %); *Brucella Spp*; Fungi *Haemophilus influenzae*; *Kingella Kingae*; *Bartonella Henselae*;
Septic arthritis	*Staphylococcus aureus*; β-Haemolytic Streptococci; *Streptococcus pneumoniae*;
Prosthetic joint infection	Early (<3 months after surgery): *Staphylococcus aureus*, *Staphylococcus epidermidis;* β-Haemolytic Streptococci and enterococci; Delayed (3-24 months after surgery): coagulase-negative *staphylococci*, *Propionobacterium acnes*; Late (>24 months after surgery): *Staphylococcus aureus*, Gram-negative bacilli.

Adapted from [15], [17].

1.1.2. Bone infection caused by *S. aureus*

S. aureus is considered to be a major virulent pathogen, capable of colonizing and infect hospitalized patients with decreased immunity, as well as health immuno-competent people in the community [17]. Therefore, this organism has a more relevant approach in this work.

S. aureus is a gram-positive facultative anaerobic bacterium and it is found naturally on the skin and in the nasopharynx of the human body. It is also found within environment hospital, mainly resistant *S. aureus* strains. Members of the staphylococci genus, as *S. aureus*, are catalase-positive and its cell wall is composed of peptidoglycan and teichoic acids, attached to which are adhesins and exotoxins [17].

This bacterium is an important human pathogen that causes several diseases, such as skin infections, scalded-skin syndrome, toxic shock syndrome, endocarditis, septic arthritis, and bone infections. This happens because, although the skin and mucous membranes are excellent barriers against local tissue invasion by *S. aureus*, after they are breached due to trauma or surgery, this bacterium can enter the underlying tissue, reaching the lymphatic channels or blood vessels as well. Once it penetrates the subcutaneous tissues and reaches the lymphatic channels or blood, it can infect almost any organ and can cause septicemia, but it mostly affects bone tissue and cardiac valves. It is responsible for about 80 % of all cases of human bone infections. Infections caused by *S. aureus* are a worldwide public health concern. The difficulty in treatment of clinical cases and the wide variety of staphylococcal disease, reflects the evolutionary versatility of this pathogen and the major challenges faced by health care providers [8], [17]–[20]. The treatment of bone infection is challenging, because the approaches are scarce, expensive and infections caused by *S. aureus* are subject to recurrence months and even years after apparently successful therapy [8].

How *S. aureus* targets bone may relate to its ability to adhere to bone. This capacity of this organism is directly related to its expression of the receptors (adhesins) for components of bone matrix such as fibronectin, collagen, and bone sialoprotein [6], [14]. Therefore, this bacterium is capable of forming an extracellular matrix, designated biofilm that adheres to bone. Bacteria in biofilm may cause persistent infection due to increased resistance to antibiotics [7], [21]. Additionally, *S. aureus* expresses toxins and exoenzymes that may defend staphylococci against immune cells and destroy host tissue, which allows the pathogens to enter in deep tissue structures [14].

Therefore, this bacterium has the capacity to persist within bone cells, as osteoblasts, facilitating the development of chronic infections, as it has been demonstrated *in vitro* and *in vivo*. This fact leads to an urgent need for development of novel therapeutic approaches in bone infection caused by this pathogen [7], [21]. Furthermore, this bacterium also expresses cytotoxic and pro-inflammatory virulence factors. [14].

The existence of highly virulent methicillin-resistance *S. aureus* (MRSA) is also an important and problematic concern in treatment of infections, whereas some of these have also acquired multi-resistance. MRSA strains are resistant to a series of antibiotics, such as β-lactam antibiotics (oxacillin,

penicillin, and amoxicillin) including cephalosporins, streptomycin, tetracycline, and sulphonamide. There are also reports that, upon exposure to vancomycin and other glycopeptide antibiotics, certain MRSA strains become less susceptible to these antibiotics [17], [20], [22].

1.1.3. Bone infections caused by *S. aureus* biofilms

The surface of implanted biomaterials may suffer attachment of a biological active layer. This includes an extracellular matrix, proteins, host cells (fibroblasts, osteoblasts, endothelial cells) and bacteria. The extracellular matrix consists in a complex mixture of macromolecules, such as fibronectin, fibrinogen, albumin, vitronectin, and collagen. This matrix serves as a substrate for the host cells as well as for bacteria, such as *S. aureus* [17].

S. aureus expresses many surface adhesins that promote attachment to plasma and to extracellular matrix proteins of host cells or those adsorbed onto metal or polymer surfaces, and possess a variety of adhesion mechanisms. These mechanisms include microbial surface components recognizing adhesive matrix molecules (MSCRAMMS-fibronectin binding proteins, clumping factor binding proteins, collagen binding protein), which facilitate its adhesion to biomaterials and to the extracellular matrix proteins deposited on the biomaterial surface. When *S. aureus* attaches to a surface, host cells are unable to dislodge it [17].

Nevertheless, many staphylococcus strains have the capacity to produce its own extracellular matrix, resulting in biofilms [17]. Biofilms are communities of microorganisms that are attached to a solid surface, such as a biomaterial used in implants, and encased in extracellular polymeric matrix (ECM). This ECM is constituted by polysaccharides, proteins and extracellular DNA, that provides structural stability and protection to bacteria within the biofilm [23]–[25].

Biofilm formation on biomaterial surface occurs in four main phases (Figure 1.1). The first is the reversible adhesion of planktonic bacteria (freely moving) on a biomaterial surface, which may be covered by a layer of proteins or other host cells. In this phase, the bacteria are still susceptible to antibiotics, followed by irreversible binding to the surface and multiplication of the bacteria. This happens in a few hours, forming microcolonies and beginning to produce their own ECM around these microcolonies. In the next phase, biofilm grows in thickness to form a mature biofilm. When *in vitro* conditions, it grows up to 50 µm and demonstrates mushroom-like or tower-like structures. At that phase, the biofilm shows maximum resistance to antibiotics. After the formation of mature biofilm, there is liberation of planktonic bacteria, which can then spread to another location where new biofilms can be formed. Biofilm can resemble to multicellular organisms due to possible existence of water-filled channels within the biofilm [17], [26], [27].

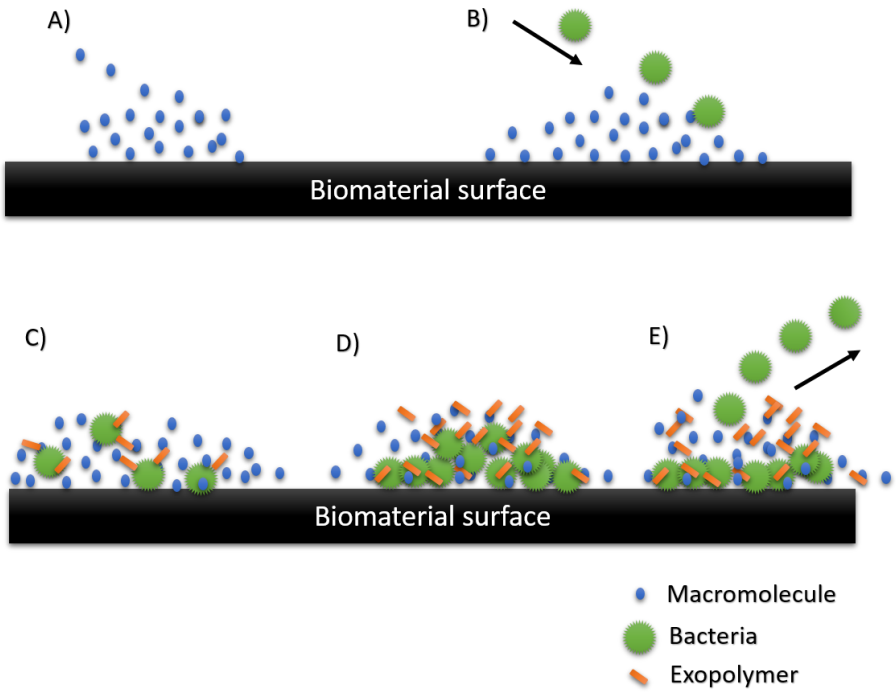

Figure 1.1 – Sequential phases of the formation of biofilms on a biomaterial surface.
A) Formation of a complex mixture of macromolecules; B) Bacteria mass transport; C) Initial bacteria reversible adhesion and anchoring through exopolymer production (extracellular matrix), first phase of biofilm formation; D) Irreversible binding to the surface and multiplication of the bacteria, and production of their own extracellular matrix, second and third phase; E) liberation of planktonic bacteria, fourth phase. Adapted from [140].

Consequently, biofilm-growing bacteria cause chronic infections. These are infections that persist despite antibiotic therapy and innate and adaptive immune and inflammatory responses of the host [27]. It is important to note that cells within a biofilm become phenotypically different from their planktonic analogs. Bacteria in biofilms have reduced mobility, distinct gene transcription patterns, and exhibit a spectrum of metabolic activity with cells at the biofilm periphery growing more rapidly than the nutrient-deprived cells in the biofilm's inner layers. This fact leads to significant consequences of diagnostic and therapy of disease [27], [28].

Traditional sampling techniques may not be sufficient to culture biofilm-growing bacteria sticking to a surface, because the biofilm is subject to an ultrasonic pre-treatment for bacteria release. Thus, these techniques reveal only the properties of planktonic bacteria, for example, the results of antibiotic susceptibility testing do not reflect the increased resistance of the bacteria living in biofilms. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of antibiotics to biofilm bacteria may be up to 100-1000 fold higher compared with free bacteria [27].It is known that bacterial biofilms develop mechanisms that provide resistance to antibiotics, disinfectant chemicals and to phagocytosis and other components of the innate and adaptive inflammatory defense system of the host [17], [23], [25]. These mechanisms consist in physical or chemical diffusion barriers to antimicrobial penetration into biofilm, in biofilm slow growth due to nutrient limitation, in activation of general stress response and the emergence of a biofilm-specific phenotype [23].

1.1.4. Intracellular *S. aureus* infection in osteoblasts

Cells such as neutrophils and macrophages, designated by “professional phagocytic cells”, are designed to engulf microorganisms and their subsequent death. Nevertheless, some pathogens have the ability to escape these phagocytic cells by their internalization in other cells, that may be called “non-professional phagocytic cells”, which possess mechanisms that permit endocytic uptake of microorganisms [20].

S. aureus is not considered a significant intracellular pathogen when compared with some microorganisms, such as *Listeria* and *Shigella*. This bacterium has been regarded as non-invasive extracellular pathogen that damages host cells after adhering to the extracellular matrix [8]. However, this organism has demonstrated to have the capacity to invade and persist within eukaryotic cells for long periods of time. Several studies have been undertaken in this scope in order to understand their intracellular mechanism and persistence. *S. aureus* internalization and intracellular persistence have been performed in endothelial cells, epithelial cells, fibroblasts, osteoblasts and keratinocytes. However, recent studies have been documenting bacterial survival also within professional phagocytes [8], [19]–[21]. Intracellular invasion and persistence of *S. aureus* provides an ideal strategy to escape from immunological defence mechanisms as well as promote protection against several classes of antibiotics, due to its limited exposure to antibiotic treatment, namely extracellular bactericides. This promotes recrudescent infection, which could explain the persistence of disease [20], [21], [29]. Thus, the internalization of *S. aureus* into osteoblast cells has led to an increase of concern in terms of public health care, because of its likely interconnection with persistent infections and chronic disease, which leads to a consequent increase in morbidity and mortality. This happens because host cells, in this case osteoblast cells, are responsible for the continual process of bone remodelling, together with another type of cells, the osteoclasts. Osteoclasts are responsible for driving the resorption of bone by acidification and release of lysosomal enzymes. Osteoclasts derive from myeloid precursors, whereas osteoblasts derive from mesenchymal bone marrow precursors and produce components of bone, mainly type I collagen. These cells also catalyse the calcification process and produce soluble factors that serve to modulate the activity and formation of osteoclasts. This way, osteoblasts are an important and essential component of bone, due to their purpose as a principal director of osteoclast function, and thus, control net bone formation or resorption [29].

However, the evidence that *S. aureus* might be a facultative intracellular pathogen is particularly demonstrated in certain subpopulations of this organism. These subpopulations, called small colony variants (SCVs), are phenotypically very different from the parent strain and naturally occurring mainly during the course of antibiotic therapy. They have a slow growth that lead to small colonies, normally these being one-tenth the size of “normal” *S. aureus.* SCVs show altered drug-resistance profiles and they are difficult to detect and treat. Therefore, they cause recrudescent infections, an intracellular niche that might then serve as a reservoir for chronic or relapsing *S. aureus* infections and may also contribute to chronic carriage. The capacity to interconvert from the SCV phenotype to wild type might be an

integral part of the *S. aureus* life cycle, although it is already known for several years that SCVs cause subacute antibiotic-resistant infections [20], [30].

Nevertheless, evidence to support the hypothesis that intracellular *S. aureus* promotes the persistent infection in humans or animals are few, because the current techniques aren't viable to prove this hypothesis. Furthermore, another recognized problem with human studies of *S. aureus* infection is the time point and duration of infection, that frequently are unknown and patients often present an acute disease [20].

In bacterial bone infections, bacteria can destroy bone by several possible mechanisms, including the production of certain compounds, such as acids or proteases, or by indirectly stimulating osteoclastogenesis. Furthermore, it has been reported that *S. aureus* surface-associated proteins are potent stimulators of bone resorption and that stimulation of osteoclast formation due to these proteins plays a role in bone destruction. Other studies have been demonstrating that exposure of mouse and human osteoblasts to *S. aureus* induces the expression of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) by these cells. This fact suggests an additional mechanism whereby *S. aureus* can mediate bone destruction via induction of apoptosis in bone-forming osteoblasts. However, the most important mechanisms in the pathophysiology of bacterium-induced bone resorption are not yet fully known [29].

Studies with osteoblast cell lines have been demonstrating that actin microfilaments, microtubules, and receptor-mediated endocytosis are required in the internalization of *S. aureus* into osteoblasts. Furthermore, other studies have revealed that the mechanism of *S. aureus* host cell invasion is mediated via fibronectin bridging between host $\alpha_5\beta_1$ integrins and staphylococcal surface proteins, FnBPA and FnBPB (Figure 1.2) [22], [29].

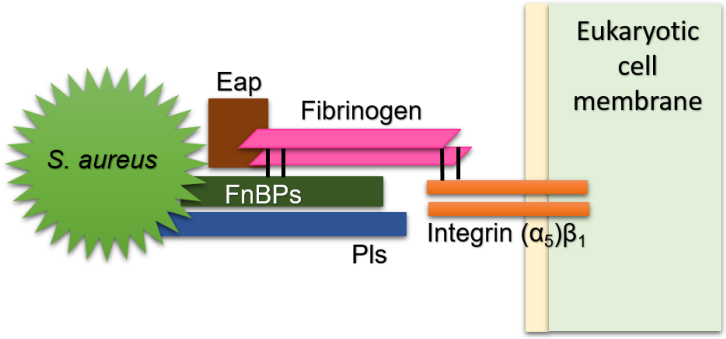

Figure 1.2 – Schematic diagram of current model of the *S. aureus* invasion mechanism.
Host cell invasion mediated via fibronectin bridging between host $\alpha_5\beta_1$ integrins and bacteria surface proteins, FnBPs (FnBPA and FnBPB). Extracellular adherence protein (Eap) contributes to invasion that it can partially compensate for loss of FnBPs function. Plasmin-sensitive surface protein (Pls) also down-modulates invasion and adherence by steric hindrance. Adptaded from [20], [22].

Moreover, there are other proteins known to be required for intracellular invasion. The Eap is a multifunctional protein, consisting of 4 to 6 tandem-repeat domains. Two tandem-repeat domains have been identified as minimal structural requirements for Eap-mediated host cell invasion. Eap may also

bind fibronectin, which promotes its capacity to partially compensate for the loss of FnBP functions. Another one is an additional surface protein, Pls (plasmin-sensitive protein). Pls is a protein of MRSA located on staphylococcal chromosomal cassette type I (Scc*mec* I), which down-modulates invasiveness of these strains and acts by steric hindrance, rather than other mechanisms for down-modulation of host cell invasion [22].

The majority of intracellular *S. aureus* is contained within vesicles in osteoblasts, although some bacteria appear free in the cytoplasm. It should be noted that, following lysis or trypsinization of the human osteoblasts, the bacteria are released while viable, which promotes the invasion onto other osteoblasts. Thus, *S. aureus* is sequestered from the host immune system within osteoblast cells, acting as a reservoir of bacteria, which may explain why chronic bacterial infections of bone are associated with multiple recurrences, even when in the presence of a proper humoral response [29].

Therefore, host cells have developed powerful mechanisms to destroy invading pathogens. These mechanisms generally consist of generation of reactive oxygen species, modulation of essential cations and nutrients, and degradation by proteolytic enzymes. However, some intracellular pathogens, such as *S. aureus*, have also developed sophisticated mechanisms to survive and persist within this intracellular environment. One of the mechanisms employed by bacteria to escape host cells defence is to avoid lysosomal killing. This way, the invading pathogen can go into the cytoplasm [22].

Destruction of the phagosomal compartment of non-professional phagocytes cells is a prerequisite for induction of host cell death. Phagosomal escape of *S. aureus* has been described as pore-forming toxins as well as phospholipases for phagolysosomal membrane destruction [22].

1.2. Conventional antibiotic treatment of bone infection

The conventional treatment for bone infection, is usually the antibiotic treatment. Antibiotics are substances that either stop bacteria from growing, designated by bacteriostatic agents, or kill them, depending on their capacity to block critical bacteria cellular processes, these being referred to as bactericidal agents [2], [31].

The introduction of antibiotics in the medical field was one of the most important interventions to reduce the onslaught of many diseases. As a result, antibiotics are the economic powerhouses of our society [2].

In the case of bone infections, antibiotics are administered to control inflammation and to prevent recrudescence in chronic infections [32]. Furthermore, in prosthetic joint infections, antibiotic therapy is usually applied after the surgical removal of all bioprosthetic components [33]. There are a variety of antimicrobial agents used in this type of disease. There is no consensus as to the best method of antibiotic therapy, but it is always based on a definitive microbiologic diagnosis [32], [33].

Generally, antibiotics can be categorized according to their principal action mechanisms. The main action mechanisms performed by antibiotics for bone infection treatment are: 1 - β-lactams and glycopeptides, agents that interfere with cell wall synthesis; 2 - macrolides, aminoglycosides, tetracyclines, and oxazolidinones which are agents that inhibit protein synthesis; 3 - fluoroquinolones and rifampin are compounds that interfere with nucleic acid synthesis; 4 - sulfamides and folic acid analogues inhibit a metabolic pathway; and 5 - compounds that disrupt the bacterial membrane structure, which include polymyxins and daptomycin [2].

The therapeutic success in bone infections is also determined by the rate and extent of antibiotic penetration in bone tissues. An important factor to take into account is the choice of the appropriate antibiotic treatment [34]. This way, the bone penetration of several antibiotics has been studied. However, methodologies have not been standardized, which makes difficult the interpretation of results, and a consequent variation of these [35]. Penetration of an antibiotic into infected bone tissue depends on its pharmacological characteristics, the degree of vascularization, good conditions of soft tissues, and the presence of foreign bodies. Some of the antibiotics used in this pathology are described in Table 1.2, as well as their bone/serum concentration ratio and respective time interval since last dose (h) [34].

Table 1.2 – Bone penetration of antibiotics.

Antibiotic	Time interval since last dose (h)	Bone/serum concentration ratio
Amoxycillin	2	0.17-0.31
Ampicillin	0.25-4	0.11-0.71
Oxacillin	1	0.11
Erythromycin	0.25-2	0.18-0.28
Rifampin	2-14	0.08-0.56
Tigecycline	4-24	0.35-1.95
Levofloxacin	0.7-2	0.36-1.0
Vancomycin	0.7-6	0.05-0.67
Linezolid	0.5-1.5	0.4-0.51
Daptomycin	2	1.08

Adapted from [34].

Furthermore, many other problems have been revealed by antibiotic treatment of bacterial infections. When there is clinical treatment failure of bacterial infectious disease, it is usually associated with low bioavailability of antibiotics and their side effects, tissue and cellular barriers, biofilm-related infections and the emergence of resistant bacteria [2]. MRSA strains, resistant to methicillin, are normally synonymous of multidrug-resistant *S. aureus*, because many of these strains are also resistant to many other commonly used antibiotics. In Europe, it was reported that approximately 20 % of *S. aureus* isolates are methicillin-resistant strains [33]. *In vitro* antibacterial activity is based on determinations of the minimum inhibitory concentrations (MIC) necessary to inhibit bacterial growth in 90 % of tested strains (MIC_{90}) [36].

