Sofia Miguel dos Reis Pinto da Silva

Licenciada em Bioquímica

Novel antibiotic-loaded orthopaedic bone cements: insights on drug release profiles and biocompatibility

Dissertação para obtenção do Grau de Mestre em Bioquímica

Orientadora: Ana Bettencourt, Professora Doutora, Faculdade de Farmácia, Universidade de Lisboa
Co-orientadora: Lídia Gonçalves, Professora Doutora, Faculdade de Farmácia, Universidade de Lisboa

Júri:

Presidente: Prof. Doutor Pedro Tavares
Arguente(s): Prof. Doutora Isabel Ribeiro
Vogal(ais): Prof. Doutora Ana Bettencourt

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

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Acknowledgements

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Abstract

Acrylic bone cement (BC) is widely used as an anchor of artificial joints. Bacterial infection due to biofilm formation and inflammation are common and difficult to treat problems associated with commercial available BC formulations. Research on novel BC compositions is urgently needed.

The main objective of this thesis was to develop a new biocompatible antibiotic-loaded BC with improved release profile. To achieve that aim several additives were incorporated, as an antibiotic (levofloxacin) to combat bacterial growth, an anti-inflammatory drug (diclofenac) to decrease the inflammatory process and two well-known and broadly used biopolymers, alginate and chitosan in order to increase matrix porosity, and in this way to intensify the amount of released drug.

Novel BC formulations were tested in order to find the most suitable one that had potential to proceed to clinical application. Numerous tests were conducted as: a) evaluation of drug release profiles in different biomimetic media, b) mechanical and surface studies, c) microbiological activity testing against *Staphylococcus aureus* and d) *in vitro* biocompatibility assays (fibroblasts and osteoblasts).

In general, the addition of biopolymers increased drug release, didn't compromised BC mechanical properties and increased BC hydrophilicity. Microbiological testing revealed that Lev[BC]Chi was the only matrix that reduced significantly biofilm formation. On the contrary, alginate and diclofenac loading into BC seemed to increase biofilm growth. Biocompatibility studies showed some decrease in cell viability, in particularly on osteoblasts, mainly due to the high amounts of released drugs.

In conclusion, the present work has shown that the matrix with more potential to proceed in further investigations was Lev[BC]Chi. Other conditions (namely additives and drugs concentrations) should be evaluated with the other tested BC matrices before being discharged.

Keywords: Bone cement; levofloxacin; diclofenac; biocompatibility; biofilm; biopolymers.

Resumo

Os cimentos acrílicos (BC) têm sido extensamente utilizados para preenchimento ósseo e fixação de próteses ortopédicas. Infeções bacterianas associadas à formação de biofilme e inflamação são complicações comuns e de difícil tratamento que podem ocorrer aquando da utilização das formulações atualmente disponíveis de cimentos acrílicos. A investigação para desenvolvimento de novas composições de cimentos é urgente e pertinente.

O principal objetivo desta tese foi o desenvolvimento dum novo cimento ósseo que apresentasse propriedades superiores aos atualmente existentes designadamente o perfil de libertação de fármacos. Para tal alguns aditivos foram incorporados, tais como, um antibiótico (levofloxacina) para inibir o crescimento bacteriano, um anti-inflamatório (diclofenac) para diminuir o processo inflamatório e ainda dois biopolimeros, o alginato e o quitosano, com o intuito de aumentar a porosidade da matriz e assim intensificar a quantidade de fármacos libertada.

As novas formulações de cimento foram testadas com o objetivo de selecionar a mais adequada com potencial para aplicação clínica. Foram realizados diversos testes: a) ensaios de libertação em vários meios biomiméticos, b) ensaios mecânicos e de superfície c) determinação da atividade microbiológica contra *Staphylococcus aureus* e d) ensaios de biocompatibilidade (fibroblastos e osteoblastos).

Em geral, a adição dos biopolímeros ao BC aumentou a libertação dos fármacos, não comprometeu as propriedades mecânicas mas aumentou a hidrofilicidade das superfícies. Os ensaios microbiológicos revelaram que a matriz Lev[BC]Chi foi a única que diminui significativamente a quantidade de biofilme formado. Pelo contrário, alginato e diclofenac pareceram aumentar a quantidade de biofilme. Os estudos de biocompatibilidade demonstraram alguma diminuição na viabilidade celular, particularmente nos osteoblastos, especialmente devido às elevadas quantidades de fármacos libertados.