1.2.1. Fluoroquinolones: Levofloxacin

Fluoroquinolones have proved to be potent antibacterial agents, demonstrating a broader spectrum of antibacterial activity, a great efficacy against resistant organisms, and a better safety profile than other antimicrobial agents, including the older quinolones [37], [38]. Fluoroquinolones have several characteristics that have led to their increased use in bone infections. They have a rapid bactericidal effect against most susceptible organisms, and show one of the highest median extents of bone penetration of all antibiotic classes, partly due to quinolone binding to calcium in the bone [39], [40]. Levofloxacin is being described as the quinolone with the higher values in this group. Levofloxacin belongs to the fluoroquinolone class, widely used in treatment of certain bacterial infections (Figure 1.3). This drug has an oral broad spectrum of activity and excellent tissue penetration, above the minimum inhibitory concentration (MIC) for susceptible pathogens generally associated to bone and joint infections. The MIC of levofloxacin, as described in literature, for MSSA and MRSA is 0.25 – 0.5 µg/mL and 0.5 -16.0 µg/mL, respectively [41]. Moreover, it has availability in both oral and intravenous formulations, with lower toxicity. It is active against Gram positive and Gram negative bacteria, as well as other pathogens such as *Mycoplasma*, *Chlamydia*, *Legionella* and *Mycobacteria* spp. Futhermore, it is being referred as the fluoroquinolone with the greater *in vitro* and *in vivo* anti-staphylococcal activity against both intracellular and extracellular pathogens. Levofloxacin is the L-isomer of the racemic drug substance ofloxacin, a quinolone antibacterial agent. In chemical terms, levofloxacin is a chiral fluorinated carboxyquinolone, the pure enantiomer of ofloxacin. However, levofloxacin is more active against bacterial pathogens than its enantiomer. [36], [40], [42].

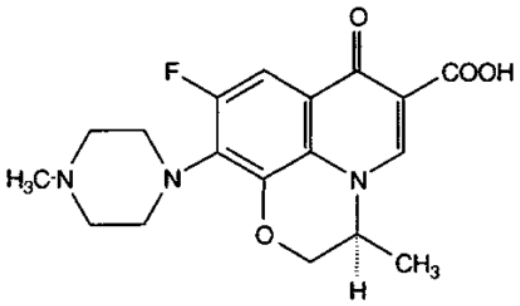

Figure 1.3 – Structure of levofloxacin.

After oral administration of 50 to 200 mg of levofloxacin in healthy volunteers, the mean maximum plasma concentrations range from 0.57 to 2.04 mg/L achieved within 0.8 to 2.4 h. These values are linearly related to dose. Levofloxacin has an oral bioavailability approaching 100 %. Furthermore, with this administration method, levofloxacin penetrates rapidly and efficiently throughout the body, achieving concentrations in tissues or body fluids which are generally higher than those in plasma. This antibiotic is approximately 24 to 38 % bound to serum plasma proteins, mainly to albumin. The plasma elimination

half-life is 4 to 7 h, further within 24 h of an administrated oral dose, about 80 to 85 % of the drug is excreted unchanged in the urine, though glomerular filtration and tubular secretion [36], [43].

Like other fluoroquinolones, levofloxacin exerts its antibacterial effects through inhibition of deoxyribonucleic acid (DNA) gyrase, a type II topoisomerase (Figure 1.4). DNA topoisomerases are a class of enzymes that alter the topology of DNA by catalysing reactions called supercoiling, relaxing, knotting and catenating. Mainly, it controls the supercoiling of DNA. DNA gyrase has two subunits A and B. The subunits A, encoded by the gyrA gene, cause strand breaks on a bacterial chromosome and then reseal the chromosome after supercoiling. The B subunits, encoded by the gyrB gene, are ATP hydrolysis-dependent and introduce negative supercoils into the DNA double strand after the initial strands are resealed by subunits A. So, the principle bactericidal activity of levofloxacin results from the inhibition of the A subunits of DNA gyrase following supercoiling, by stabilizing the DNA-DNA gyrase complex. This stabilized complex blocks movement of the replication fork, causing formerly reversible DNA-DNA gyrase complexes to became irreversible, leading to inhibition of bacterial DNA replication and transcription [36], [43], [44].

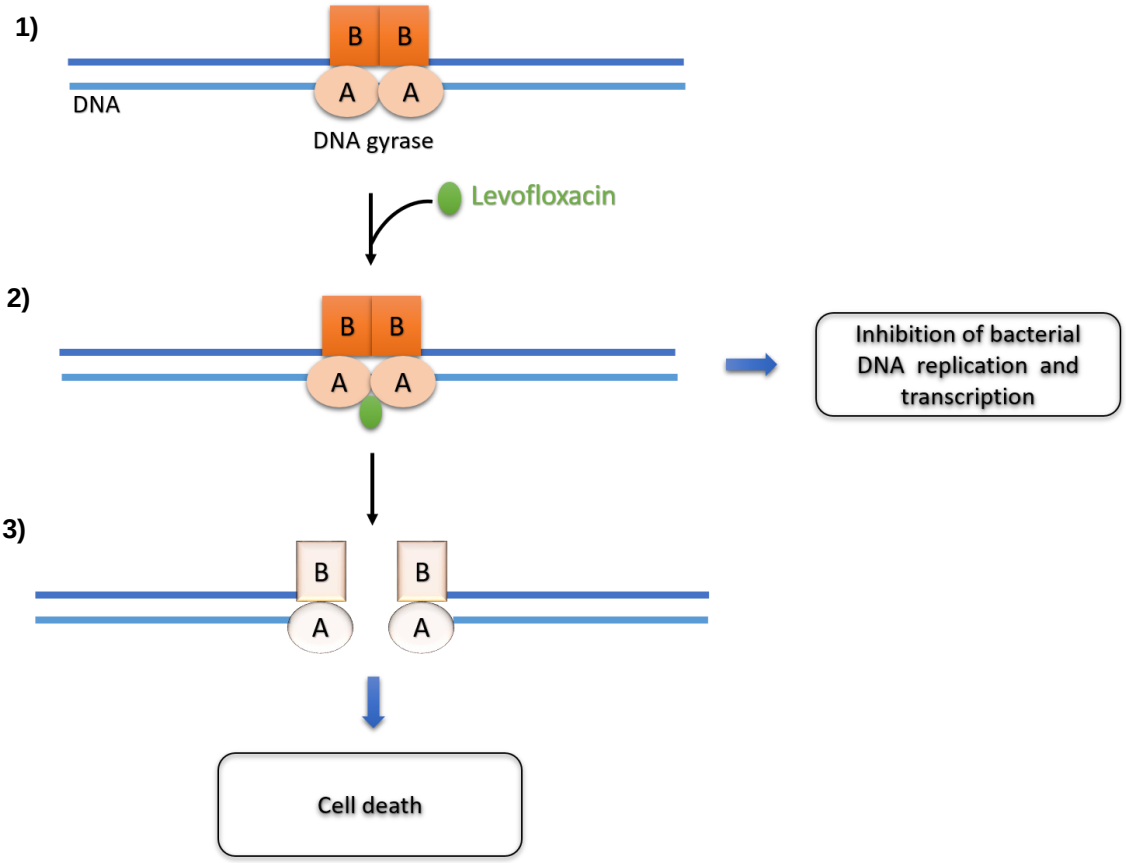

Figure 1.4 – Mechanism of action of levofloxacin.
1) DNA double strain with the enzyme DNA gyrase, DNA-DNA gyrase complex; 2) Introduction of the antibiotic levofloxacin which binds to the A subunits of the enzyme. Levofloxacin stabilizes the DNA-DNA gyrase complex, resulting in an irreversible complex; broken strands cannot be released leading to inhibition of DNA replication; 3) Broken strands are released resulting in cell death. A – Subunits A of DNA gyrase; B –Subunits B of DNA gyrase. Adapted from [36], [43], [44].

1.2.2. Glycopeptides: Vancomycin

Vancomycin is a glycopeptide antibiotic used clinically to treat serious Gram-positive bacterial infections that are resistance to other antibiotics, such as β-lactams (Figure 1.5). Vancomycin is a first-line treatment for many bone and joint infections caused by typical organisms, including methicillin-resistant *S. aureus* (MRSA). However, *S. aureus* reduced susceptibility to vancomycin may be partially due to its ability to produce biofilms, which may facilitate resistance by promoting horizontal gene transfer. The frequency of resistance to the glycopeptide antibiotics has increased significantly, which represents a serious threat to public health. Furthermore, vancomycin has poor bone penetration [33], [45].

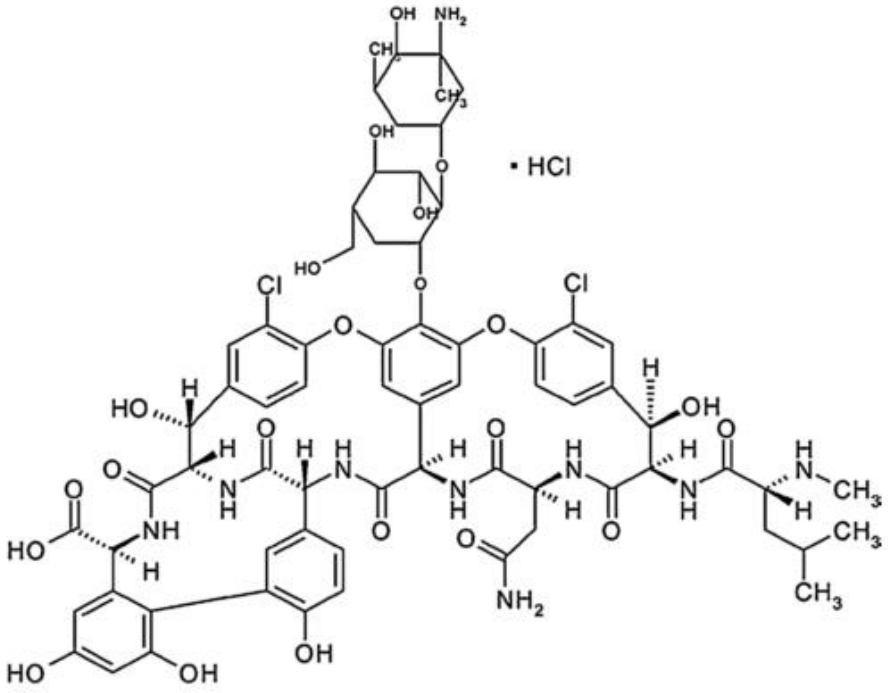

Figure 1.5 – Structure of vancomycin.

Vancomycin mechanism of action consists in blocking steps in the biosynthesis of the peptidoglycan layer of bacterial cell walls (Figure 1.6). Bacterial cells are surrounded by layers of peptidoglycan that provide the mechanical support necessary to prevent osmotic pressure oscillations, as well as cell lysis. Peptidoglycan is a rigid polymer of alternating units of *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) connected by peptides cross-links. Peptidoglycan biosynthesis takes place in three stages. The first two stages lead to the production of lipid II, where the first step involves synthesis of the wall precursors in the cytoplasm, and the second consists in formation of the wall subunit on a mobile lipid in the membrane followed by its transfer to the outer surface of the membrane. The final stage involves glycan polymerization and cross-linking by transglycosylation and transpeptidation. Cell wall active antibiotics, such as vancomycin, inhibit the final step of peptidoglycan biosynthesis, by binding to the substrates, mainly of transglycosylases reaction. The enzymes involved in this step are

extracellular and are thus accessible to vancomycin, that needs not penetrate to the cytoplasm to exert its antibiotic effects. Vancomycin interacts with terminal acyl-$_D$-Ala-$_D$-Ala residues in peptidoglycan precursors. It involves blocking the utilization of a substrate rather than acting directly on a biosynthetic enzyme. The heptapeptide backbone of the drug assumes a rigid conformation and forms a carboxylate bonding pocket that binds acyl-$_D$-Ala-$_D$-Ala residues via five hydrogen bonds and hydrophobic interactions. The result is a shielding of precursor substrates from the enzymes that catalyze transglycosylation and transpeptidation. Inhibition of this reaction leads to the accumulation of lipid intermediates in the biosynthetic pathway and of UDP-MurNAc-pentapeptide in the cytoplasm [45]–[47].

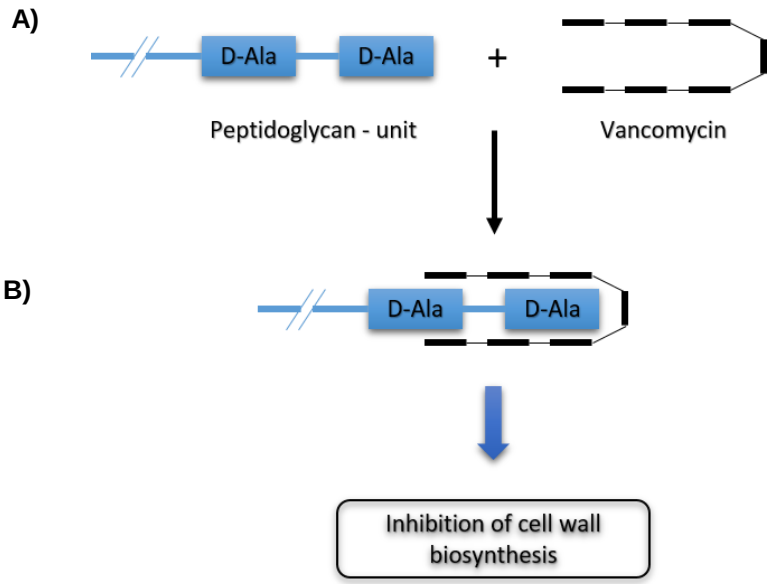

Figure 1.6 – Mechanism of action of vancomycin.
A) Peptidoglycan unit and antibiotic vancomycin; B) Interaction between vancomycin and acyl-$_D$-Ala-$_D$-Ala residues of peptidoglycan unit – inhibition of cell wall biosynthesis. Adapted from [45]–[47].

1.2.3. Lipopeptides: Daptomycin

Daptomycin is a novel cyclic lipopeptide antimicrobial agent that was developed as an alternative therapy in bone infections treatment after vancomycin failure. The drug has excellent bactericidal activity against most Gram-positive organisms, including multiple antibiotic-resistant and -susceptible strains, such as methicillin-resistant strains, beta-hemolytic groups A, B, C, and G streptococci and enterococci, and ampicillin- and vancomycin-resistant strains (Figure 1.7) [16], [48]–[51]. Its bactericidal activity is concentration dependent and very fast. Furthermore, it also retains this advantage in biofilms. Several studies have been demonstrating that daptomycin penetrates rapidly into biofilms. Due to its unique mechanism of action on a cell membrane, daptomycin retains antibacterial activity against both stationary-phase cultures of staphylococci within the biofilm and bacteria in the multiplication phase [51], [52]. Moreover, daptomycin penetrates bone effectively [51].

With a half-life of 8 h allowing for once daily dosing results in linear pharmacokinetics at doses up to 12 mg/Kg, with minimal drug accumulation. Daptomycin distributes primarily in the plasma, with penetrations to vascularized tissues. This antibiotic is highly protein-bound (92 %) [33], [50]. Its excretion occurs first via the kidneys. Approximately 80 % of the total dose, of which two-thirds is intact drug, is recovered in the urine. The daptomycin single mechanism of action and its lack of metabolization by cytochrome P450 or other hepatic enzymes results in an absence of drug interactions between itself [33].

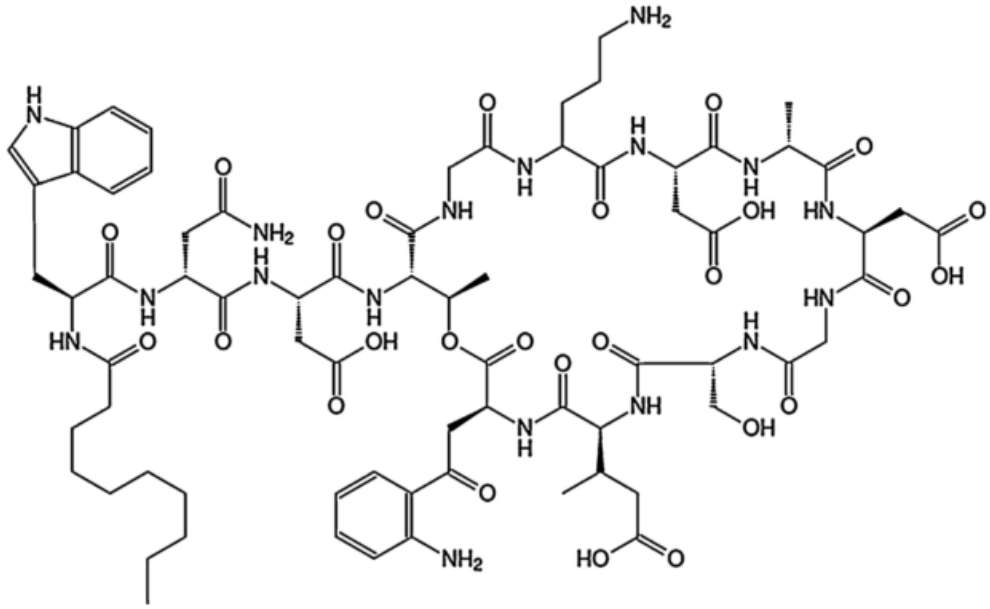

Figure 1.7 – Structure of daptomycin.

Daptomycin has an unique structure, which consists of a 13-member amino acid cyclic lipopeptide with a decanoyl side-chain. This structure confers a novel mechanism of action. The mechanism involves the calcium-dependent insertion of the lipophilic daptomycin tail into the bacterial cytoplasmatic membrane, causing rapid membrane depolarization and a potassium ion efflux. This is followed by arrest of DNA, RNA and protein synthesis resulting in bacteria cell death (Figure 1.8) [49], [53]. Calcium binding between two of the aspartate residues on daptomycin decreases its net negative charge and increases the area of its hydrophobic surface, improving the interaction with the membrane [53]. In addition, recently, studies have been demonstrated that calcium, as Ca^{2+} ions, is needed to generate two structural transitions in daptomycin [54]. Calcium binds to daptomycin in solution and there is an aggregation of drug, resulting in more tightly defined family of structures of the apo-form of this drug. This is consistent with the suggestion that Ca^{2+} is needed to lock the molecule into an active conformation. Furthermore, calcium ions promote deeper insertion of daptomycin into the membrane by bridging the residual negatively charged amino acids on daptomycin and the negatively charged phospholipids that are typically found in the cytoplasmic membrane of Gram-positive bacteria. However, for this, the micellar structure may need to dissociate [53], [54]. The second structural transition

observed requires the presence of both Ca^{2+} and lipids with negatively charged headgroups (e.g., phosphatidyl glycerol). The effect of daptomycin on these lipid bilayers in the presence of Ca^{2+} is to perturb the membrane further and induce leakage, leading to cell death. It is also possible that daptomycin aggregation in the membrane would interfere with membrane associated processes including synthesis of cell wall components, energetics, cell division, and other [54].

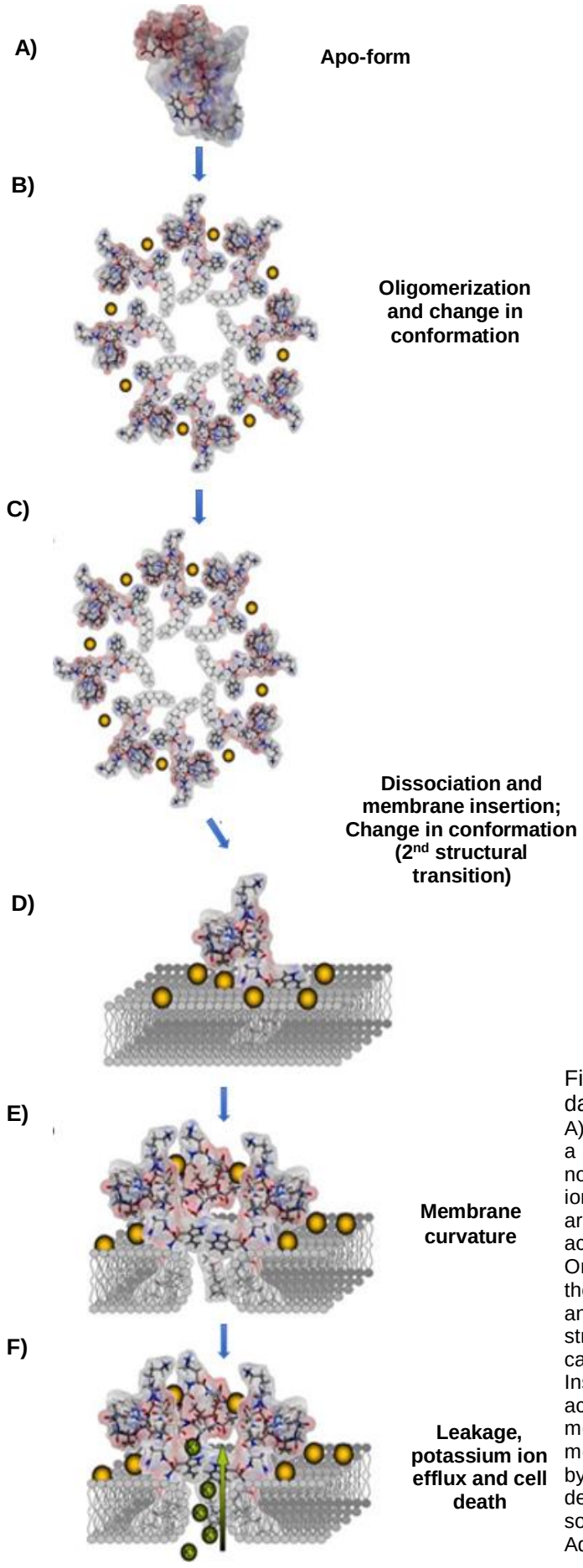

Figure 1.8 – Mechanism of action of daptomycin.
A) Daptomycin molecule without calcium adopts a structure which is reasonably well defined but not highly amphiphilic; B) In presence of calcium ions, the lipopeptide oligomerizes and most likely arranges itself into a micelle. This process is accompanied by change of conformation; C) Once daptomycin comes into close proximity with the bacterial membrane, the multimer dissociates, and drug inserts into the bilayer and the second structural transition is formed; D) Daptomycin causes the rupture of the bacterial membrane; E) Insertion of daptomycin into the membrane is accompained by the induction of positive membrane curvature and oligomerization in the membrane may occur; F) Bacterial cells are killed by membrane perforation (assessed as depolarization), and potassium ions efflux, or some other membrane associated event. Adptaded from [54].