Em conclusão, o presente trabalho demonstrou que a matriz com mais potencial para prosseguir em futuras investigações é a Lev[BC]Chi. Outras condições devem ser reavaliadas com as outras matrizes de cimento antes de serem consideradas totalmente inadequadas.

Palavras-chave: cimento ósseo; levofloxacina; diclofenac; biocompatibilidade; biofilm; biopolimeros.

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List of Abbreviations

Alg – Alginate
ATCC - American Type Culture Collection
BC – Bone Cement
BHI – Brain Heart Infusion
BSE - Back-Scattered Emission
CFU - Colony Forming Unit
Chi – Chitosan
CLSI - Clinical and Laboratory Standards Institute
CV - Crystal Violet
Diclo – Diclofenac
DMSO - Dimethylsulfoxide
EDS - Energy Dispersion Spectroscopy
FBS - Fetal Serum Bovine
FDA – Food and Drug Administration
HKAI - Hip or Knee Arthroplasty Infections
HMW – High Molecular Weight
HPLC - High Performance Liquid Chromatography
ISO - International Organization for Standardization
Lev – Levofloxacin
LMW – Low Molecular Weight
MHB - Mueller-Hinton Broth
MBIC – Minimum Biofilm Inhibitory Concentration
MIC – Minimum Inhibitory Concentration
MMA – Methyl methacrylate
MMW – Medium Molecular Weight
MRSA - Methicillin-Resistant *Staphylococcus aureus*
MRSE - Methicillin-resistant *Staphylococcus epidermis*
MTT - 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-Tetrazolium Bromide
NPs – Nanoparticles
PBS – Phosphate Buffered Saline
PDI – Polydispersive Index
PMMA – Poly(methyl methacrylate)
SEM – Scanning Electron Microscopy
SD – Standard Deviation
SDS – Sodium Dodecyl Sulphate
SSI - Surgical Site Infections
TEA - Triethylamine

TPP - Tripolyphosphate Pentasodium

TSA - Tryptone Soya Agar

TSB - Tryptic Soy Broth

TTC - 2,3,5-triphenyl tetrazolium Chloride

UV – Ultraviolet

VRE - Vancomycin-Resistant *enterococci*

Z-Ave – Average size

ZP – Zeta potential

Objectives and Thesis Structure

The thesis main objective was to develop a novel biocompatible antibiotic-loaded bone cement with improved drug release profile.

Specific aims were:

- Development of novel bone cements formulations by incorporation of drugs as levofloxacin and diclofenac as well as porogens namely chitosan and alginate;
- Evaluation of the effect of the additives in *in vitro* drug release profiles;
- Assessment of microbiological activity on the new bone cement formulations;
- Evaluation of bone cement biocompatibility by *in vitro* cellular assays.

The thesis is structured in four chapters including: *Introduction*, *Materials and Methods*, *Results and Discussion* and *Conclusions and Future Work*.

Chapter 1 – *Introduction*

Contains a brief clinical context of the current use of polymethylmethacrylaye (PMMA) bone cement and the problems involving its application in cemented prosthetic implants. In particularly, the adverse reactions triggered by the bone cement implantation like inflammation and bacterial infections are summarized. Novel approaches under research including the incorporating of new additives to the cement are also described. Finally the tests that should be conducted to evaluate the potential for a novel bone cement to be clinically used, namely drug release, surface, mechanical, microbiological and biocompatibility studies, are briefly described.

Chapter 2 – *Materials and Methods*

First chapter describes nanoparticles preparation and characterization. Second chapter details all *in vitro* drug release experiments. Third chapter focus on mechanical and surface studies. Fourth and fifth chapters describe the microbiological assays and the *in vitro* cellular studies, respectively. All the reagents, materials, equipment, methods and assay conditions used in this work are presented in detail.

Chapter 3 – *Results and Discussion*

This chapter is also subdivided and presents the results and respective discussion about all the assays concerning nanoparticles preparation and characterization, drug release profiles from different bone cement matrices, followed by a detailed discussion of the bone cement effects on microbiological activity and biocompatibility profiles. Whenever available, a comparison is made between the obtained results consistency and the existent literature over the evaluated subjects on this work.

Chapter 4 – *Conclusions and Future Work*

In this chapter, the main conclusions of the project are summarized and a reflection is made over the possibilities for future work, as well as possible improvements on the established methodologies on this thesis.

Chapter 1. Introduction

1.1. The Clinical Context

Over the last decades a large number of researchers across the world have been committed to discover solutions to the major health problems affecting society. These studies led to an increase in human life quality and average life expectancy. One of the most promising medical areas involves orthopaedic procedures to restore function lost as a result of injury or disease of bones, joint, muscles, tendons, and ligaments. For example hip and knee arthroplasty (Fig. 1) are a highly effective type of intervention that significantly improves patient's quality of life, providing symptom relief, restoration of joint function, improved mobility and independence (*Grammatico-Guillon et al*., 2008).
However, associated to these successful surgical procedures are complications as surgical site infections (SSI), which can become very costly, due to prolonged hospital stays, complicated treatment protocols and frequent readmissions; in these cases hip or knee arthroplasty infections (HKAIs) remains one of the most serious complications of prosthetic joint implantation. The treatment to these infections often involves surgical intervention and prolonged courses of intravenous or oral antimicrobial therapy (*Grammatico-Guillon et al*., 2015; *Osmon et al.,* 2012 and *Uckay et al.,* 2013).
In recent years, surgical techniques were improved by adopting efficient anti-septic pre-operative and intra-operative procedures, as special enclosure using laminar flow and systemic antibiotic prophylaxis, that lead to a significant reduction of deep infections; however in hip and knee arthroplasty the presence of a biomaterial involves a high risk of developing severe bone infections once bacteria adheres to biomaterial surface and forms biofilm of that bacterial strain (*Bistolfi et al*., 2011).
Gram-positive pathogens (including *Staphylococcus aureus* and coagulase-negative staphylococci (CoNS) are the most common organisms implicated in peri-prosthetic joint infection (*Chang et al.,* 2013).

Once HKAIs are among the most difficult to manage, and with the spread of these type of surgeries in recent years, it is imperative to find new solutions to keep the number of infections at the very low rates and also to prevent total removal of the implant.

Figure 1 – Schematic representation of joint replacements in knee and hip (adapted from http://www.ciosortho.com/joint-pain/).

One approach that is used to reduce the incidence of joint infections is to add antibiotics to the bone cements (BC) used to fix the implants in cemented arthroplasties (*Bistolfi et al*., 2011). These types of bone cements will be detailed in the next sections.

1.2. Bone Cement and bone infections

Poly(methylmethacrylate) (PMMA) bone cement (BC) has been widely used for implant fixation and trauma surgery and acts as a space-filler that creates a tight space which holds the implant against the bone (*Bettencourt et al.,* 2012). Its primary function is to transfer load from bone to prosthesis.

PMMA is an acrylic polymer (Fig. 2) and it is one of the most widely explored biomaterials because of its biocompatibility, versatility and low cost. Besides its use as a bone cement, it is also applied as spacer, bead and in dental applications with a high rate of success (*Vaishya et al.*, 2013).

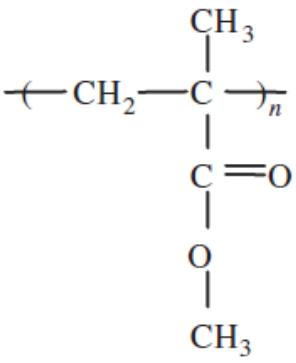

Figure 2 – Chemical structure of PMMA repeating unit.

PMMA bone cement is formed by *in situ* polymerization, mixing two sterile components, a liquid monomer – methylmethacrylate (MMA) - and a powered PMMA polymer. When the two components are mixed, the liquid monomer polymerizes around the pre-polymerized powder particles to form hardened PMMA. In this process, heat is generated, due to an exothermic reaction reaching up to 80 ºC (*Arora et al*., 2013; *Rothrock et al*., 2014 and *Vaishya et al.*, 2013).

Table 1 - Example of the components included in bone cement formulation.