1.3. Local drug delivery systems as new therapeutic strategies

Due to problems of the conventional treatment of bone infection, new approaches are required. Drug delivery systems arise as new therapeutic strategies for treatment of bone infections, which achieve elevated antibiotic concentration at the site of infection without exceeding the systemic toxicity [13]. Furthermore, drug delivery systems can provide drugs more effectively and conveniently than those conventionally used, increase patient compliance, extend the antibiotic life cycle, provide product differentiation and reduce healthcare costs [55]. These controlled drug delivery systems usually include particulate carriers composed primarily of lipids and/ or polymers, and their associated therapeutics [56].These systems may be divided into non-biodegradable and biodegradable carriers. The non-biodegradable delivery systems have been approved for use in treatment of osteomyelitis in Europe, an example are the polymethylmethacrylate (PMMA) beads containing gentamicin. However, this product although effective, suffers from the major drawback of requiring subsequent removal of the beads at completion of drug release. Furthermore, in recent years biodegradable systems have also been developed and evaluated for local delivery of antibiotics in the treatment of bone infections [13]. Examples of these systems are shown in Table 1.3.

Table 1.3 – Local drug delivery systems.
Carriers used for local delivery and antibiotics released.

Carrier system	Antibiotic released
Non-biodegradable	
Acrylic bone cement	Oxacillin [57] Cefazolin [57] Gentamycin [58]–[60] Fucidin [58] Cephalosporin [61]
Cement of BIS-GMA/TEGDMA resin Plaster of Paris pellets/beads	Cephalexin [62] Teicoplainin [63] Gentamicin [64]–[66] Fucidin [64]
Polymethylmethacrylate (PMMA) beads	Gentamicin [59], [60], [67]–[70] Tazocin [71] Vancomycin [72]
Polymethylmethacrylate (PMMA) cement	Vancomycin [73] Vancomycin and Tobramycin [74] Minocycline [75] Daptomycin [76] Gentamicin [77], [78]
Biodegradable	
Collagen-gentamicin sponge	Gentamicin [59], [70], [79]–[81]
Hydroxyapatite blocks	Vancomycin [82], [83] Gentamicin [84], [85] Arbekacin [86]
Hydroxyapatite cement	Vancomycin [87]
Nano-HA-PHBV/PEG-GM microsphere	Gentamicin [88]
Bone cement	Ciprofloxacin [89]
Hydroxyapatite-β-tricalcium phosphate composite	Gentamicin [90]
B-tricalcium phosphate-chitosan scaffold	Gentamicin [91]
Chitosan bar	Gentamicin [92]

	Vancomycin [93]
Apatite-wollastonite glass ceramic blocks	Cefmetazole [94] Isepamicin sulfate [94]
Bioglass reinforced plaster of Paris, hydroxyapatite and sodium alginate	Cephazoline [95]
Polylactide and/or polyglycolide implants	Gentamicin [96]–[100] Ciprofloxacin [101], [102] Vancomycin [103]–[106] Tobramycin [107], [108] Sodium fusidate [109]
Poly(acrylic acid) and gelatin crosslinked	Gentamycin or Vancomycin [110]
Polyanhydride and polylactide blend	Ofloxacin [111]
Polycaprolactone	Tobramycin [112]
Polyanhydride implant (Septacin)	Gentamicin [113]
Injectable gelling polymer	Gentamicin [114]
Fibrin clots/ sealant	Arbekacin [115] Tobramycin [116] Ciprofloxacin [117]
Fibrin gel (vanco-AB-FG) with bone marrow-derived mesenchymal stem cells (BMMSCs)	Vancomycin [118]
Dilactate polymers	Teicoplanin [119] Tobramycin [120] Sulperazone [121] Fleroxacin [122] Ciprofloxacin [123] Pefloxacin [123]
Bone xenograft	Gentamicin [124]
Bone graft/ demineralized bone matrix	Tobramycin [125]
B-tricalcium phosphate	Gentamicin [126] Vancomycin [126]
Calcium sulphate	Tobramycin [127] Daptomycin [128]
Calcium sulphate with demineralized bone matrix (DBM)	Vancomycin [129]
Calcium phosphate cement (CPC)/ injectable CPC	Gentamicin [130], [131] Teicoplanin [132]
Biomedical polyurethanes	Flucloxacillin [133] Ciprofloxacin [133] Fosfomycin [133] Gentamicin [133]
Fibres	Tetracycline [134], [135]
Cross-linked hyaluronic acid (HA) gel	Gentamicin [136]
Monoolein-water gels	Gentamicin [137]

1.3.1. Antibiotic-loaded acrylic bone cement (ALABC)

Acrylic bone cement has an important role in orthopedic surgery. It is used for fixation of prosthetic implants, for remodeling osteoporotic, neoplastic and vertebral fractures repair [138]. It is usually a polymer-based material composed of polymethylmethacrylate (PMMA) (Figure 1.9) or copolymers [139]. PMMA was one of the first materials produced by the chemical industry to be used as a biomaterial [140]. Indeed, PMMA bone cement is one of the products of study in this work, thus with more focus in this section. PMMA bone cement acts as a space-filter that creates a tight space which holds the implant against the bone (Figure 1.10) [141]. PMMA is an acrylic polymer that is marketed in two phases: a

liquid (monomer) and a solid phase (powder). The solid phase is characterized by the polymer (PMMA), the catalyst of the polymerization reaction and by the ratio-opacifier, while the liquid phase is characterized by the monomer (MMA), by the reaction accelerator and by the stabilizer (Table 1.4). The solid and the liquid phase components are usually in a 2:1 ratio [138], [141]. The two components are mixed, the liquid monomer polymerizes around the pre-polymerized powder particles to form hardened PMMA. This is an exothermic reaction, where the cement heats up, initiated by the decomposition of a catalyst (e.g. benzoyl peroxide) producing free radicals that set off additional polymerization of the MMA. The exothermic reaction reaches temperatures of around 82-86 °C in the body [140], [141]. Thus, the exposure of bone to these high temperatures have been mentioned as a cause of necrosis and tissue damage, resulting in failure of the prosthetic fixation [142].

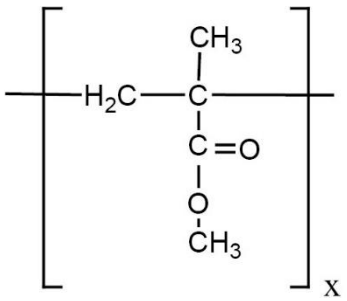

Figure 1.9 – Structure of PMMA.
X represents a repetition of its building units: monomers.

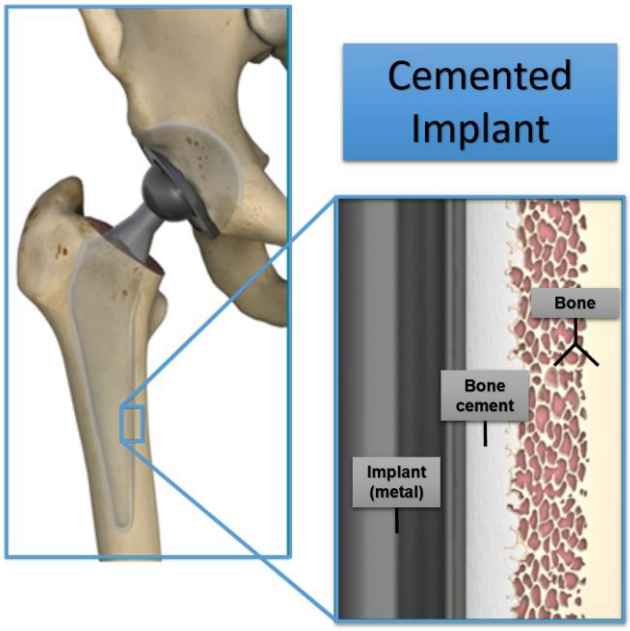

Figure 1.10 – Artificial hip replacement – cemented prosthesis.
Adapted from [178].

Table 1.4 – Components of bone cement.

Powder	**Liquid**
Poly (methyl methacrylate) (PMMA) – Polymer	Methyl methacrylate (MMA) – Monomer
Benzoyl peroxide (BPO) – Initiator	N, N-Dimethyl para-toluidine (DMPT)/dimethyl para-toluidine (DMpt) – Accelerator
Barium sulphate ($BaSO_4$)/ Zirconia (ZrO_2) – Radio-opacifier	Hydroquinone – Stabilizer

Adapted from [141]

Bone cement mixed with active agents, such as antibiotic, releases the agents slowly, thus serving as a vector for specific controlled *in situ* therapy [143]. PMMA bone cement with antibiotics incorporated

reduces the infection rates in orthopedic surgery. These drug delivery systems are already used for primary and revision surgery, while antibiotic-loaded PMMA beads are part of a more complete treatment of infection and supplement other interventions (mostly surgical). Antibiotic-loaded PMMA bone cement is normally used in multistage revision of infected implants, where they have not only an antibacterial effect, but also prevent contraction of ligaments, and scar tissue from growing into the joint space. Several studies indicate that antibiotic released from PMMA bone cements is a surface phenomenon, but the mechanism by which these drugs are released is still debated. Moreover, studies reported that *S. aureus* biofilm formation was reduced on different gentamicin-loaded bone cements, when compared to unloaded bone cements only during a short period, which depends on the initial drug release of the bone cement [140].

As described by Arora et al., several kinds of additives started to be added to BC, besides antibiotics, with different aims, while still maintaining structural and mechanical integrity [144]. An example of this is the addition of release modulators, as a means to increase the drug release rate from BC matrices. In the case of PMMA BC the rate of antibiotic release is low, since *in vitro* and *in vivo* studies have demonstrated that, in this type of cement, the initial liberation of the drug is a surface phenomenon, due to the BC matrix being impermeable to drugs. The antibiotics must be released through an interconnecting series of voids and cracks in the cement. Therefore, an increase of superficial porosity of the cement is required to increase the efficiency of drug release from bone cement matrices, since the sustained liberation of antibiotics is largely affected by the penetration of fluids into the polymer matrix. So, the inclusion of water soluble compounds (release modulators) promotes the increase of porosity of the bone cement and consequently the drug release [145]. Previous data published by Matos and colleagues using levofloxacin-loaded PMMA bone cement describes the study of addition of lactose as enhancer of drug release. In this study, 10 % of this release modulator was added to a novel levofloxacin-loaded bone cement matrix. The results (during a 7-week period) demonstrated an improve of the amount of levofloxacin released from lactose-loaded BC matrices of 3.5-fold higher than from plain BC matrices [40].

1.3.2. Antibiotic-loaded polymeric microparticles

Polymeric nanoparticles and microparticles, made of natural and synthetic polymers, have revolutionized the administration of medicines, due to the important impact in the treatment and management of several conditions with high social and economic effect, such as cancer, respiratory and metabolic diseases, infections and tissue regeneration. These shown several advantages as drug carriers, such as high stability both *in vitro* and *in vivo*, multifunctionality and good biocompatibility. In this way, the interest of these systems has increased in the medical field [2], [146], [147].

The novel polymeric drug delivery system discussed in this study is composed by PMMA microparticles, since PMMA is a biocompatible polymer, used in bone cement preparation, as described in previous section (1.3.1). Thus, at the present, PMMA is described as a non-toxic polymer as it possesses a very

good toxicological safety record in biomedical applications. PMMA as a particulate carrier material was firstly used in the development of nanoparticles for vaccination purposes and on beads to fill defects related to surgical resection of chronic bone infections. Furthermore, this polymer has been used as a local drug delivery system of drugs in antibiotic impregnated bone cement applied in arthroplasties and antibiotic impregnated bead chains for musculoskeletal infections. However, its use as an antibiotic particulate carrier system has been somehow neglected, due to its bioinert and non-biodegradable properties. Even so, PMMA is still an important candidate as a biomedical material because of its biocompatibility. The diameter of polymeric nanoparticles ranges between 10 and 1000 nm, while the diameter of microparticles ranges from 1 to 250 µm. PMMA particles (either spheres or capsules), can be prepared either by direct polymerization of the MMA monomer using polymerization reactions or from pre-formed PMMA polymer (Figure 1.11). Each technique has its own advantages and disadvantages that need to be evaluated before selecting the adequate preparation method for the intended PMMA particulate carriers [148].

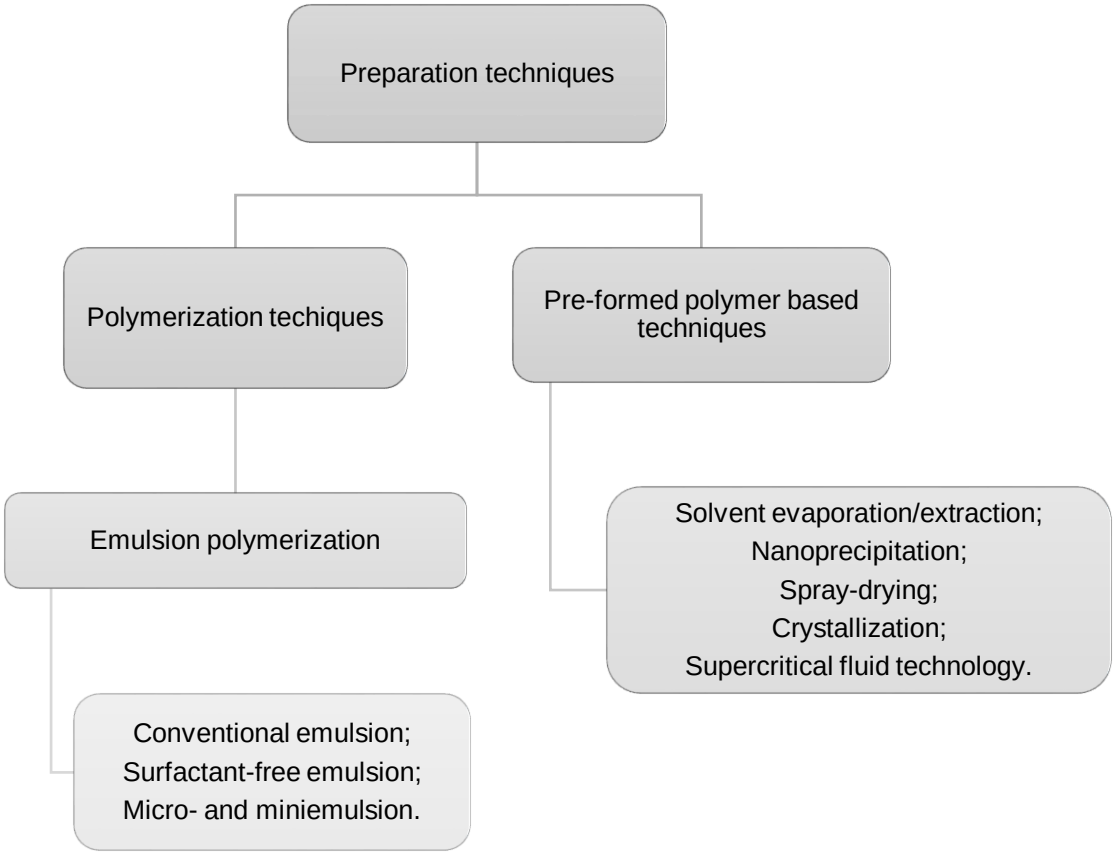

Figure 1.11 – Schematic representation of the techniques commonly used in the preparation of PMMA particulate drug carriers.
Adapted from [148].

1.4. Compliance of novel local drug delivery systems

Development of novel local drug delivery systems requires a series of studies, such as antibiotic release and stability studies, surface and mechanical properties, antimicrobial activity and biocompatibility assays. Although the biomaterial used as carrier is already being used in medical environment, the addition of new compounds or some changes in the protocol of preparation require the conducting of these studies again. These assays are needed and they are of utmost important, shedding some light concerning the potential of these systems, to verify that its characteristics were not compromised, as well as a means to choose the best systems for the intended application. In the present work, drug release studies, surface properties evaluation, namely contact angle and surface energy estimation, microbiological and antibacterial intracellular activity assays were conducted.

1.4.1. Surface studies

Contact angle and surface energy determination are imperative tests performed to evaluate surface characteristics of a biomaterial. Regarding bone cement drug delivery systems, the cement surface is in close connection with living bone, being the first component to be in contact with cells. So it is possible to assume that surface properties such as surface composition and surface energy will play a role in the biocompatibility problems related to the bone cement performance [75], [149]. However, it is important to take into account that cells are not the only ones adhering to the implant surface, as infectious organisms often tend to as well. This leads to other problems besides biocompatibility behaviour, such as the characteristics of the interacting bacterial and biomaterial surfaces, crucial properties potentially leading to an infection [140].

When altering the biomaterial in study, the obtained bone cement matrix surface energy revealed that its surface characteristics did not change with these alterations, such as drug loading, meaning that biological interactions between bone cement and biological tissues will not be compromised due to changes in the surface proprieties of the bone cement matrix [75]. Contact angle determination provides information about the level of hydrophobicity and hydrophilicity of the biomaterial. This assay was performed according to the Wilhelmy plate method, according to which the biomaterial is partially immersed vertically in a test fluid along one of the larger dimensions and the force intensity acting on the plate is measured. The surface energy of the biomaterial is an estimate through the values of the contact angle obtained from two different test fluids with different polarity as water and propilenoglicol [150].

1.4.2. *In vitro* drug release studies

In vitro release studies are also important tests that must be performed when novel drug delivery systems are being developed. These assays aim to quantify and evaluate the released drug behaviour

from systems in different conditions, i.e., to mimic physiological conditions, using a biological model fluid (phosphate buffer saline solution: PBS; pH=7.4, 25 ºC), as well as the conditions of microbiological and intracellular assays, using a culture medium and complete cell medium, respectively. Also, to optimize the techniques for monitoring and quantification of released drug, both High Performance Liquid Chromatography (HPLC) and fluorescence methods can be used.

1.4.3. Microbiological assays

In order to evaluate the antibacterial activity of novel drug delivery systems, a battery of microbiological tests should be performed using the main pathogenic organisms as test model (in this work *S. aureus* strains were used to test BC matrices formulations efficacy). In these assays, the efficacy of free antibiotic is compared to the same antibiotic liberated from formulations against selected bacteria strains in its planktonic form or in structures organized within biofilms. The microbiological assays allow the minimum inhibitory concentration (MIC) determination and also the minimum biofilm inhibitory concentration (MBIC), defined as the minimum antibiotic concentration required to inhibit growth of planktonic bacteria or bacteria organized within biofilm, respectively [151]. Furthermore, it is possible to observe the anti-biofilm ability of formulations. Bacterial biofilm-forming ability can be measured by crystal violet staining technique.

1.4.4. Antibacterial intracellular activity assays

When pathogenic organisms have the ability to invade and persist within cells, like *S. aureus* strains, that have been demonstrated to be facultative/opportunist intracellular pathogens, the antibacterial therapy is more complex than in an extracellular target [152]. In this context, infections caused by intracellular pathogens require the evaluation of the intracellular effect of novel drug delivery systems. The antibacterial intracellular activity of these systems was evaluated using a human osteoblast infection model. This infection model is performed by using osteoblast cells infected with pathogenic organisms (*S. aureus* strains), which are then left to incubate with the novel formulations (usually 24 h), after which the survival intracellular bacteria are determined by enumeration of colony forming units (CFU).