Components:	Aim:
- Liquid component (MMA)	-
N,N-Dimethyl *p*-toluidine (DMPT)	Polymerization Initiator
Hydroquinone	Stabilizer
- Powder component (PMMA)	-
Benzoyl Peroxide (BPO),	Polymerization Initiator
Zirconium Dioxide (ZrO_2) / Barium Sulphate ($BaSO_4$)	Radio-Opacifer

1.2.1. Antibiotic loaded bone cements

It was in middle 70's when joint replacement surgeries become a common procedure and infection rate was high that researchers started to add antibiotics to BC. They found that aminoglycosides were the most suitable ones due to their antibiotic spectrum and chemical properties. It was necessary to have an antibiotic that (*DiMaio et al*., 2002):

a) presented stability during polymerization, sterilization and manufacturing processes;

b) have a long shelf life;

c) readily delivered from the biomaterial;

d) maintained its activity against bacteria and

e) acts locally in high concentrations without any systemic effect.

Gentamicin was one of the first antibiotics to be incorporated as an additive in PMMA BC. It showed to act as a local drug delivery system able to deliver the drug directly at the surgical site decreasing the amount of antibiotic in the organism when compared to the intravenous route. Since then other antibiotics have been mixed with BC namely tobramycin, erythromycin, cefuroxime and vancomycin. Also, combinations of antibiotics have been used (*DiMaio et al*., 2002).

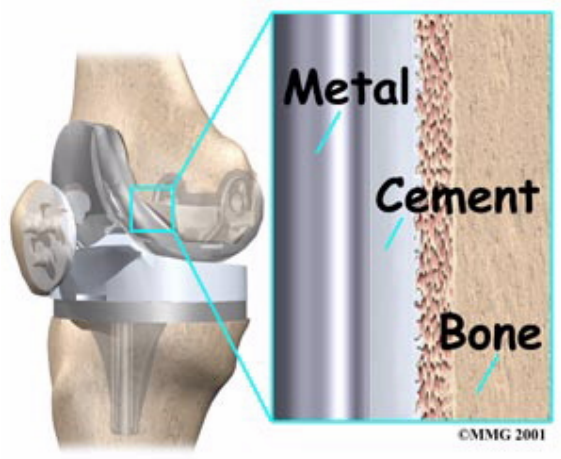

Figure 3 – Schematic representation of joint replacement with bone cement inclusion (adapted from http://www.eorthopod.com/artificial-joint-replacement-of-the-knee/topic/59).

Besides antibiotics various types of additives started to be added to BC with different aims as reviewed by *Arora et al*., (2013). For example, vitamin E aiming to improve cement biocompatibility and reduce peak temperature (Méndez *et al.,* 2002); polymers as chitosan and barium sulphate to improve bone cement mechanical strength (*Ricker et al*., 2008 and *Shi et al*., 2006); nanoparticles of magnesium oxide or barium sulphate to improve osteoblasts adhesion, minimize tissue necrosis and improve mechanical strength (*Makita et al*., 2008); chitosan nanoparticles to improve BC antibacterial activity against *S. aureus* and *S. epidermidis*, and silver nanoparticles that presented also antibacterial activity against *S. epidermidis*, MRSE and MRSA (*Shi et al.,* 2006).

Although antibiotic-loaded BC are often used, especially in Europe, in hip and knee arthorplasty (*DiMaio et al*., 2002), its use raises many questions.

In fact, once the PMMA is prepared with the selected antibiotic and polymerizes, it is of utmost importance to have adequate release kinetics, (Fig. 4) *i.e*, the amount of drug loading in BC has to be tested as well as the released amount. Too much antibiotic loading can release toxic amounts of drug, and low loading or inadequate release can lead to the development of multiresistant bacterial strains if the antibiotic concentration is below the minimum inhibitory concentration (MIC) (*Matos et al.*, 2014) - Fig 4.