Chapter 2. Materials and methods

2.1. Preparation of biomaterials

2.1.1. Acrylic bone cement

Commercial acrylic bone cement (BC) CMW1® Radiopaque (DePuy Synthes Portugal), a high viscosity bone cement intended for digital application, was used to prepare the BC composite specimens. Its composition is described in Table 2.1.

Table 2.1 – Composition of the commercial acrylic bone cement DePuy CMW1®.

Bone cement powder	Bone cement liquid
Poly (methyl methacrylate) – 88.85 % w/w;	Methyl Methacrylate – 98.50 % w/w;
Benzoyl Peroxide – 2.05 % w/w;	N, N-Dimethyl-p-toluidine - ≤ 1.50 w/w;
Barium Sulphate – 9.10 % w/w.	Hydroquinone – 75 ppm.

Levofloxacin (Lev on specimens designation) was purchased from Sigma-Aldrich and lactose monohydrate (Lac on specimens designation) was acquired from Merck.

Six different specimens of BC were prepared in parallelepiped form, at room temperature (23±1 °C) and atmospheric pressure. The six specimens had different content composition (Table 2.2), referred throughout the study by BC (Control 1), BCLac (Control 2), BCLev0.5%, BCLev1%, BCLev1.5% and BCLev2.5%. All BC matrices were prepared according the commercial supplier recommendations, that is, 5 g of CMW1® powder for 2.5 mL of monomer (liquid). In all matrices, except Control 1, it was added 10 % (w/w) of lactose to improve levofloxacin release, due to increase of porosity of BC matrix [40]. In brief, CMW1® powder, levofloxacin and lactose (when part of the formulation) were carefully mixed. After, the monomer was added. Then, the components were all mixed again to obtain a dough with the desired consistency. The obtained cement was transferred to aluminium moulds (Figure 2.1.), and it was allowed to set, for the process of cure to take place, at room temperature for 20 min. After, all BC specimens were carefully polished and cut according with the necessary size for each assay.

Table 2.2 – Composition of different BC formulations.

BC Matrices	% Levofloxacin (w/w)	% Lactose (w/w)
BC (Control 1)	0.0	0.0
BCLac (Control 2)	0.0	10.0
BCLev0.5%	0.5	10.0
BCLev1%	1.0	10.0
BCLev1.5%	1.5	10.0
BCLev2.5%	2.5	10.0

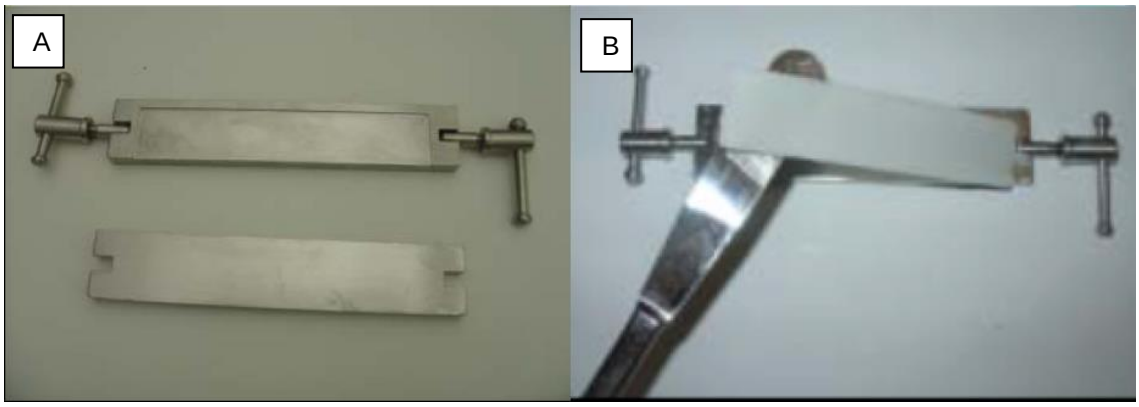

Figure 2.1 – A) Aluminium moulds used for preparing the BC plates; B) Example of a BC matrix.

2.1.2. Polymeric microparticles

Poly(methyl methacrylate) (PMMA; M_w = 350000) and poly(vinyl alcohol) (PVA; M_w = 13000-23000, 87-89 % hydrolysed) were purchased from Sigma-Aldrich. Poly(ethyl acrylate-co-methyl methacrylate-co-trimethylammonioethyl methacrylate chloride) (Eudragit® RL 100, EUD; average M_w = 32000) was kindly provided by Evonik Industries AG and dichloromethane (DCM) from Fisher Scientific. D(+)-sucrose and glucose were purchased from AppliChem GmbH. The antibiotics were used without purification, daptomycin (Dapto on formulations designation; Cubicin, 350 mg) was provided by Novartis and vancomycin hydrochloride (Vanco on formulations designation; vancomicina 100 mg) was purchased from Farma APS Produtos Farmacêuticos, Lda. All other reagents were analytical grade.

Three formulations of polymeric microparticles were prepared, referred throughout the study by PMMA-EUD (control), PMMA-EUD-Dapto and PMMA-EUD-Vanco.

Polymeric microparticles were prepared by double-emulsion w/o/w-solvent evaporation method [16], [148], [153]. Briefly, polymer blends (PMMA and EUD, 70 w%) were dissolved in 5 mL of DCM and emulsified by homogenization using an Ultra-Turrax T10 basic during 3 min with a 10 % (w/w) PVA solution, where the antibiotics were previously solubilized with a final concentration of 15 %. To the first o/w emulsion 30 mL of 1.25 % (w/w) PVA solution was added and further emulsified by homogenization using a Silverson Laboratory Mixer Emulsifier L5M (Silverson Machines Inc.) for 10 min at 9999 rpm (w/o). The resulting double emulsion (w/o/w) was magnetically stirred at room temperature for 4 h to evaporate the organic solvent. Particles were harvested by centrifugation (7500 rpm, 10 min, 4 °C; Allegra 64R High Speed Centrifuge, Beckman Couter Inc., Fullerton), washed trice with 10 % (w/V) sucrose solution and resuspended in a 0.5 % (w/V) sucrose solution. Particles were freeze-dried (Christ Alpha 1-4, B. Braun Biotech International) to obtain a fine, free-flowing dry powder.

2.2. Characterization of biomaterials

2.2.1. Acrylic bone cement

2.2.1.1. Contact angle and surface energy determination

Contact angle determination was performed according with our teamwork-established protocol [75], [154]. BC matrices were cut in parallelepipeds with measures approximately 1.2×25.0×15.0 mm. Measurements were conducted in a Kruss K12 tensiometer (Kruss GMBH) according to the Wilhelmy Plate method [150]. BC matrices were immersed, about 5 mm, into the test liquids (water and 1,2-propanediol) at a speed of 3 mm/min, at 25.0±0.1 °C. Advancing contact angles were used for surface energy (γ), dispersive (γ_d) and polar components (γ_p) estimation of all BC matrices. This estimation was based on the harmonic mean method proposed by *Wu* (1971), and expressed by the Eq.2.1, where γ_{12} is the interfacial tension between phases 1 and 2, and each have a surface tension consisting of polar and dispersive component [155]. Six replicates were performed for each BC formulation. Equations for surface tension estimation were solved using the equation handling KRUSS-software program: contact angle measuring system K12 (version 2.05).

$$\gamma_{12} = \gamma_1 + \gamma_2 - \left(\frac{4\gamma_1^d\gamma_2^d}{\gamma_1^d+\gamma_2^d}\right) - \left(\frac{4\gamma_1^p\gamma_2^p}{\gamma_1^p+\gamma_2^p}\right)$$

Eq.2.1

2.2.1.2. *In vitro* release studies

2.2.1.2.1. Levofloxacin release assays in PBS

In vitro levofloxacin release was conducted in 96-well flat-bottom cell culture plates (Greiner bio-one) as well as 24-well flat-bottom cell culture plates (Standard F, Sarstedt). For 96-well plates BC matrices with an average area of 40.19±3.85 mm^2 and weight of 15.90±1.98 mg were used, and for 24-well plates BC matrices with 82.46±5.35 mm^2 and 39.20±5.34 mg (Figure 2.2.). Evaluated specimens were: control (BCLac), BCLev1% and BCLev2.5% levofloxacin loaded-BC.

For specimens tested in the 96-well plates, 200 µL of PBS (0.01 M phosphate buffered saline; Sigma-Aldrich) was added, and for 24-well plates, 500 µL of PBS was added. All specimens were incubated at 37 °C with 5 % of CO_2 (the same temperature and CO_2 concentration used in cell assays). Drug release was determined in three cumulative time-points, 1 h, 20 h, and 40 h. Here, at each time-point, aliquots were collected and levofloxacin content was determined by high-performance liquid chromatography coupled to a UV detector (HPCL-UV) (Shimadzu LC-6A and SPD-6A). Chromatography analysis was performed using an adjusted method described in literature [156]. Chromatographic conditions were:

125-4, 5 µm, LiChrosphere® 100 RP-18 (Merck) column, a degassed mobile phase of water:acetonitrile and triethylamine (85:15(V/V), 0.6 %(V/V)) adjusted to pH 3 with ortho-phosphoric acid, a 1.2 mL/min flow rate and UV detection at 284 nm. Chromatography analysis was carried out at 25 °C. Orthophosphoric acid (analytical grade) and triethylamine were purchased from Panreac. The deionized water used for mobile phase preparation was obtained from a Millipore analytical deionization system (F9KN225218) and further filtered under vacuum (Vacuum pump V-700, Büchi) with 0.45 µm hydrophilic cellulose filters.

For the tests in 96-well plates, six replicates of BCLev1% and BCLev2.5% were made for each time-point, with the exception of controls (BC and BCLac) (n=1). In 24-well plates, triplicates of each matrix were tested in each time-point, also with the exception of controls (n=1). Calibration curves (Figure 2.3.) were prepared in water with eight standards (i.e. 0.3; 0.5; 1.0; 2.0; 3.0; 4.0; 6.0 and 8.0 µg/mL). The calibration curves were repeated in each day.

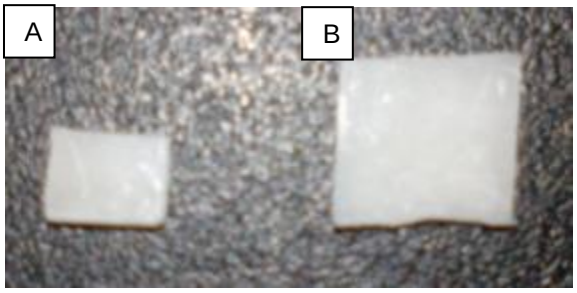

Figure 2.2 – Bone cement matrices.
A) BC matrices for 96-well plates assays; B) BC matrices for 24-well plates assays.

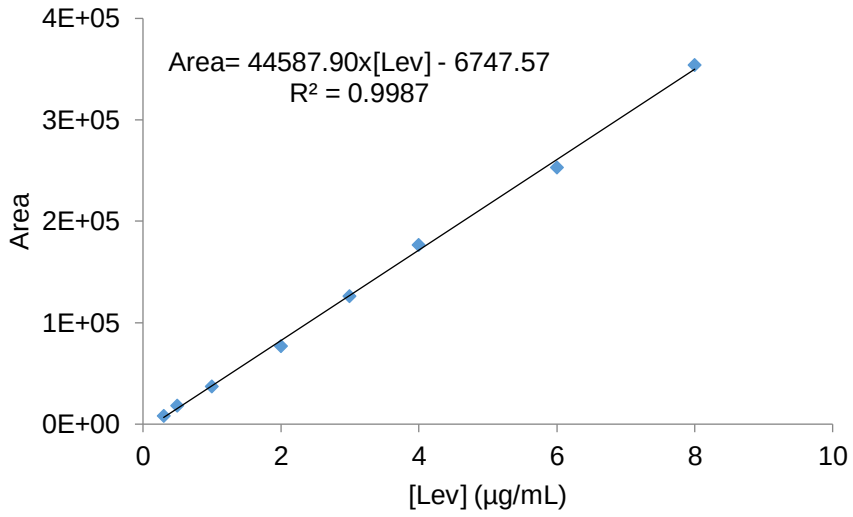

Figure 2.3 – Example of calibration curve of levofloxacin in water, by HPLC measurement.

2.2.1.2.2. Levofloxacin release assays in culture media

In vitro levofloxacin release by fluorescence method was performed in 96-well flat-bottom cell culture as well as 24-well flat-bottom cell culture plates. For 96-well plates BC matrices with an average area of 36.57±3.93 mm^2 and weight of 14.02±2.02 mg were used, and for 24-well plates BC matrices with 83.83±6.85 mm^2 and 38.44±4.49 mg. Evaluated specimens were: control (BCLac), BCLev0.5%, BCLev1%, BCLev1.5% and BCLev2.5%. The smaller BC matrices were incubated with 200 µL of Müeller-Hinton (MH) broth medium (Oxoid) or RPMI 1640 medium (Lonza), and the biggest with 500 µL, at 37 °C with agitation (Mini incubator, Labnet) for 24 h. After incubation, 100 µL of each well was collected and levofloxacin content was determined by fluorescence at 280 nm of excitation wavelength and 460 nm emission wavelength in Microplate Reader (FLUOstar Omega, BMGLabtech). Calibration curves (Figure 2.4) were prepared in MH broth or RPMI medium with nine standards (i.e. 25.0; 12.5; 6.25; 3.12; 1.56; 0.78; 0.39; 0.20 and 0.098 µg/mL). The respective incubation medium was also measured to be used as blank. Three replicates of each BC matrices were assayed. The calibration curves were repeated in each day.

(A) (B)

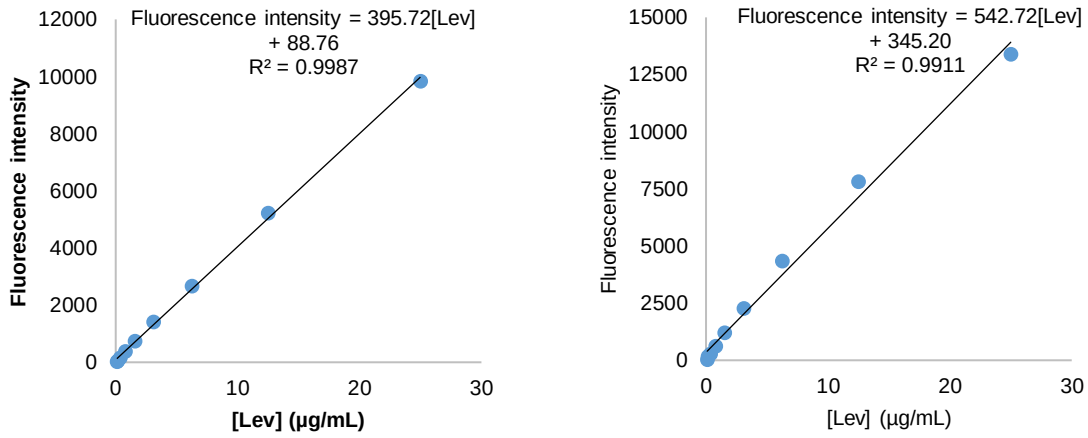

Figure 2.4 – Example of calibration curve of levofloxacin, by fluorescence measurement.
(A) Levofloxacin in MH broth; (B) Levofloxacin in RPMI medium.

2.2.1.2.3. Release of lactose

Lactose release from BC matrices was assessed by the dinitrosalicylic acid (DNS) method. This technique estimates the concentration of reducing sugars and involves mixing dinitrosalicylic acid reagent (sodium hydroxide from EKA and 3,5-dinitrosalicylic acid from Sigma-Aldrich and sodium potassium tartrate (Panreac)) with the samples, heating to catalyse the reaction (Figure 2.5), and measuring the visible absorbance [157], [158]. It was performed a method adapted from Wood and Bhat (1988) [159]. For 96-well plates BC matrices with an average area of 37.48±3.83 mm^2 and weight of 14.66±2.00 mg were used, and for 24-well plates BC matrices with 82.22±7.51 mm^2 and weight of

37.69±5.53 mg. Three and six replicates, for incubation in PBS and in MH broth respectively, of BC, BCLac, BCLev1% and BCLev2.5% were tested. The BC matrices were incubated with 200 µL of PBS or MH broth, at 37 °C with agitation for 24 h. After incubation, it was collected 50 µL of each well and added 50 µL of DNS reagent, previously prepared, for another microplate (96-well flat-bottom cell culture plate – Nunc). This microplate was heated for 5 min at 100 °C in a water-bath (VWB). The well-plate was allowed to cool to room temperature, and 75 µL of each well was transferred to another microplate. The absorbance was measured at 545 nm, using a Microplate Reader (FLUOstar Omega, BMGLabtech).

Standard solutions of lactose were prepared in PBS or in MH broth (i.e. 4.0; 2.0; 1.0; 0.5; 0.25; 0.12; 0.062; 0.031; 0.016; 0.0078 and 0.0039 mg/mL) and their concentrations covers the concentration of lactose release (Figure 2.6).

3,5-Dinitrosalicylic + Reducing sugar → 3-Amino-nitrosalicylic acid + Oxidised sugar

Figure 2.5 – Representation of the oxidation reaction of a general reducing sugar, in the presence of 3,5-Dinitrosalicilic.
Adapted from [158].

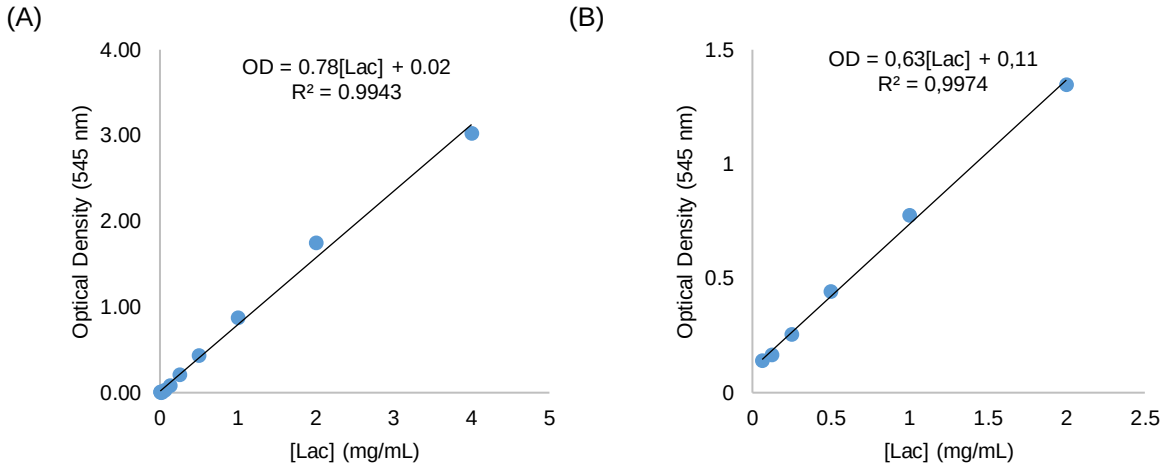

Figure 2.6 – Example of calibration curve of Lactose, by DNS method.
(A) Lactose in PBS; (B) Lactose in MH broth.

2.2.2. Polymeric microplarticles

2.2.2.1. Encapsulation efficiency and drug loading

Encapsulation efficiency (EE) was determined spectrophotometrically, using a Microplate Reader (FLUOstar Omega, BMGLabtech), by quantification of the antibiotics in the supernatants (non-encapsulated antibiotic) obtained during microparticles preparation. Supernatants were previously centrifuged for 10 min (B. BraunBiotech International GmbH, Sigma 112) and transferred to an UV 96-well plate (Greiner Bio-one). Antibiotic detection was conducted at 260 nm for daptomycin and 280 nm

for vancomycin [16]. The EE is expressed as the percentage of antibiotic encapsulated in particles reported to the initial amount of antibiotic used for particle preparation.

Standard solutions of daptomycin and vancomycin were prepared in filtered distilled water (i.e. 1.0; 0.5; 0.250; 0.125; 0.065; 0.0313; 0.0156; 0.0078; 0.0039; 0.00195 and 0.00098 mg/mL) (Figure 2.7.).

Furthermore, drug loading (DL) was determined according to the following formula:

$$\frac{\text{Amount of antibiotic in particles}}{\text{Theoretical amount of particles}} \times 100$$

Eq.2.2.

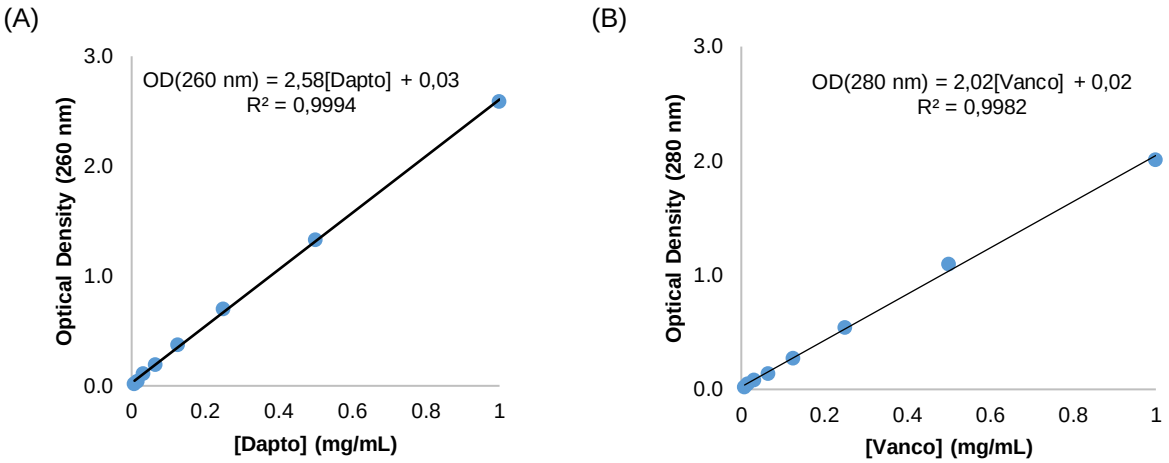

Figure 2.7 – Examples of calibration curves for determination of encapsulation efficiency of antibiotics. (A) Calibration curve for daptomycin; (B) Calibration curve for vancomycin.

2.3. Microbiological assays

2.3.1. Bacterial strains and cell line

Two reference bacterial strains and one clinical isolate of *S. aureus* were evaluated. Among the reference strains are a methicillin susceptible *S. aureus* ATCC®25923 (MSSA), and a methicillin resistant *S. aureus* ATCC®12600T, (MRSA-1), obtained from American Type Culture Collection (ATCC). The clinical isolate is also methicillin resistant (MRSA-2). Bacterial stocks prepared from over-night cultures on Müeller-Hinton (MH) agar (Oxoid) with 20 % glycerol and stored at -20 °C until further use. For each assay fresh cultures were prepared from frozen stocks on MH agar incubated overnight at 37 °C (Agitorb 200, AraLab).

When required, strains were stained with Oregon green (Molecular Probes). The bacterial suspensions were incubated with 1 mg/mL of Oregon green in 0.1 M phosphate buffer pH 7.4 (PBS) for 30 min at 5 °C, protected from the light. Fluorophore excess was removed by washing with PBS. This procedure

was performed twice being the centrifugations performed at 3500 rpm for 10 min (centrifuge A14, Jouan SA).

All procedures involving handling of bacteria were performed in laminar flow chamber (ESI Biocyt 95 Standard, Flufrance).

Human osteoblast cell line MG63 (ATCC®CRL-1427^{TM}) was used. Cells were cultured in RPMI 1640 growth medium (Lonza) supplemented with 10 % heat inactivated fetal bovine serum (V/V; FSB) (Lonza), 100 units/mL penicillin G (sodium salt) (Gibco), 100 µg/mL streptomycin sulphate (Gibco) and 2 mM L-glutamine (Gibco), at 37 ºC with 5 % CO_2 (Brinder), until confluence levels reached at least 75 %. At this point cell lines were dislodged from surface with 1 mL of EDTA-Trypsin (Gibco) and subsequently transferred to new T-flasks at a tenth of its original volume.

2.3.2. Biofilm assembly

The assay was performed in triplicate using 96-well flat-bottom cell culture plates (standard F, Sarstedt) as described previously with small modifications [160]. Briefly, bacterial suspensions with a final concentration of 10^8 bacteria/mL were prepared in sterile saline solution. 20 µL of the bacterial suspension were distributed by each well with 180 µL of MH broth, being MH broth used as negative control. The plates were incubated at 37 ºC to allow biofilm formation for different time periods. Then, the content of each well was removed, and each well was vigorously washed three times with sterile distilled water to remove non-adherent bacteria. The attached bacteria were then stained for 15 min with 100 µl violet crystal (Merck) at room temperature, washed with distilled water three times to remove dye in excess and allowed to dry at room temperature. The violet crystal was dissolved in 100 µl of 96 % ethanol (Merck) and the optical density at 570 nm was read using a microplate reader (Multiskan Ascent, Thermo Labsystems).

2.3.3. Bacteria susceptibility to levofloxacin

2.3.3.1. Standard levofloxacin

The antimicrobial activity of levofloxacin was evaluated by microdilution method according to the described by guidelines of the Clinical & Laboratory Standards Institute (CLSI), with exception of inoculum concentration. It was twenty times higher than the recommended concentration. The assay was performed in duplicated, using 96-well flat-bottom cell culture plates (Nunc) for both planktonic bacteria and biofilm. Briefly, the antibiotic was diluted in MH broth to produce a two-fold dilution in the concentrations range of 500 – 0.00095 µg/mL. Bacterial suspensions were prepared as described in the previous section (2.3.2.), being inoculated approximately 2×10^6 bacteria/well. A positive control containing a suspension of bacteria in MH broth without antibiotic and a negative control containing only MH broth, were performed in parallel. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of antibiotic resulting in the absence of turbidity after 24 h of incubation at 37 ºC.

The minimum inhibitory concentration for biofilm (MBIC) was performed using the same concentration range for 48 h old biofilms (mature biofilm). Briefly, after removing non adherent bacteria as described in section 2.3.2., levofloxacin solutions in the concentration range described above were added being the biofilm further incubated 24 h at 37 °C. The wells of MBIC and twice MBIC were washed with sterile distilled water three times, and sonicated for 5 min (ultrasonic cleaner Bransonic 2510E-MTH) with 200 µL sterile distilled water to recover bacteria from biofilms. The MBIC, and twice the MBIC were serial diluted in sterile distilled water, platted on MH agar and incubated at 37 ºC until CFU were visible (approximately 24 to 48 h).

2.3.3.2. Levofloxacin released from BC matrices

Effluent sample with 155.12±1.86 µg/mL levofloxacin determined by fluorescence assay (section 2.2.1.2.2.), was obtained from smaller BCLev2.5% plates with an average area of 37.37±3.39 mm^2 and weight of 13.64±1.70 mg. Each BC plate was incubated with 200 µL of MH broth at 37 °C for 24 h. After this, total volume was collected and stored at -20 °C until further use.

Levofloxacin susceptibility was determined as described in section 2.3.3.1., for 48 h old biofilms using different concentration ranges. For reference MSSA was used a concentration range of 39.78 – 0.30 µg/mL and for the remaining strains 155.12 – 1.21 µg/mL.

2.3.3.3. Levofloxacin-loaded BC matrices

The antibacterial activity of BC matrices, namely BCLac (control), BCLev0.5%, BCLev1%, BCLev1.5% and BCLev2.5% was evaluated against 48 h old biofilms, prepared as described in section 2.3.2. Briefly, after removing non-adherent bacteria by washing, fresh culture medium and the smaller BC plates with an average area of 37.37±3.39 mm^2 and weight of 13.64±1.70 mg were added and further incubated at 37 ºC for 24 h. The positive control, the BC matrix with the lowest levofloxacin concentration resulting in the absence of turbidity and the BCLev2.5% were treated in the same way as MBIC and twice MBIC as described in section 2.3.3.1.

2.3.4. Biofilm assembly by scanning electron microscopy (SEM)

Biofilm assembly on BC matrices was monitored by scanning electron microscopy (SEM). The two reference *S. aureus* strains were allowed to assemble biofilms on BCLac (control), BCLev0.5% and BCLev2.5% plates with an average area of 37.37±3.39 mm^2 and weight of 13.64±1.70 mg, attached to the bottom of 96-well plates with silicon, during 48 h at 37 ºC. Biofilms were washed as described previously (section 2.3.2.) and fixed with a solution of 2.5 % glutaraldehyde (EMS) and 4 % paraformaldehyde (Sigma-Aldrich) in PBS over-night at 4 ºC (±20 h) protected from the light. The samples were washed twice with PBS for 10 min and post-fixed with 1 % osmium tetroxide (EMS) in

PBS at room temperature protected from the light during 2 h. After being washed twice with PBS as described before and once with distilled water during 10 min samples were dehydrated using once 50 %, 70 % and 95 % ethanol for 30 min and thrice absolute ethanol for 30 min. The samples were then transferred to glass slides (BioMérieux) coated with carbon tape and allowed to dry at room temperature, sputter-coated with a gold–palladium film (thickness of 20 nm) using a QISOT ES sputter coater (Quorum Technologies) and were analysed with a JSM-7100F SEM (JEOL).

2.4. Antibacterial intracellular activity

2.4.1. Survival assay

2.4.1.1. Acrylic bone cement

The assay was performed as described in the literature with some modifications [161]. The human osteoblast cells were inoculated into 24-well flat-bottomed cell culture plates (Nunc), with a number of $1x10^5$ cells per well, and incubated for 48 h at 37 °C with 5 % CO_2. In order to assure a multiplicity of infection (MOI) of 25 bacteria per cell osteoblasts were infected with a *S. aureus* suspension of OD_{600nm} ≈ 0.02 (Genesys 20), for 30 min at room temperature followed by additional 3 h at 37 °C with 5 % CO_2. Growth medium was removed and cells were washed three times with PBS to remove extracellular bacteria. In order to remove adherent methicillin susceptible or resistant *S. aureus* infected cultures were incubated for 10 min with 20 µg/mL of lysostaphin (Sigma-Aldrich) alone or 20 µg/mL of lysostaphin and 100 µg/mL gentamicin (Gibco) for 1 h at 37 °C with 5 % CO_2, respectively. Fresh culture media with 10 µg/mL gentamicin (MSSA) or 20 µg/mL of lysostaphin (MRSA) were added together with BC matrices (BCLac, BCLev1% and BCLev2.5% - with 83.47±6.47 mm^2 and weight of 34.96±10.86 mg) and further incubated at 37 °C with 5 % CO_2. After 24 h and 48 h growth medium was removed, cells were washed one time with PBS and lysed with aqueous solution of 1 % igepal (v/v; Sigma-Aldrich) for 5 min at 37 °C with 5 % CO_2. Cell lysates were serially diluted in sterile distilled water platted on MH agar and incubated at 37 ºC until CFU were visible (approximately 24 to 48 h). Furthermore, the levofloxacin amount in the collected media (MSSA assay) was assessed by fluorescence method described in section 2.2.1.2.2.

2.4.1.2. Polymeric microparticles

The assay was performed as described in section, 2.4.1.1., with some exceptions. In this assay only one *S. aureus* strain, MSSA, was used. The protocol previously described in section 2.4.1.1 for MSSA was used but instead of adding BC matrices, PMMA-EUD-Dapto or PMMA-EUD-Vanco microparticles were added at 2 mg/mL, 1 mg/mL and 0.5 mg/mL. Empty PMMA-EUD microparticles at the same concentrations were used as control. Also, the growth medium for PMMA-EUD-Dapto was supplemented with 50 mg/L Ca^{2+}. Growth medium was removed only after 24 h.

2.4.2. Intracellular distribution of *S. aureus*

Using the same MOI described under survival assay (section 2.4.1) osteoblasts grown on glass coverslips were infected with *S. aureus* stained with Oregon green (section 2.3.1).

Cells were fixed with 4 % paraformaldehyde (w/V; Sigma-Aldrich) at room temperature protected from light for 15 min, and then were washed three times with PBS. After, cells were incubated with 50 mM ammonium chloride for 15 min, permeabilized with 0.1 % Triton X-100 (V/V; Sigma-Aldrich) in 200 mM glycine-PBS for 30 minutes, and washed thrice with PBS. After 30 min blocking with 0.4 % skin fish gelatine (Sigma-Aldrich) in PBS at room temperature, samples were incubated with a 1:50 dilution of Alexa Fluor 568 phalloidin (Life technologies) in blocking solution for 30 min. Cells were washed thrice with PBS, and was added 5 µg/mL Hoechst 33258 (Sigma-Aldrich) in PBS, incubated for 3 min and washed thrice with PBS. Cell slides were mounted by inverting the glass coverslips on fluorescent mounting medium (Dako). Confocal microscopy images were collected using the SP2 microscope from Leica, and data analysis was performed using ImageJ 1.50i software (Image Processing and Analysis in Java).

2.4.3. Statistical analysis

All experiments were performed in duplicates and by independent assays (minimum n=2, depending on the assay). All data are presented as mean and standard deviation (mean±SD). Statistical significance was assessed by the student t-test. The level of statistical difference was defined at a $p < 0.05$ level.

Chapter 3. Results and discussion

3.1. Characterization of biomaterials

3.1.1. Acrylic bone cement

3.1.1.1. Contact angle and surface energy determination

Contact angle and surface energy are important tests, because surface properties of a biomaterial strongly influence its biocompatibility behaviour, as well as the bacteria adherence, and subsequent formation of biofilms [75]. Biocompatibility problems associated to bone cement application limit the clinical success of cemented arthroplasties. Being the cement surface in close connection with the living bone and in direct contact with blood, it is reasonable to assume that surface properties, such as surface composition and surface energy, will play a role in the biomaterial performance [140], [149]. Surface energy is related to the hydrophilic degree of the surface, which is accountable for the adhesion of proteins, bacteria and cells like osteoblasts, and their proliferation [75]. The surface of a biomaterial is defined as hydrophobic when a water droplet spreads poorly over a surface to form a high contact angle and low surface energy. While if water spreads, the contact angle is low and surface energy is high, and in this case the biomaterial surface is hydrophilic (Figure 3.1) [140]. The hydrophobicity of a material surface has been measured mainly by contact angle measurement. Depending on the hydrophobicity of both bacteria and material surfaces, bacteria adhere differently to materials with different hydrophilicities. Overall, hydrophilic materials are more resistant to bacterial adhesion than the hydrophobic [162].

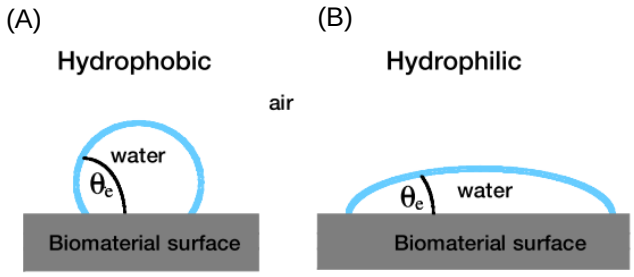

Figure 3.1 – A liquid droplet on the biomaterial surface.
(A) Liquid spreads poorly to form a high contact angle (hydrophobic surface); (B) Liquid spreads well on a hydrophilic surface. Adapted from [140].

However, usually polymeric biomaterials have low surface energy and are hydrophobic [162]. Thus, the use of PMMA bone cement entails the risk of attracting infectious microorganisms [140]. In this way, the incorporation of antibiotic in PMMA bone cements to reduce the infection rates in orthopaedic surgery arises as a solution. Nevertheless, when there are changes to the biomaterial surface, such as additive or antibiotic loading into the polymer matrix, it is essential to ensure that these changes will not compromise the interaction between bone cement and biological tissues [75].

In order to estimate the surface energy based on the harmonic mean method proposed by Wu (1971), it was necessary to determine the contact angle between the BC matrices and two liquids, water (polar compound) and 1,2-propanediol (less polar compound) (section 2.2.1.1.) [155] The results are shown in Table 3.1. The advancing contact angle values in water do not present relevant differences in relation to the ones described in the literature for CMW1 BC (85 °) [140].

Table 3.1 – Results obtained for advancing contact angle surface in water and in 1,2-propanodiol (mean±SD; n=6).

BC matrix	Advancing contact angle (°)	
	water	1,2-propanodiol
BCLac	78.27±4.11	39.96±2.67
BCLev0.5%	81.30±2.04	33.68±5.49
BCLev1%	76.79±2.32	28.52±4.54
BCLev1.5%	79.50±1.66	35.16±4.69
BCLev2.5%	80.82±4.76	39.75±5.24

The results obtained of total (γ), dispersive (γ^d) and polar (γ^p) surface energy are presented in Figure 3.2 and in Annex A (Table 6.1).

The results of total, dispersive and polar surface energy did not show statistical difference ($p > 0.05$) between the control (BCLac) and the antibiotic loaded BC formulations. The data obtained for BCLac and BCLev2.5% are in agreement with previously published data by Matos and colleagues (2015), in the same experimental conditions [40]. Thus, biological interaction between bone cement and biological tissues will not be compromised. Moreover, the data leads to the conclusion that the modified BC matrices show a hydrophobic behaviour.

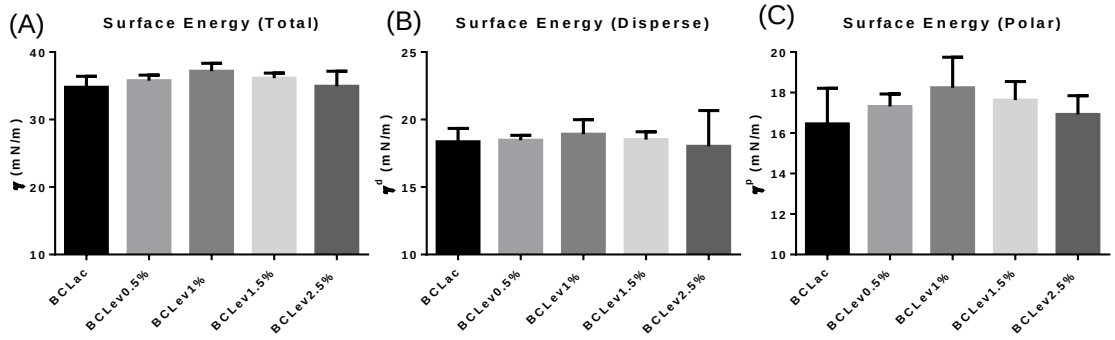

Figure 3.2 – Results obtained for the surface energy of the BC matrices. (A) Total (γ); (B) Dispersive (γ^d); (C) Polar (γ^p). (mean±SD; n=6)

3.1.1.2. *In vitro* release studies

In general studies of drug release provide important information about drug behaviour in different media and what to expect in further studies. Different types of BC plates, as well as different release media were tested.

3.1.1.2.1. Levofloxacin release assays in PBS

First assay of levofloxacin release quantification was performed by HPLC. This test aimed to evaluate the percentage of levofloxacin eluted from BC matrices over time with different concentrations of drug loading and different size of plate matrices. The release assay was conducted with BCLev1% and BCLev2.5%. BCLac was used as control. Two sizes of matrices were used (section 2.2.1.2.1.). As incubation medium, a biological model fluid (phosphate buffer saline solution: PBS; pH=7.4, 25 ºC) was used. The results obtained are shown in Table 3.2, and in Annex B (Figure 6.1).

Table 3.2 – *In vitro* results of levofloxacin cumulative release (µg/mm^2) in PBS.
Measurements were performed by HPLC, at time-points of 1 h, 20 h and 40 h

	1 h	20 h	40 h	1 h	20 h	40 h
BC matrix	**µg/mm² BC**			**CV (%)**		
96-well plates*						
BCLev1%	0.21±0.04	0.25±0.06	0.31±0.04	21.23	22.26	11.29
BCLev2.5%	0.51±0.09	0.74±0.10	0.85±0.19	17.06	14.30	22.57
24-well plates**						
BCLev1%	0.16 ± 0.02	0.29±0.02	0.24±0.05	9.55	8.61	18.97
BCLev2.5%	0.38 ±0.05	0.60±0.01	0.75±0.06	13.16	1.29	8.14

Note: *(mean±SD; n=6); **(mean±SD; n=3).

In all tested antibiotic-loaded BC matrices, it was observed a slight increase of levofloxacin release over time and with the increase of loaded drug. However, the results obtained for both sizes of BC matrices were different in respect to reproducibility. The levofloxacin released from smaller BC matrices (assay in 96-well plates) demonstrated higher coefficient of variation than from larger BC matrices (assay in 24-well plates), mainly in BC with the lowest concentrations of antibiotic. This fact may be due to the possibility of the drug not being homogeneous dispersed throughout the BC matrix. So, the smaller the tested plates, the less reliable the reproducibility of the assays. Furthermore, a slight decrease of levofloxacin release with the increase of BC dimensions was observed. It is known that matrix geometry, such as shape and size, affects the drug release profiles [163]. This fact is associated to differences in surface area of the BC matrices with different sizes. The small matrices have a higher relative surface area and usually the release from this matrices is faster than from large matrices [163]. Overall, the amount of drug released was lowest compared to total loaded amount. Some authors may pose the hypothesis that some free radicals produced due to reactivity conditions in BC setting, might react with vicinal levofloxacin molecules during BC curing, and it may have relevance in the release of antibiotic. Previous studies, by Matos and colleagues (2015), leaded to conclusion that levofloxacin established covalent and non-covalent interactions with PMMA during polymer setting, which may responsible for the antibiotic retention inside the BC matrix and consequently release inhibition [40].

The average of levofloxacin concentrations (µg/mL) released from different tested matrices are shown in Table 3.3. In both doses of drug and sizes of matrices the values obtained were above that of levofloxacin minimum inhibitory concentrations (MIC) for *S. aureus* strains, described in literature (MIC_{90} Levofloxacin: 0.25 – 0.5 µg/mL for MSSA and 0.5 – 16.0 µg/mL for MRSA) [41]. It is important that the

concentrations of levofloxacin released from BC matrices exceed the MIC of pathogens causing bone infection *in vivo* conditions.