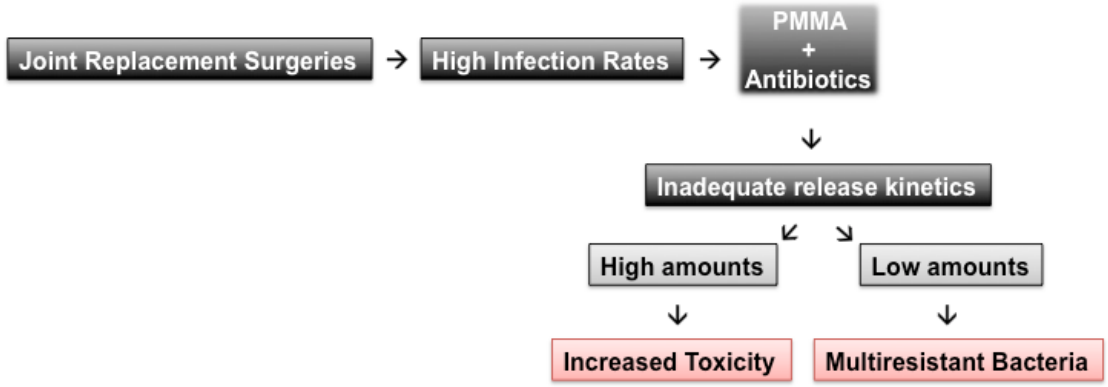

Figure 4 – Schematic representation of the problems associated to inadequate antibiotic release from BC.

There are several aspects that can influence drug release from the cement, namely (*Bistolfi et al*., 2011):

- type of antibiotic – physicochemical characteristics like charge and size;
- amount of loaded drug – inappropriate amounts can lead to toxicity or bacterial multiresistance;
- cement porosity - porosity of the polymer matrix depends on air entrapment during the wetting and stirring of the cement powder, this allows the passage of fluids, allowing the release of the incorporated antibiotic (*Bistolfi et al*., 2011 and *De Belt et al*., 2000);
- surface of contact/exchange - antibiotics release is largely influenced by the penetration of the dissolution fluids into the polymer matrix and from cracks and voids.

It was shown that the initial antibiotic release is directly proportional to the roughness of the surface: the higher the roughness, the wider the area of release; also a linear correspondence after a week between the porosity of the cement and the release of antibiotic was demonstrated: the continuous release after several days would depend on the deep penetration of antibiotic in the cement previously determined by the porosity (*Bistolfi et al*., 2011 and *Moojen et al.,* 2008). Inadequate release kinetics of antibiotics from the BC matrix occurs and often led to the development of multiresistant bacterial strains, especially methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), multiresistant *Acinetobacter baumannii* and extended spectrum beta-lactamase producing *Enterobacteriaceae (Matos et al.,* 2014).

1.3. Bone Cement and the Inflammatory Process

PMMA bone cements are materials that are implanted into living tissue initiating a host response to the biomaterial (Fig. 5) that can include injury, blood-material interactions, acute inflammation, chronic inflammation, granulation tissue development, foreign body reaction and fibrosis/fibrous capsule development (*Anderson et al.*, 2008 and *Franz et al.*, 2011). Also, when PMMA bone cements are implemented, erosion processes through time will happen so microcracking of the cement may lead to particle release inducing an additional localized immune response and osteolysis (*Lopes et al.*, 2013). Frequently anti-inflammatory drugs are used in the treatment of inflammation. High oral doses are usually necessary due to reduced blood supply to the bone with the concomitant aggressive side effects on gastrointestinal tract (*Guevara-Fernandez et al.,* 2003 and *Lopes et al.*, 2013).

As for antibiotics, a locally controlled drug release system could be more suitable and could minimize the undesired side effects. Anti-inflammatory drugs, namely ibuprofen, started then to be added to bone cements in order to achieve a controlled drug release aiming to decrease the implant related inflammatory process. This is already being study as well as the adjacent inflammatory response and promising results are observed, indicating a mild inflammatory reaction around the implanted material (*Arcos et al.*, 1997 and 2001). However, studies revealed inconclusive and controversial results in terms of anti-inflammatory drug effects in bone formation and bone mineral density. It is not proven if the drug potentiates bone regeneration or if on the contrary delays or decreases it, so the use of anti-inflammatory drugs must be deeply studied (*Gupta et al.*, 2010).