Table 3.3 – *In vitro* results of levofloxacin cumulative release (µg/mL) in PBS.
Measurements were performed by HPLC, at time-points of 1 h, 20 h and 40 h

Concentration of levofloxacin released from BC matrix (µg/mL)			
BC matrix	1 h	20 h	40 h
96-well plates*			
BCLev1%	44.03±10.28	50.31±12.72	60.92±7.32
BCLev2.5%	103.66±13.41	146.24±20.48	158.91±31.13
24-well plates**			
BCLev1%	28.32±4.53	49.54±5.90	40.08±6.19
BCLev2.5%	59.28±7.69	97.81±5.59	115.50±8.25

Note: *(mean±SD; n=6); **(mean±SD; n=3).

Several *in vitro* studies of antibiotic-loaded BC elution have been described in literature, with several drugs, such as gentamicin, tobramycin, vancomycin, and others [74], [78], [164], [165]. These studies were conducted in saline solution and the release was measured by other methods, for example by fluorescence polarization immunoassay, and in other experimental conditions [74], [78], [164], [165]. In these works the amount of released antibiotic was also lower in relation to the total theoretical amount, as well as in previously published data by Matos and colleagues with levofloxacin-loaded PMMA BC [40]. However, another study, by de same author (Matos and colleagues), with minocycline revealed a complete drug release from PMMA BC in the same experimental conditions [75].

3.1.1.2.2. Levofloxacin release assays in culture media

To better mimic the release during cell and microbiological *in vitro* assays, levofloxacin liberation was also tested in complete cell medium (RPMI) and in bacteria culture medium (MH broth). As HPLC method requires the precipitation of proteins present in the medium another analytical method was used – fluorescence technique (section 2.2.1.2.2.). The fluorescent method is faster and more economic (no organic solvents are necessary) than HPLC.

Four BC matrices with different concentrations of levofloxacin were tested (BCLev0.5%, BCLev1%, BCLev1.5% and BCLev2.5%). Plates were incubated during 24 h with RPMI medium or MH broth. The data obtained are shown in Table 3.4 and Annex B (Figure 6.2).

Table 3.4 – *In vitro* results of levofloxacin cumulative release (µg/mm^2) after 24 h of incubation in RPMI or MH broth obtained by fluorescence technique.

	RPMI medium*				MH broth*			
	96-well plates		24-well plates		96-well plates		24-well plates	
BC matrix	**µg/mm^2**	**CV (%)**	**µg/mm^2**	**CV (%)**	**µg/mm^2**	**CV (%)**	**µg/mm^2**	**CV (%)**
BCLev0.5%	0.04±0.003	5.74	0.07±0.004	5.26	0.13±0.04	33.49	0.13±0.04	31.90
BCLev1%	0.08±0.009	11.05	0.11±0.008	7.74	0.30±0.06	21.78	0.28±0.03	9.80
BCLev1.5%	0.12±0.008	6.92	0.16±0.006	3.86	0.80±0.08	10.42	0.59±0.10	17.76
BCLev2.5%	0.12±0.003	2.84	0.17±0.005	3.17	0.98±0.17	16.91	0.82±0.16	20.20

Note: *(mean±SD; n=3).

The values of levofloxacin released from BC matrices in MH broth were similar to values obtained in PBS solution for BCLev1% and BCLev2.5% at 20 h of incubation (section 3.1.1.2.1.). Furthermore, the same profile for both sizes of matrix was observed in MH broth. The smaller matrices revealed less drug release than larger matrices. However, the liberation of levofloxacin in RPMI medium showed the opposite. Moreover, in this medium the BC specimens had lower release than in the other media. These variances are associated to different composition of the media (described in detail in Annex C, Tables 6.2 and 6.3). The RPMI medium has several components including high amount of proteins which may interfere with levofloxacin, decreasing the drug release in this medium, while the MH broth has only acid hydrolysate of casein, beef extract and starch in its composition.

In relation to different concentrations of levofloxacin-loaded BC, the release is higher with increase drug loading until BCLev1.5% in both sizes and media, with exception of matrices for 24-well plates in MH broth that continued to have the same behaviour. For other conditions, the liberation of drug between the two BC matrices with higher concentration of levofloxacin (BCLev1% and BCLev2.5%) had no substantial differences.

The average of levofloxacin released (µg/mL) from different tested matrices are show in Table 3.5. The concentration of levofloxacin released from BC matrices in both tested incubation medium are also above MIC values described for *S. aureus* strains (MIC_{90} Levofloxacin: 0.25 – 0.5 µg/mL for MSSA and 0.5 – 16.0 µg/mL for MRSA, also discussed in the previous section (3.1.1.2.1.) [41]. Furthermore, in this work, the MIC and MBIC of three *S. aureus* strains was evaluated in MH broth (section 3.2.2.1.). However, only the two BC matrices with higher doses of drug (BCLev1.5% and 2.5%) showed concentrations of released levofloxacin in MH broth above that of the results for MIC and MBIC of these strains (range of 0.5 – 62.5 µg/mL). With these data is possible to conclude that even in RPMI medium, where the release of drug is lower, the amount of drug liberated from BC with smaller loaded dose is enough to kill some *S. aureus* strains, but not all.

Table 3.5 – *In vitro* results of levofloxacin cumulative release (µg/mL) after 24 h of incubation in RPMI or MH broth obtained by fluorescence technique.

BC matrix	Concentration of levofloxacin released from BC matrix (µg/mL)			
	RPMI medium*		MH broth*	
	96-well plates	24-well plates	96-well plates	24-well plates
BCLev0.5%	8.66±0.71	11.03±1.10	25.98±10.17	28.42±13.93
BCLev1%	15.05±3.21	17.51±0.55	52.27±18.30	52.41±5.44
BCLev1.5%	20.43±1.38	27.72±1.12	124.94±11.39	97.20±27.14
BCLev2.5%	21.95±1.53	28.19±0.79	206.37±43.34	124.36±31.89

Note: *(mean±SD; n=3).

The next step was to evaluate the levofloxacin concentration in the medium (RPMI) obtained after the exposure of human osteoblast cells infected with MSSA (section 2.4.1.1) to BC. In this assay, 24-well plates of BCLac (control), BCLev1% and BCLev2.5%, exposed to human osteoblast infection model during 24 and 48 h were tested. The results are shown in Table 3.6.

Table 3.6 – *In vitro* results of levofloxacin cumulative release (µg/mm^2).
Levofloxacin concentration values measured in the effluents collected from the antibacterial intracellular activity assays (at 24 and 48 h), by fluorescence measurement.

BC matrix	µg/mm^2*		CV (%)	
	24 h	48 h	24 h	48 h
BCLev1%	0.31±0.02	0.30±0.02	5.86	5.76
BCLev2.5%	0.69±0.13	0.91±0.03	18.86	3.51

Note: *(mean±SD; n=3).

Curiously, the release values of these effluents at 24 h of incubation were similar to results obtained in PBS and in MH broth, in opposite to results obtained only in RPMI medium (sections 3.1.1.2.1 and 31.1.2.2.). However, at 48 h of incubation an increase of drug liberation was observed only in BC with higher concentration of loaded antibiotic (BCLev2.5%). The difference between this assay (Table 3.6) and the assay in RPMI medium (Table 3.4) was the presence of osteoblast cells and the bacteria (MSSA). This fact leads to supposition that the presence of cells or bacteria affect the release of levofloxacin from BC matrices, in a positive way.

The average of concentration values (µg/mL) of levofloxacin released are shown in the Table 3.7. Once again the liberated concentrations of levofloxacin were above that of MIC described in literature for some strains of *S. aureus* (MIC_{90} Levofloxacin: 0.25 – 0.5 µg/mL for MSSA and 0.5 – 16.0 µg/mL for MRSA) [41]. However, only BCLev2.5% at 48 h of incubation revealed concentrations above the MIC and MBIC values for the strains tested in the present work.

Table 3.7 – *In vitro* results of levofloxacin cumulative release (µg/mL).
Levofloxacin concentration values measured in the effluents collected from the antibacterial intracellular activity assays (at 24 and 48 h), by fluorescence measurement.

Concentration of levofloxacin released from BC matrix (µg/mL)*		
BC matrix	**24 h**	**48 h**
BCLev1%	25.86±1.68	25.21±3.60
BCLev2.5%	54.23±7.61	71.02±2.76

Note: *(mean±SD; n=3).

3.1.1.2.3. Release of lactose

Both *in vivo* and *in vitro* studies have shown that the initial release of the antibiotics from BC is a surface phenomenon. The BC is impermeable to antibiotics; the drug must be released through an interconnecting series of voids and cracks in the polymer matrix [145]. In this way, the sustained release of drug from BC matrices is largely affected by the penetration of fluids into the polymeric matrix, which requires a certain degree of superficial porosity of the cement. The porosity of the cement may be increased by inclusion of water soluble components, designated by release modulators, which will consequently increase the release of drugs [145]. In previous studies, lactose was added to the PMMA BC formulation to increase drug release [40], [145]. According to Matos and colleagues (2015) the elution of levofloxacin held throughout a 7-week time period was strongly influenced by lactose loading into BC; higher release of levofloxacin (around 35 %) from levofloxacin-loaded BC with therapeutic concentration of 2.5 % (w/w) and with 10.0 % (w/w) of lactose was observed in comparison with BC without lactose [40]. In this scope, the same amount of lactose was added to BC formulations tested in the present work. Therefore, it was considered important to determine the release of this component from BC, as it can influence bacterial activity. It is known that bacteria can use glucose or other sugars, such as lactose, as source of carbon and energy [166]. So, the released of this sugar from BC may influence the antibacterial activity of levofloxacin in *in vitro* assays.

Thus, to measure the lactose released from BC matrices, DNS method was used. This method aims at estimating the concentration of reducing sugars (section 2.2.1.2.3.). Lactose release assay was conducted with BCLac, BCLev1% and BCLev2.5%, all with two different sizes. Plain BC was used as control. The incubation was performed by 24 h in PBS and MH broth. Release results in PBS and MH broth are shown in Table 3.8 and Annex B (Figure 6.3).

Table 3.8 – *In vitro* results of lactose cumulative release (µg/mm^2) after 24 h of incubation in PBS or MH broth obtained by DNS method.

	PBS*				MH broth**			
	96-well plates		24-well plates		96-well plates		24-well plates	
BC matrix	µg/mm^2	CV (%)	µg/mm^2	CV (%)	µg/mm^2	CV (%)	µg/mm^2	CV (%)
BCLac	15.97±1.64	10.29	4.59±0.50	10.83	17.89±8.33	46.59	10.22±1.51	14.77
BCLev1%	14.95±7.75	51.85	4.69±0.13	2.81	18.50±5.86	31.66	5.12±1.05	20.53
BCLev2.5%	10.71±5.21	48.68	4.01±0.26	6.45	25.20±5.73	22.75	5.74±0.60	10.49

Note: *(mean±SD; n=3); **(mean±SD; n=6).

The medium of release plain BC does not interfere with the measurement of lactose by DNS method. The effect of plate size on lactose is shown to be similar to those of levofloxacin (sections 3.1.1.2.1.and 3.1.1.2.2.). As in levofloxacin release assays, with different sizes of matrices in PBS and MH broth, the lactose released from smaller BC matrices demonstrated higher coefficient of variation than from larger BC matrices. This points again to the possible inadequate homogenization of the additives during the cement preparation. Furthermore, similar to levofloxacin liberation, a slight decrease of lactose release with the increase of BC dimensions was observed. The amount of lactose released in MH broth was slightly higher than in PBS. For both BC plate sizes, it was observed that lactose release is independent of concentration of levofloxacin loaded in BC matrices. However, it was possible to note that the amount of lactose liberated from BC matrix without antibiotic was slightly higher, with the exception of matrices for 96-well plates in MH broth.

3.1.2. Polymeric microparticles

3.1.2.1. Preparation and characterization

The preparation of daptomycin and vancomycin loaded PMMA-EUD microparticles using a $w_1/o/w_2$ double emulsion-solvent evaporation method and high molecular weight polymers (section 2.1.2.) yielded a particle size distribution within the micrometre range (around 1 µm). These particles usually revealed a spherical shape with rather smooth surfaces and positive surface charge [16].

The inclusion of a second polymer, EUD, in the formulations of PMMA particles was previously studied by Ferreira and colleagues [16]. This second polymer is a cationic non-biodegradable acrylic polymer commonly used for preparation of controlled release pharmaceutical dosage forms, and it is added with the aim to optimize the encapsulation efficiency of daptomycin in PMMA [16]. The results obtained of encapsulation efficiency and drug loading are show in Table 3.9.

Table 3.9 – Encapsulation efficiency (EE) and drug loading (DL) for PMMA-EUD microparticles formulations (mean±SD; n=3).

Formulations	EE (%)	DL (%)
PMMA-EUD (control)	-	-
PMMA-EUD-Dapto	93.14±1.50	13.90±0.22
PMMA-EUD-Vanco	31.71±2.18	4.59±0.31

Results are in agreement with previously published data by Ferreira and colleagues (PMMA-EUD-Dapto: EE (%) = 95.6±1.2; DL (%) = 12.4±0.3; PMMA-EUD-Vanco: EE (%) = 53.5±1.6; DL (%) = 6.9±0.3) [16]. As expected, EE of daptomycin was higher due to the addition of EUD to the formulations. The positively charged polymer will attract the negative charge of micelles formed by this drug, thus increasing the EE of this antibiotic into particles, while the opposite was observed in relation to vancomycin encapsulation. Adding EUD to the particles decreased vancomycin EE, due to the repulsion between positive charges of the PMMA-EUD and those of vancomycin [16]. The same behavior was observed in relation to DL results.

3.2. Microbiological assays

It is known that bone infections are frequently caused by *S. aureus*, and became very difficult to eradicate due to biofilm formation [167]. For this reason, the activity of BC matrices with different concentrations of levofloxacin on *S. aureus* biofilms was evaluated. Three *S. aureus* strains were selected for the assays. MSSA was chosen since it is a recognized strong biofilm producer [168] and MRSA were select as representatives of highly antibiotic resistant bacteria usually responsible for healthcare associated diseases (HAIs) such as device associated infections.

3.2.1. Biofilm assembly

Biofilm assembly ability for the three strains was followed over 120 h (section 2.3.2.). Biofilms were assembled on cell culture plates, since plastic surfaces are reported as suitable for bacterial attachment, due to its hydrophobic nonpolar nature with little or no surface charge [169]. After 2, 6, 24, 48, 72 and 120 h biofilm assembly was evaluated using the violet crystal assay.

The results of biofilm assembly assay are shown in Figure 3.3. The three *S. aureus* strains were able to assemble biofilms although following diferent kinetics. A feature commom to the three strains was the absence of an adaptation phase. The best biofilm assembler is the reference strain MSSA reaching the higher biomass level at 72 h. After 72 h the biomass starts decreasing leading us to the conclusion that the mature biofilms enter its dispersion phase. MRSA strains although presenting lower biomass than MSSA reached biofilm maturation phase 24 h earlier (48 h) although following different kinetics. The MRSA-1 (orange line in Figure 3.3) is a better biofilm producer showing a fast increase in biomass until 48 h and then enters in dispersion phase characterized by a drop in biomass. On the other hand the

MRSA-2 (Green line in Figure 3.3) increases its biomass slowly but after 48 h seems to reach a steady state since for this strain biofilm dispersion was not observed until 120 h.

Based on these data, bacteria were ranked concerning their biofilm assembly ability. As previously stated MSSA is the best biofilm assembler, followed by MRSA-1 and MRSA-2. However, even the best biofilm assembler exhibits low levels of biofilm assembly when compared with another etiological agent of HAIs *Klebsiella pneumoniae* in the same experimental settings [170].

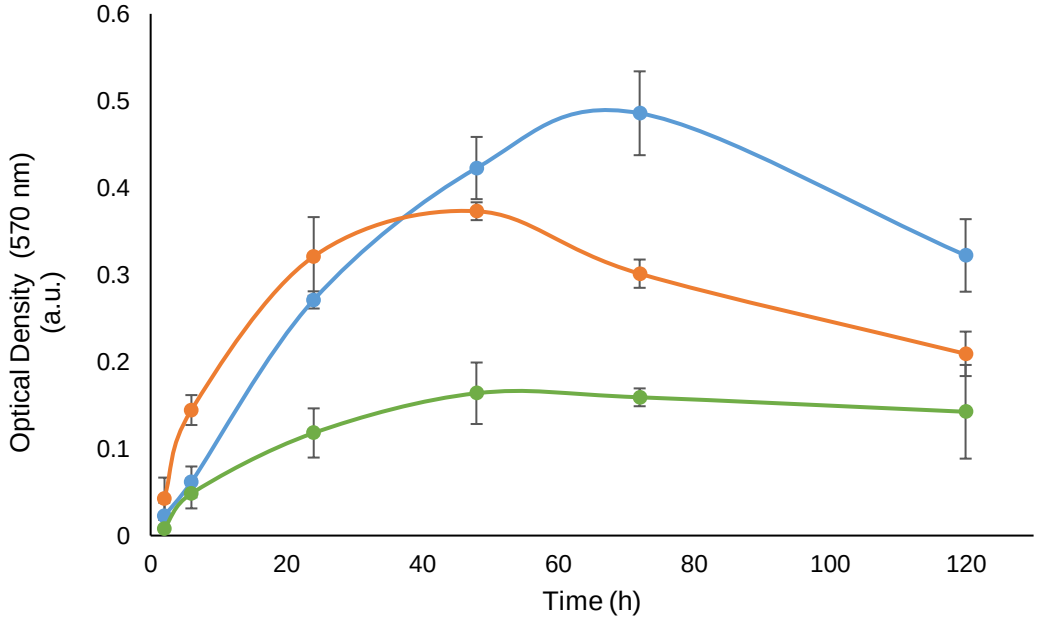

Figure 3.3 – Kinetic of biofilm assembly for *S. aureus* strains.
Kinetic of biofilm assembly for MSSA (blue line), MRSA-1 (orange line) and for MRSA-2 (green line) (mean±SD; n=3). OD values were measured at 570 nm at time-points of 2, 6, 24, 48, 72 and 120 h.

3.2.2. Bacteria susceptibility to levofloxacin

The emergence of antibiotic resistant bacteria, particularly multi-resistant bacteria, is a public health concern world-wide contributing to the persistence of infections such as medical devices associated infections. One of the factors contributing to an increase bacterial resistance to antimicrobial agents is biofilm assembly. The minimum inhibitory concentration (MIC) for a bacteria within biofilm known as minimum biofilm inhibitory concentration (MBIC) can be up to 1000-fold higher than the respective planktonic counterpart [27], [169]–[171]. In order to evaluate antibacterial activity of BC matrices mature biofilm were used, since at this stage the biofilm shows maximum resistance to antibiotics [27]. Thus, with observation of biofilm assembly assay data (Figure 3.3) it was possible to select 48 h old biofilms for the subsequent assays.

3.2.2.1. Standard levofloxacin

Firstly, levofloxacin standard activity against planktonic and biofilm organized bacteria was evaluated by the MIC and MBIC, respectively (section 2.3.3.1.). The MIC is defined as the minimum antibiotic

concentration required to inhibit growth of planktonic bacteria or bacteria organized within biofilm (MBIC) [151]. This assay aims to evaluate the role played by biofilms in increased antibiotic resistance by *S. aureus* strains. The data obtained is shown in Table 3.10.

Table 3.10 – Results obtained for *S. aureus* strains susceptibility to free levofloxacin (mean±SD; n=2).
MIC (minimum inhibitory concentration) and MBIC (minimum biofilm inhibitory concentration) values of free levofloxacin against 48 h old biofilms.

Bacteria	MIC (µg/mL)	MBIC (µg/mL)
MSSA	0.50	3.91
MRSA-1	62.5	31.25
MRSA-2	62.5	7.81

The MIC and the MBIC values found were different according to *S. aureus* strains. For planktonic bacteria, the MSSA revealed to be less resistant to levofloxacin than MRSA strains (0.5 µg/mL), this value is in accordance to what is described in literature (MIC ≥ 0.25 µg/mL) [40]. To our knowledge for MRSA strains there is no previous reported data, however the same MIC value (62.5 µg/mL) was observed for both strains.