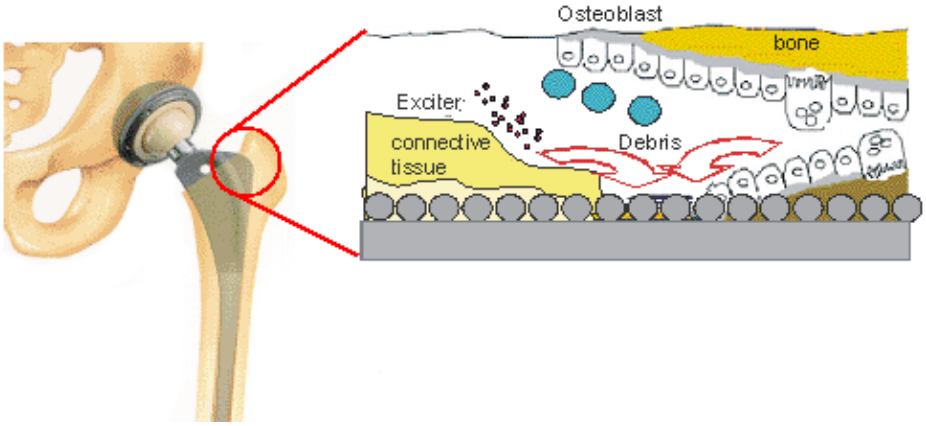

Figure 5 - Scheme of inflammatory response in a hip arthroplasty (adapted from http://www.ciosortho.com/joint-pain/).

1.4. Novel Approaches – New Additives In Bone Cements

As previously mentioned, PMMA is a very versatile carrier material that allows the loading of different additives and the delivery of drugs.
In the present study four additives were chosen to add to bone cement, two drugs namely an antibiotic (levofloxacin), and an anti-inflammatory drug (diclofenac); and two polymeric porogens – (chitosan and alginate).

The selection of the polymers was based on their common biomedical use. Alginate is approved as pharmaceutical excipient and chitosan biocompatibility is proven (*Goy et al.*, 2009). And when chitosan was loaded in bone cement its antimicrobial activity (*Arora et al.*, 2013 and *Shi et al.*, 2006) was observed being then of utmost interest to this work.

Levofloxacin has been recently evaluated as a promising antibiotic to be loaded into BC (*Matos et al.*, 2015a,b). Diclofenac has never been incorporated in a bone cement. From the best of our knowledge the incorporation of both drugs in bone cement has never been tried. The idea is to develop a novel BC formulation with enhanced antimicrobial and anti-inflammatory activity. Also, incorporation of the chosen biopolymers will constitute a novelty once that incorporation of chitosan was tested (*Shi et al.,* 2006 and *Tunney et al.*, 2008) but not with these drugs. Loading of alginate wasn't tested at all.
Chitosan and alginate, widely used biocompatible polymers, were selected as porogens aiming to increase drug release. Furthermore chitosan has already demonstrated antimicrobial activity (*Goy et al*., 2009) including when incorporated in bone cement (*Arora et al*., 2013; *Shi et al*., 2006 and *Tunney et al.*, 2008). To the present, loading alginate into BC has not been tested nor the loading of both drugs and chitosan at the same time.

1.4.1. Levofloxacin And Sodium Diclofenac

Levofloxacin is the active L-isomer of ofloxacin (Fig. 6), a fourth-generation fluoroquinolone derivative. It has broad-spectrum antimicrobial activity against gram-positive (methicillin-sensitive but not methicillin-resistant *Staphylococcus aureus*, *Streptococcus pyogenes*) and gram-negative (*Escherichia coli*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Legionella pneumophila*, etc) strains responsible for respiratory, urinary tract, gastrointestinal and abdominal infections (*Gupta et al.*, 2010 and *Lafredo et al.*, 1993).
Levofloxacin presents low toxicity and an adequate penetration into osteoarticular tissues above the MIC for susceptible pathogens generally present in bone and joint infections, being then an adequate choice to insert in PMMA cement (*Matos et al*., 2015b and *Rimmele et al*., 2004).

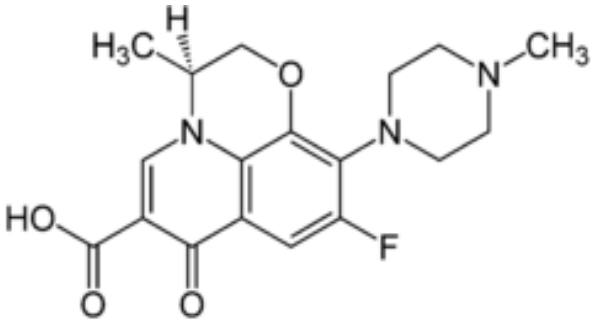

Figure 6 - Levofloxacin chemical structure.