As expected the MBIC of MSSA is higher than the MIC, indeed an eight-fold increase was observed for bacteria within biofilm when compared to planktonic bacteria. The opposite phenomenon was observed for MRSA strains. A decrease in antibiotic resistance of two-fold and eight-fold was observed for MRSA-1 and MRSA-2, respectively within biofilm in comparison to the planktonic counterparts. Although surprising, this phenomenon has been previously described by other authors who claim that bacteria in biofilm form is not necessarily more resistant than the planktonic form [172]. In some cases, biofilm does not grow better than planktonic bacteria in the presence of a broad range of antimicrobials, which may explain this result. Some authors point three main factors when considering biofilm cell resistance to antibiotics: the presence of a diffusion barrier to antibacterial agents formed by the biofilm, slower cell growth rate, or even expression of certain resistance genes. Some antibiotics, like vancomycin and fluoroquinolones (such as levofloxacin), were shown to freely diffuse into biofilms, bypassing its natural defences [173]. Furthermore, the three *S. aureus* strains exhibited different kinetics of biofilm assembly, which may also explain these results. The less resistant strain (MSSA) showed to be the best biofilm assembler, as previously discussed (section 3.2.1.), this show that biofilm assembly is not an exclusive feature of intrinsically antibiotic resistant bacteria.

Antibacterial activity of an antibiotic can be classified as bacteriostatic or bactericidal. Usually, the action mechanism of bacteriostatic drugs involves blocking a specific metabolic pathway, inhibiting the growth of susceptible bacteria but do not result in bacterial death. Thus, the treatment using this type of drugs entails a major disadvantage: in the absence of the antibiotic, bacteria may resume growth [31], [174]. While the bactericidal agents involve disruption of the cell wall or cell membrane, or interfere with essential bacterial enzymes, leading to bacterial death. Nevertheless, bactericidal activity of an antibacterial agent can be strain dependent, as well as, concentration and time dependent [174]. In order to classify the antibacterial activity of levofloxacin, CFU enumeration of MBIC and twice the MBIC was performed. For MSSA and MRSA-2 an approximately 99 % decrease of CFU counts was observed

(compared to the control). Thus, the MBIC corresponding to the minimum bactericidal concentration (MBC), that is a measure of the concentration at which bacteria are killed by the antibacterial agent [174]. All together these data support the bactericidal activity of levofloxacin. Furthermore, a rapid bactericidal effect against most susceptible microorganisms mediated by interference with nucleic acid synthesis, by inhibition of an essential type II topoisomerase (bacterial DNA gyrase) has been described for fluoroquinolones [2], [39], [41], [174].

3.2.2.2. Levofloxacin released from BC matrices

The next step was the evaluation of antibacterial activity of levofloxacin released from BC matrices. Effluent samples of BC specimens after 24 h of incubation within MH broth at 37 °C were collected (section 2.3.3.2.). Effluent of BCLev2.5% was chosen to obtain a solution with concentration of levofloxacin sufficient to perform this assay. Effluent sample from BCLac matrix was used as control. Since no antibacterial activity was detected, the antibacterial activity observed for BC matrices can be attributed to levofloxacin. The results obtained are shown in table 3.11.

Table 3.11 – *S. aureus* strains susceptibility to levofloxacin released from BC matrices (mean±SD; n=2). MBIC (minimum biofilm inhibitory concentration) values of BCLev2.5% effluent (155.12±1.86 µg/mL of levofloxacin) against 48 h old biofilms.

Bacteria	MBIC (µg/mL)
MSSA	2.42
MRSA-1	19.39
MRSA-2	4.85

The MBIC for MSSA (2.42 µg/mL) is in good agreement with those previously obtained by Matos and colleagues (MBIC ≥ 1.99 ± 0.7 µg/mL), despite the fact that the effluents were obtained in different conditions (7-week levofloxacin released from BCLev2.5%) [40].

For the MRSA strains there is no previous data and the obtained results are 19.39 µg/mL and 4.85 µg/mL, for MRSA-1 and MRSA-2, respectively. The minimal inhibitory concentration obtained for bacteria organized within biofilms is two-fold bellow (one dilution) than those of standard levofloxacin (Table 3.10). This fact shows that levofloxacin does not lose antibacterial activity due to the polymerization reaction of BC. During polymerization a large amount of heat is released that could affects the antibacterial activity of levofloxacin [143].

In order to confirm that levofloxacin released from BC matrices keeps its bactericidal activity, CFU enumeration of MBIC and twice the MBIC was performed. In accordance to standard levofloxacin a decrease of approximately 99 % in CFU counts compared to the control (BCLac) was observed, confirming the bactericidal effect of levofloxacin.

Antibacterial susceptibility results disclosed that levofloxacin released from the BC matrices retained the antibacterial properties against *S. aureus* strains in biofilm form, in agreement to the described in a previous study [40].

3.2.2.3. Levofloxacin-loaded BC matrices

Next the advantage of using levofloxacin released from BC matrices *in situ* was evaluated. It is known that biofilm infections are of difficult treatment due to an increased antibiotic resistance. Furthermore, the presence of medical devices, *e.g.* bone implants, within the body increase the chances of biofilm assembly. Biofilms are assembled on the medical device surface and could generate serious infections known as medical device associated infections [17]. This public health problem should be kept in mind when novel drug delivery systems are developed. The major advantage of using these systems is providing local antibiotic delivery in high concentrations for an extended period of time without exceeding systemic toxicity, and preventing biofilm formation by killing early colonising bacteria [24], [175].

So, antibacterial activity of BC matrices was tested, in direct contact with mature biofilm, which are the most difficult to eradicate (section 2.3.3.3.).

The results revealed that BC matrices loaded with levofloxacin concentrations of 1.5 % or higher exhibited anti-biofilm activity for all *S. aureus* strains tested. More than 90 % decreased in CFU when compared to the control support the bactericidal activity of the tested matrices. The results obtained for lower concentrations of levofloxacin loaded matrices (0.5 and 1.0 %) were strain dependent. For the MSSA even the lowest concentration was effective but for the other strains the results were not reproducible. This fact demonstrates that, in the case of lower concentrations of drug incorporated in BC matrices, the reproducibility of the assays is lower, since the homogenization of antibiotic throughout the BC matrix is much more challenging.

3.2.2.4. Biofilm assembly by scanning electron microscopy (SEM)

Concerning the need for anti-biofilm approaches, the next step was evaluating the ability of BC matrices to prevent biofilm assembly (section 2.3.4.). The ability of *S. aureus* to assemble biofilm on BC matrices loaded with either 0.5 or 2.5 % levofloxacin was evaluated by SEM. The assay was conducted with low (BCLev0.5%) and high (BCLev2.5%) levofloxacin-loaded BC to observe the differences between doses. For this assay, MSSA and MRSA-1 were chosen for their higher biofilm formation ability (section 3.2.1.).

As expected, SEM images (Figure 3.4) revealed differences in biofilm assembly between strains and BC matrices. The MSSA on BCLac surface (control) (Figure 3.4. A) revealed biofilm assembly, as extracellular matrix between bacteria was observed. In the case of reference strain MRSA-1 in any of BC matrices surface (Figure 3.4. D/E/F), only adherent bacteria without extracellular matrix between them were observed. However, SEM images with MSSA (Figure 3.4. A/B/C) showed that levofloxacin-loaded BC plates reduced the biofilm assembly even at low concentrations of levofloxacin. Furthermore, a reduction of adherent bacteria in both strains was observed with the increase of levofloxacin concentration incorporated in BC plates. In order to confirm this result, enumeration of CFU on BC matrices surface would be necessary, however it would be difficult to execute, so the analysis was done only by SEM.

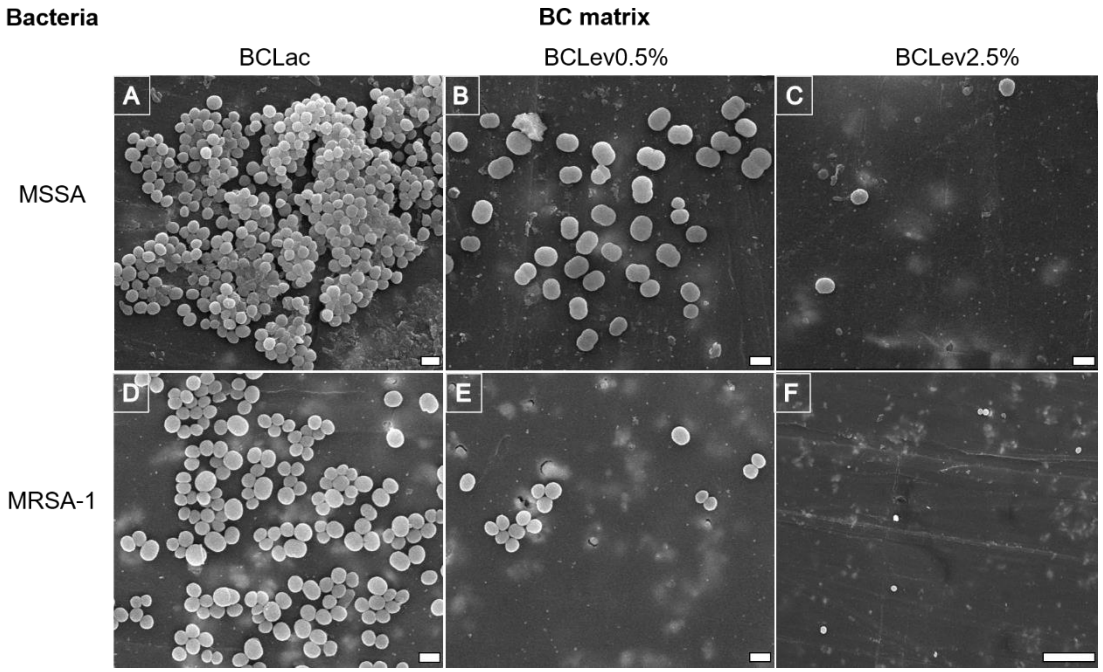

Figure 3.4 – SEM images of biofilm assembly.
A) MSSA on BCLac (control); B) MSSA on BCLev0.5%; C) MSSA on BCLev2.5%; D) MRSA-1 on BCLac (control); E) MRSA-1 on BCLev0.5%; F) MRSA-1 on BCLev2.5%.
(scale bars = 1 µm)

3.3. Antibacterial intracellular activity

Another important health concern with *S. aureus*, besides biofilm formation, is its ability to invade and persist within osteoblast cells for long periods of time, classifying this bacterium as a facultative/ opportunistic intracellular pathogen [8], [19], [152]. The persistence and recurrence of infection may be explained by intracellular presence of the bacteria [152]. This behaviour was implied as an immune-evasion strategy to escape host defence mechanisms, such as bacterial recognition by professional phagocytes, antibodies and cationic peptides. Additionally, intracellular persistence also impairs an effective treatment using several classes of antibiotics [21], [176]. Furthermore, an intracellular target for antibacterial therapy is more complex than an extracellular target, due to intracellular antibacterial activity further depending on the penetration into and accumulation within the cell, cellular metabolism, the subcellular distribution, and the bioavailability of the antibiotic [152]. In this scope, it is important to evaluate the activity of conventional and novel antibiotic delivery systems against intracellular bacteria.

3.3.1. Intracellular distribution of *S. aureus*

Primarily, the *S. aureus* ability to invade osteoblast cells and their intracellular distribution was evaluated by confocal microscopy. The human osteoblast infection models were used, with MSSA and MRSA-2 strains (section 2.4.2.), being representative images shown in Figure 3.5. As control, uninfected MG63

cells were used (Figure 3.5. A). Both *S. aureus* strains revealed ability to be phagocytized by osteoblast cells (Figure 3.5. B/C). However, differences in intracellular distribution between strains were observed. MRSA-2 strain (Figure 3.5. C) revealed an increased ability to invade cells than MSSA (Figure 3.5. B).

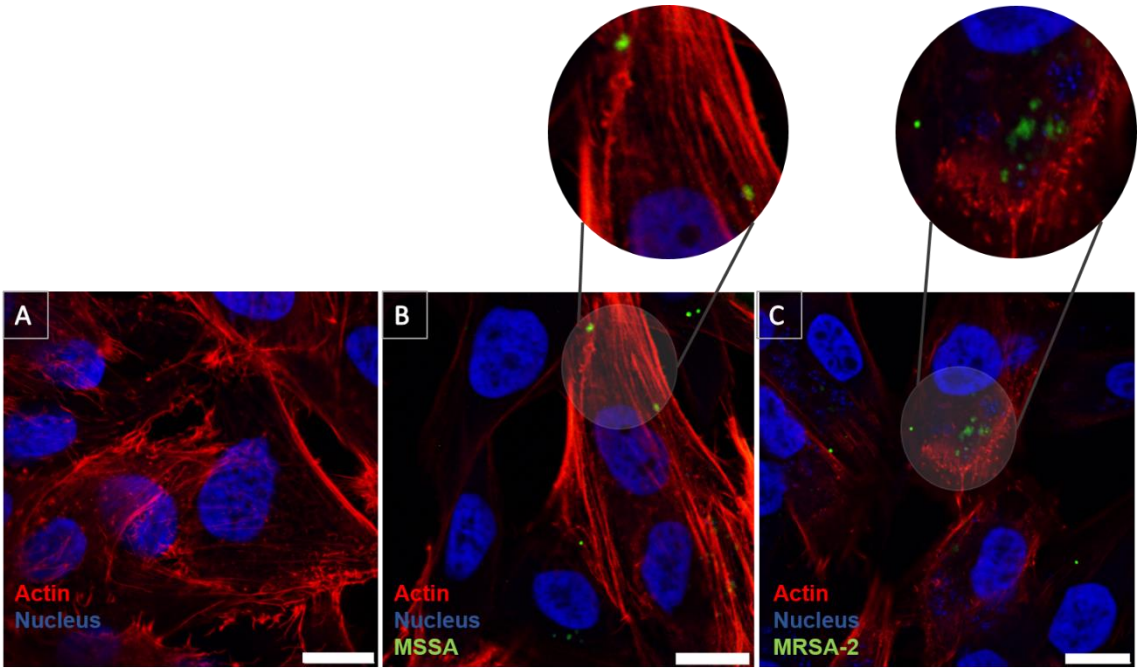

Figure 3.5 **–** Intracellular distribution of *S. aureus*.
Red: actin cytoskeleton of cells; Blue: nucleus of cells; Green: *S. aureus* strains.
A) MG63 cells (control; uninfected); B) MG63 cells infected with reference strain MSSA; C) MG63 cells infected with clinical isolate MRSA-2.
Scale bars = 100 µm

3.3.2. Acrylic bone cement

Aiming to evaluate the antibacterial intracellular activity of levofloxacin-loaded BC plates a bacterial survival assay was performed (section 2.4.1.1.). In this assay, human osteoblasts were infected with different *S. aureus* strains. The low (BCLev1%) and high (BCLev2.5%) levofloxacin-loaded BC activity against intracellular bacteria was tested.

The data obtained for intracellular MSSA and MRSA strains survival are shown in Figures 3.6 and 3.7, respectively.

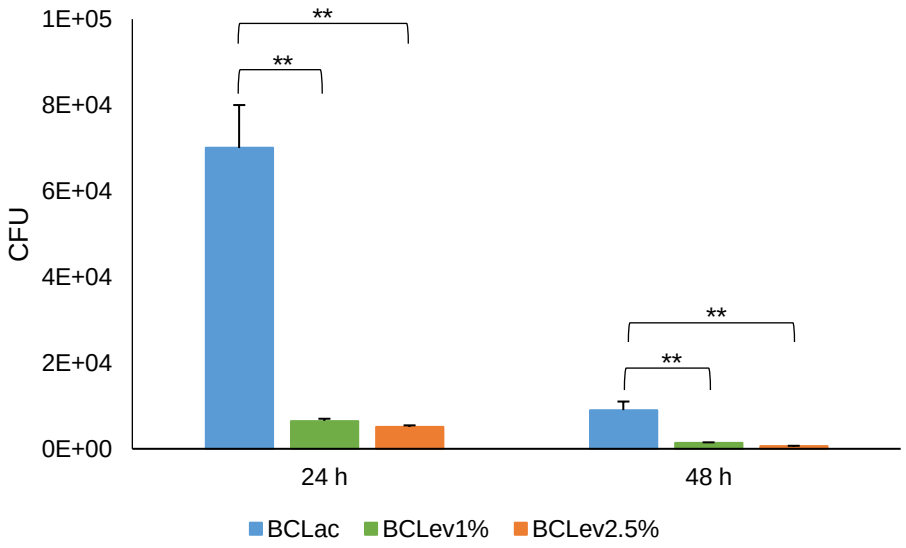

Figure 3.6 – *In vitro* survival assay of intracellular MSSA.
Human osteoblast infection model (MOI= 25 bacteria/cell) after 24 and 48 h of incubation with BCLac (blue bar), BCLev1% (green bar) and BCLev2.5% (orange bar) (mean±SD; n=3). Note: **, significantly different ($p < 0.01$).

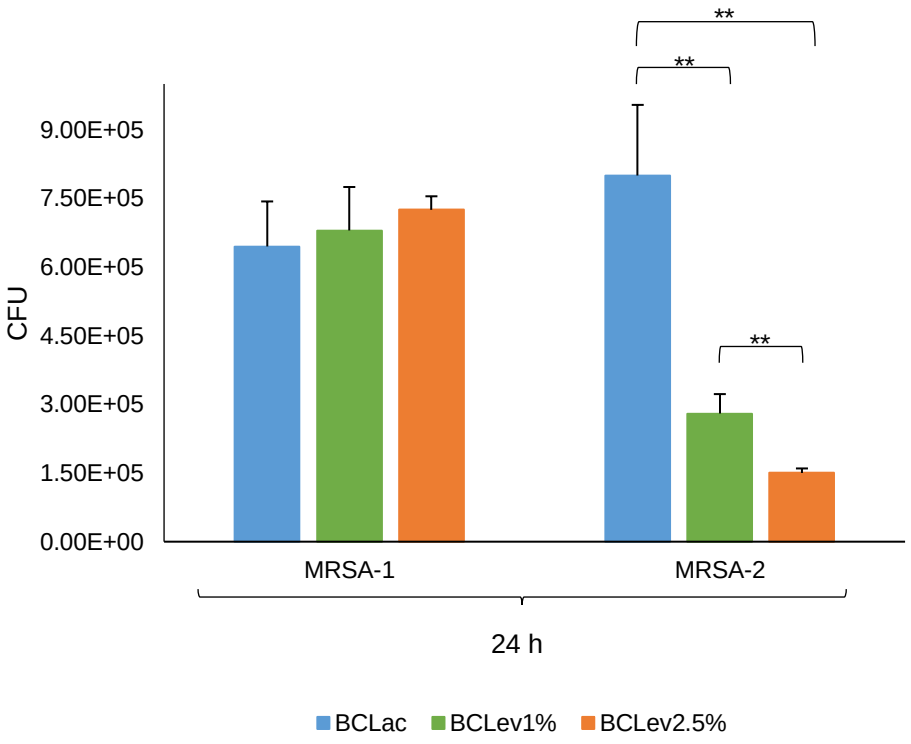

Figure 3.7 – *In vitro* survival assay of intracellular MRSA strains.
Human osteoblast infection model (MOI= 25 bacteria/cell) after 24 h of incubation with BCLac (blue bar), BCLev1% (green bar) and BCLev2.5% (orange bar) (mean±SD; n=3). Note: **, significantly different ($p < 0.01$).

The obtained results show different responses to BC matrices for the tested *S. aureus* strains. However, in all cases, in absence of BC matrix, *S. aureus* effectively invades, survives, and proliferates within

human osteoblast cells, as previously described [21], [176]. This event seems to be unaffected by the presence of BCLac, which does not prevent *S. aureus* proliferation. The number of intracellular bacteria progressively increased until 24 h post-infection. However, MSSA demonstrated an intracellular growth decline at 48 h post-infection. In the case of MRSA strains, containing the extracellular replication of bacteria at 48 h post-infection following the procedure described in section 2.5.1.1 was not possible, as bacteria replication continued even after treatment with lysostaphin and gentamicin. Therefore, results from intracellular MRSA strains survival at 48 h post-infection were not obtained.

The Human osteoblast cells infected with MSSA at both infection times reveal a significant decrease of viable intracellular bacteria that were exposed to levofloxacin-loaded BC matrices when compared to those who were in contact with BCLac (control). However, no significant differences between doses were observed (BCLev1% and BCLev2.5%). In the case of infection with MRSA-1, no statistical differences were observed between levofloxacin-loaded BC matrices and control (BCLac). This indicates that concentrations of levofloxacin released from BC matrix were not sufficient to kill intracellular bacteria. The data from infection with MRSA-2 (clinical isolate) revealed statistical differences between BC matrices. When compared to BCLac, a clear reduction of viable intracellular bacteria was observed.

Data suggests that levofloxacin released from BC matrices could penetrate the cell membrane of osteoblast cells and kill MSSA and MRSA-2 strains in the intracellular environment, but not MRSA-1 strain, since it proved to be the most resistant to the drug used. Furthermore, regarding the clinical isolate strain (MRSA-2), the effect is dose-dependent.

3.3.3. Polymeric microparticles

Bacterial survival assay was also performed in order to evaluate the antibacterial intracellular activity of vancomycin- and daptomycin-loaded microparticles. Empty microparticles were used as controls (section 2.4.1.2.). Human osteoblast infected with MSSA were treated with three different concentrations of particles (0.5, 1.0 and 2.0 mg/mL) being intracellular activity accessed at 24 h post-infection. For the assay with PMMA-EUD-Dapto a supplement of (50 mg/L) calcium was added to assure daptomycin antibacterial activity. Calcium ions are required for daptomycin uptake by bacterial cells [177].

The results obtained for PMMA-EUD (empty) and PMMA-EUD-Vanco microparticles are shown in Figure 3.8.

Regarding to human osteoblast infection model exposed to PMMA-EUD-Vanco, the results demonstrated different behaviours for each condition. The test with the lower concentration of vancomycin-loaded microparticles (0.5 mg/mL) is the only one that showed a decrease of viable intracellular bacteria in relation to control (empty PMMA-EUD in the same concentration). The other two conditions (2 and 1 mg/mL of particles) demonstrated the opposite (increase of intracellular bacteria in relation to the control). Particle aggregation at higher concentrations might occur, inhibiting the release of drug and therefore its antibacterial activity. However, it is also important to take into account that EE

(%) of PMMA-EU- Vanco is relatively low (section 3.1.2.1) and further in the previous study by Ferreira and colleagues was revealed that no significant release of vancomycin was detected for PMMA-EUD formulation after 24 h of drug liberation (in PBS solution) [16]. The proliferation of bacteria also appears to be influenced by the presence of vancomycin-loaded particles in these concentrations. It was possible to observe a slight increase in number of intracellular bacteria at 24 h post-infection, when exposed to highest concentrations of PMMA-EUD-Vanco (2 and 1 mg/mL), in relation to controls in the same concentration. This increase presented a statistically significant difference in case where 1 mg/mL of loaded microparticles was used.

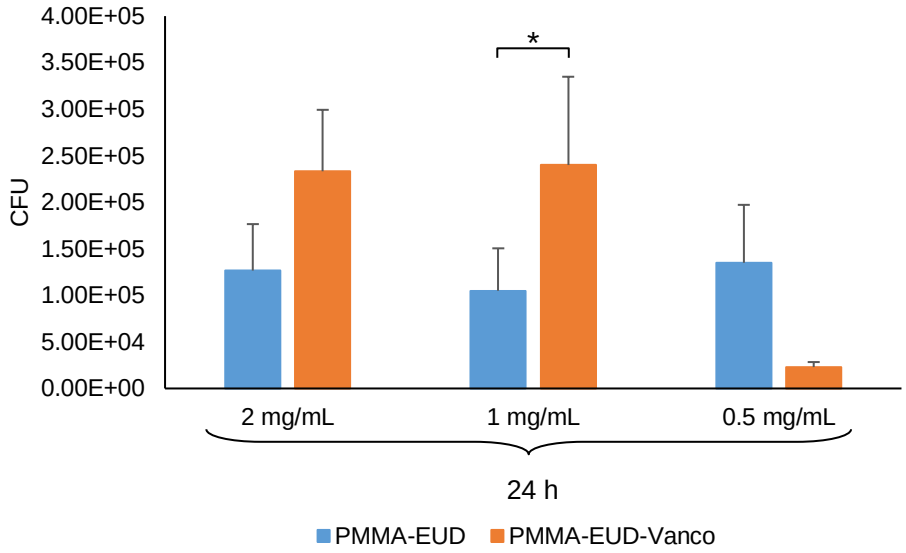

Figure 3.8 **–** *In vitro* survival assay of intracellular MSSA.
Human osteoblast infection model (MOI= 25 bacteria/cell) after 24 hours of incubation with different concentrations of PMMA-EUD (blue bar) and PMMA-EUD-Vanco (orange bar) (mean±SD; n=3). Note: *, significantly different ($p < 0.05$).

The most interesting result was obtained for PMMA-EUD-Dapto intracellular bacteria growth since after 24 h exposure to these microparticles, independently of the concentration used, intracellular *S. aureus* were eradicated. More assays must be performed to confirm this preliminary result, but it could be explained by previously published data by Ferreira and colleagues. The authors characterized daptomycin release from the formulation used in the present work after 24 h incubation in PBS. Daptomycin concentrations in PBS was always higher than the experimentally determined MICs and MBCs values in the same study (both 0.25 µg/mL for MSSA and MRSA strains) [16]. Thereby, this data show that PMMA-EUD-Dapto microparticles are able to release enough drug to achieve intracellular concentrations effective against the *S. aureus* strains tested. On the other hand, microparticles of PMMA-EUD with vancomycin were found not to be a good choice for application in treatment of bone infection. Although results are preliminary, requiring confirmation and optimization of the method, they revealed to be very promising.

Chapter 4. Conclusion and future work

The main objective of this work is the evaluation of microbiological and intracellular activity of novel acrylic polymeric systems, namely bone cement and microparticles composed mainly of polymethylmethacrylate (PMMA). Levofloxacin (Lev) was the selected antibiotic for bone cement formulations, and daptomycin (Dapto) and vancomycin (Vanco) were chosen for microparticles-loading. The selected antibiotics show a high anti-staphylococcal activity and high rate of antibiotic penetration in bone tissues. These novel drug delivery systems were developed as a possible solution for treatment of bone infection, the Lev-loaded BC for application in an initial stage of the disease, and Dapto- and Vanco-loaded MP for application in an advanced stage. Indeed, a main innovative aspect of this thesis work is the evaluation of the antibacterial intracellular activity of both systems. Once the recrudescent and chronic infections have been associated with *S. aureus* ability to invade and persist within osteoblast cells, an increase of concern in terms of public health occurred.

In this context, the present work was started by preparing and characterizing different drug delivery systems namely antibiotic-loaded bone cement and microparticles.

Surface characterization of BC specimens, by contact angle and surface energy determination, demonstrated that the additives used did not incurred in significant changes in surface energy values, which lead to the conclusion that biocompatibility behaviour was not compromised. Moreover, the BC matrices tested showed a tendency for hydrophobic behaviour. Encapsulation efficiency of MP revealed that daptomycin encapsulation was significantly higher than vancomycin.

Drug release studies of Lev-loaded BC formulations show differences in the amount of levofloxacin liberated from bone cement matrices, which is size- and composition medium-dependent. Although the assays with smaller plates reveal lower reproducibility, a slight decrease of levofloxacin release with the increase of BC plate dimensions was observed. This lower reproducibility is associated with the preparation method of BC that does not allow the correct homogenization of drug across the plate. Regarding the incubation media, in MH broth and PBS solution the values of drug release were similar while in RPMI medium the values were lower. However, a different assay with RPMI medium collected from an intracellular assay was performed and the results were already similar to those obtained for others incubation media. Nevertheless, the drug release is low compared to initial loaded levofloxacin. Furthermore, the fluorescence method proved to be a good option to quantify the released levofloxacin from BC matrices, in place of High Performance Liquid Chromatography (HPLC). Once lactose was incorporated in the bone cement formulations as a release enhancer, the lactose liberated from these was also determined, and a significant amount of this compound was quantified.

The antibacterial activity of BC specimens was also evaluated against three different *S. aureus* strains. The results of MBIC of free levofloxacin shown to be surprising, as in MRSA strains a decrease in antibiotic resistance was observed, contrary to what would be expected. However, strains studied exhibit low levels of biofilm assembly. BC matrices loaded with Levofloxacin concentrations of 1.5 % or higher exhibited anti-biofilm activity for all *S. aureus* tested strains, but the results for lower concentrations of

Lev-BC matrices (0.5 and 1.0 %) were strain dependent. Through SEM images it was possible to realize that only MSSA showed biofilm formation on BC surfaces, as MRSA-1 showed only adherent bacteria. However, Lev-BC plates reduced the adherent bacteria, as well as the biofilm assembly even at low Lev-loaded concentrations. In general, throughout the microbiological assays, the BC formulations tested presented significant biofilm inhibitory activity, mainly the BC formulation loaded with levofloxacin concentrations of 1.5 % or higher (BCLev1.5%; BCLev2.5%). It was also possible to conclude that levofloxacin did not lose its antibacterial activity due to polymerization reaction during bone cement preparation.

Concerning antibacterial intracellular activity assays, firstly it was possible to verify that *S. aureus* strains (MSSA and MRSA-1) have the ability to invade and persist in human osteoblast cells. In this assay, the tested BC formulations (BCLev1% and BCLev2.5%) showed to be effective against intracellular MSSA and MRSA-2, since a significant decrease of viable intracellular bacteria was observed. Nevertheless, the same assay was ineffective against MRSA-1. Microparticles were tested only against osteoblast infection model infected with MSSA strain, however the results have proven to be very promising. PMMA-EUD-Dapto microparticles presented a high efficacy against intracellular MSSA, as no viable intracellular bacteria were observed. However, the results for vancomycin-loaded microparticles (PMMA-EUD-Vanco) demonstrated inconsistency, due to a lower amount of drug released from the particles, as determined in a previous study.

Although vancomycin is quite used in the treatment of bone infection, the increase of vancomycin-resistant strains is a serious concern. In this way, daptomycin comes in as a new approach to replace vancomycin. The development of such a drug delivery system, ensuring the local release of higher doses of drug without exceeding the levels of toxicity, is an added value in the treatment of these infections.

Further studies must be evaluated to better understand these novel drug delivery systems and their capacity and success in terms of antibacterial activity for application in the treatment of bone infection. Despite the numerous results obtained, some are still requiring additional data, which, by variety of reasons such as time, available reagents and equipment or even high cost-associated assays, was not obtained.

In the case of release studies much is yet to be assessed, such as assays with more incubation times, in different incubation media used in microbiological assays to better understand the drug release behaviour from the biomaterial carrier. It is also important to improve the release of levofloxacin from polymeric BC, since the incorporation of lactose seems not to be enough to release high levels of this drug, due to the interaction of levofloxacin with BC. Therefore, other release modulators should be tested. Furthermore, the influence of lactose in microbiological and intracellular assays should be more extensively studied. The improvement of drug homogenization throughout the BC plate should also be considered in future work. An optimization of the biofilm assembly method should also be taken into account, since the results were very low when compared with other biofilm producer bacteria responsible for healthcare associated diseases (HAIs), which may have influenced the results of other microbiological tests. In future studies, the culture medium can be supplemented with glucose in order

to optimize biofilm assembly, and the remaining microbiological assays should then be repeated. The antibacterial intracellular activity assays were still preliminary, mainly in terms of tests with MP. The number of survival assays should be increased for both systems, in order to evaluate the reproducibility and reliability of the method. Regarding this assay using bone cement formulations, a new assay should be performed using MRSA-1 and either higher concentration of levofloxacin or a different antibiotic that can be effective against all three strains should be tested. For tests with microparticles it will be required to perform the assay against osteoblast infection model infected with MRSA strains, namely with PMMA-EUD-Dapto. In this assay, it is also important to verify if there are MP aggregates, which may be masking the obtained results. This can be made by microscopic observation.

Looking back at all the assays performed and data collected, it may be concluded that the BC formulations with levofloxacin concentrations of 1.5 % or 2.5% revealed more potential for clinical application than BC matrices with lower concentrations of drug, regarding both microbiological and intracellular assays. Furthermore, the Dapto-loaded MP were very effective, demonstrating enormous potential for further studies and applications. So, in conclusion, this work suggets that bone infection treatment using local drug delivery systems with antibiotics capable of penetrating eukaryotic cells after orthopaedic surgery could prove beneficial in reducing the rate of postoperative bone infection.

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Chapter 6. Annex

A – Contact angle and surface energy determination

Table 6.1 – Results obtained for determination of contact angle and surface energy of the BC matrices.
Values of BC matrices for total (γ), polar (γ^p) and dispersive (γ^d) surface energy (mean±SD; n=6).

BC matrix	γ^d (mN/m)	γ^p (mN/m)	γ (mN/m)
BCLac	18.4±1.0	16.4±1.8	34.8±1.6
BCLev0.5%	18.5±0.4	17.3±0.6	35.8±0.8
BCLev1%	18.9±1.1	18.2±1.5	37.2±1.2
BCLev1.5%	18.5±0.6	17.6±0.9	36.1±0.8
BCLev2.5%	18.0±2.7	16.9±0.9	34.9±2.2

B – *In vitro* release studies

- **Levofloxacin release assays in PBS**

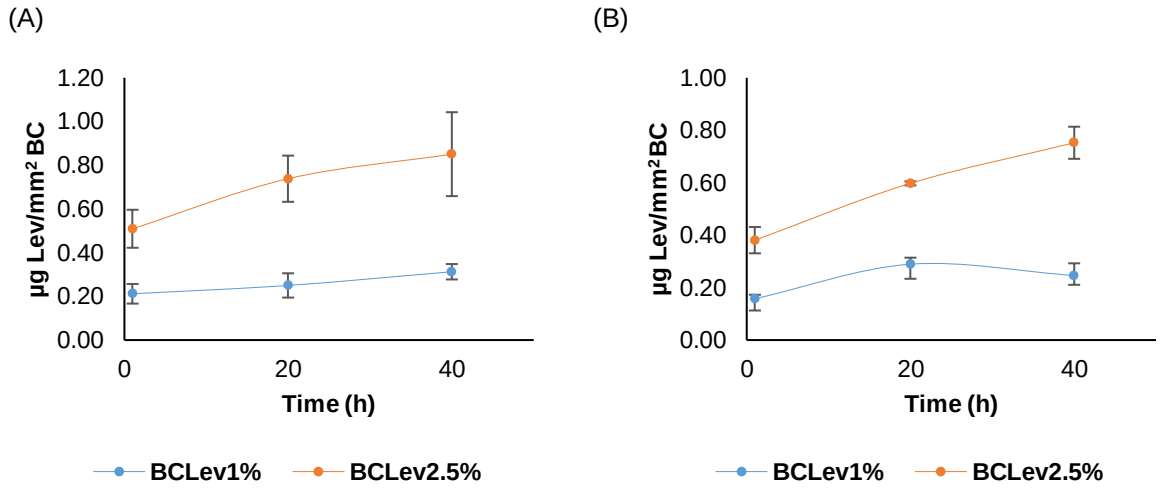

Figure 6.1 – *In vitro* accumulative release profiles of levofloxacin.
BCLev1% (blue line) and BCLev2.5% (orange line), at 40 hours of incubation with PBS solution (mean ± SD; n=6). Measurements were performed by HPLC, at time-points of 1 h, 20 h and 40 h. results are expressed as µg of levofloxacin released per mm^2 of BC matrix (A) 96-well plates; (B) 24-well plates.

- **Levofloxacin release assays in culture media**

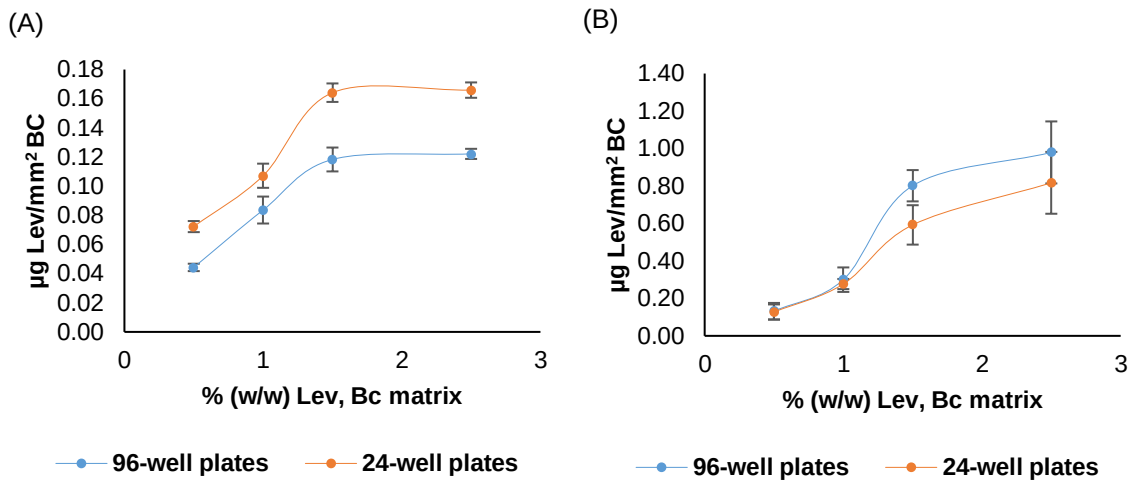

Figure 6.2 – *In vitro* release of levofloxacin.
BCLev0.5%, BCLev1%, BCLev1.5% and BCLev2.5%, at 24 hours of incubation with 96- (blue line) (mean ± SD; n=6) and 24-well plate (orange line) (mean ± SD; n=3), by fluorescence measurement. Results are expressed as µg of levofloxacin released per mm^2 of BC matrix. (A) Incubation with RPMI medium; (B) Incubation with MH broth.

- **Release of lactose**

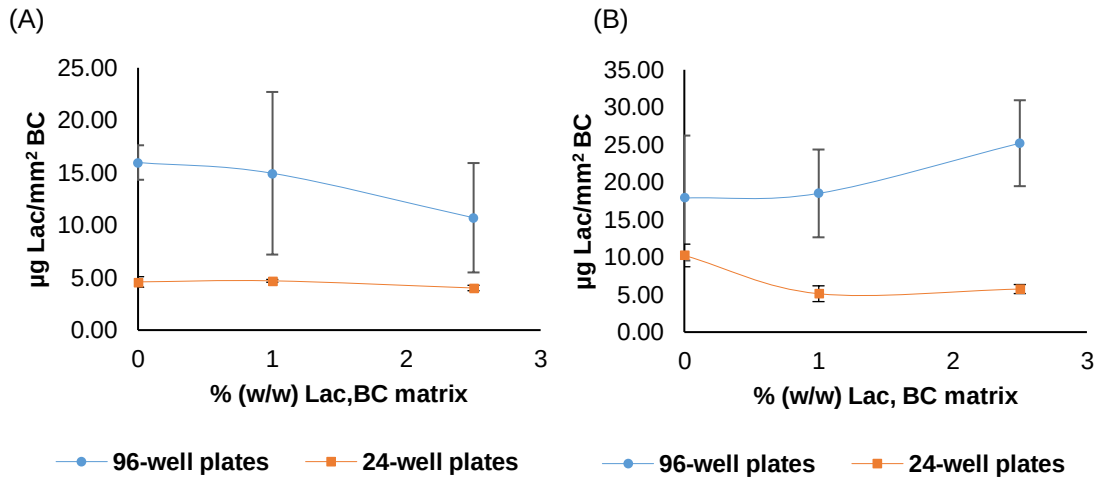

Figure 6.3 – *In vitro* release of lactose.
BCLev0.5%, BCLev1%, BCLev1.5% and BCLev2.5%, at 24 hours of incubation with 96- (blue line) and 24-well plate (orange line), by DNS method. Results are expressed as µg of lactose released per mm^2 of BC matrix. (A) Incubation with PBS (mean ± SD; n=3); (B) Incubation with MH broth (mean ± SD; n=6).

C – Composition of culture media

Table 6.2 – Composition of MH broth.

Compounds	g/L Purified water
Acid hydrolysate of casein	17.5
Beef extract	3.0
Starch	1.5

Table 6.3 – Composition of RPMI 1640 medium.

Compounds	mg/L
Sodium Bicarbonate	2.000E+03
Sodium Chloride	6.000E+03
Ca(NO3)2.4H2O	100.000
Choline Chloride	3.000
D-Biotin (Vitamin H) (00129)	0.200
D-Calcium Pantothenate (Vitamin B5)	0.250
D-Glucose anhydrous	2.000E+03
Folic Acid	1.000
Glutathione Reduced	1.000
Glycine	10.000
Potassium Chloride	400.000
L-Arginine Hydrochloride (00095)	241.860
L-Asparagine Monohydrate	56.810
L-Aspartic Acid	20.000
L-Cysteine Dihyrohydrochloride	65.190
L-Glutamic Acid	20.000
L-Histidine Monohydrochloride Monohydrate	20.270
L-Hydroxyproline	20.000
L-Isoleucine	50.000
L-Leucine	50.000
L-Lysine Monohydrochloride	40.000
L-Methionine	15.000
L-Phenylalanine	15.000
L-Proline	20.000
L-Serine	30.000
L-Threonine	20.000
L-Tryptophane	5.000
L-Tyrosine Disodium Salt, Dihydrate	28.830
L-Valine	20.000
Magnesium Sulfate Anhydrous	48.830

Myo-Inositol	35.000
Sodium Phosphate Dibasic, Anhydrous	800.490
Niacinamide (Nicotinamide)	1.000
P-Aminobenzoic Acid	1.000
Phenol Red	5.100
Pyridoxine Monohydrochloride	1.000
Riboflavin (Vitamin B2)	0.200
Thiamine Monohydrochloride (Vitamin B1)	1.000
Cyanocobalamin (Vitamin B12)	5.000E-03