In relation to the selection of an anti-inflammatory drug, a well-known one was selected – sodium diclofenac, a very widely studied drug that in literature also presented antimicrobial properties (*Reśliński et al.,* 2013). This fact is of major interest once that in combination with antibiotic could present prosperous results in combat or even eradicate biofilm formation.

Diclofenac sodium is a non-steroidal anti-inflammatory (Fig. 7), antipyretic, analgesic with potent cyclooxygenase inhibition activity, normally used to treat inflammatory disorders as rheumatoid arthritis, osteoarthritis, and gout attacks. It is also adequate to treat post-operative or post-traumatic pain particularly when inflammation is present (*Salmann* 1986).

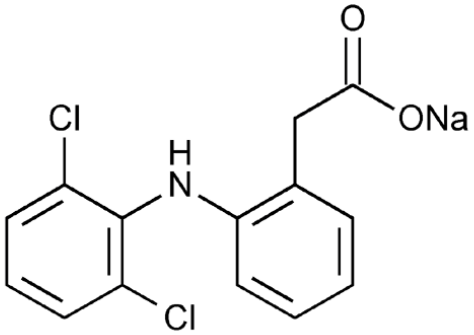

Figure 7 – Sodium Diclofenac chemical structure.

Furthermore, some studies suggest a potential diclofenac antibiofilm effect (*Reśliński et al.,* 2013). The mechanism is not fully understood and it is controversial. Studies suggest that diclofenac may act as inhibitor of bacteria fimbriae or by decreasing the production of virulence factors dependent from quorum sensing systems. The combination of diclofenac with an antibiotic as levofloxacin could then be useful in decreasing or even eradicating biofilm formation.

1.4.2. Chitosan And Alginate Polymers

Antibiotic-loaded PMMA bone cement can present major drawbacks such as incomplete and inadequate drug kinetic elution (*Matos et al*., 2015b). As such one of the strategies adopted to improve the release performance of bone cements refers to the incorporation of biodegradable polymers aiming to change cement porosity and allow a controlled and higher drug release through time, without compromising the mechanical properties of the matrix.

Many porogens have already been tested, as mentioned above and reviewed by *Arora et al.,* 2013.
In the present research work chitosan and alginate were selected to be tested.
Chitosan is an abundant bio co-polymer of glucosamine and N-acetylglucosamine deacetylated from the natural polymer chitin (Fig. 8). Chitin is the main component of the shells of crustacean, and can be fabricated into film, fibber, bead and powder forms. Chitosan is known to be biodegradable and nontoxic. It has a high resistance to heat due to its intramolecular hydrogen bonds having a great potential as a biomaterial to be loaded into bone cement. It is also often used as tissue engineering scaffold, and as drug delivery carrier for antibiotics. Furthermore, chitosan has been shown to have antibacterial activity in controlling bacteria growth and inhibiting viral multiplication (*Shi et al.,* 2006).

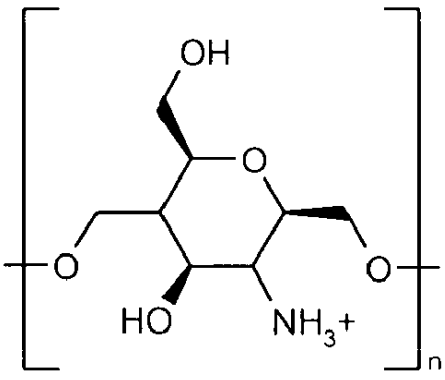

Figure 8 – Chitosan monomer structure.

Alginate (Fig. 9) is a naturally occurring anionic polymer typically obtained from brown seaweed. It has been extensively investigated and used for many biomedical applications, due to is biocompatibility, low toxicity, low cost and easy handling. It presents wide applications namely in wound healing once it minimize bacterial infection at the wound site, delivery of bioactive agents as small chemical drugs and proteins, and cell transplantation. Alginate gels are also promising for cell transplantation in tissue engineering (*Lee et al.,* 2012). Alginate has also been reported as a bone regenerator polymer (*Cecoltan et al*., 2015).

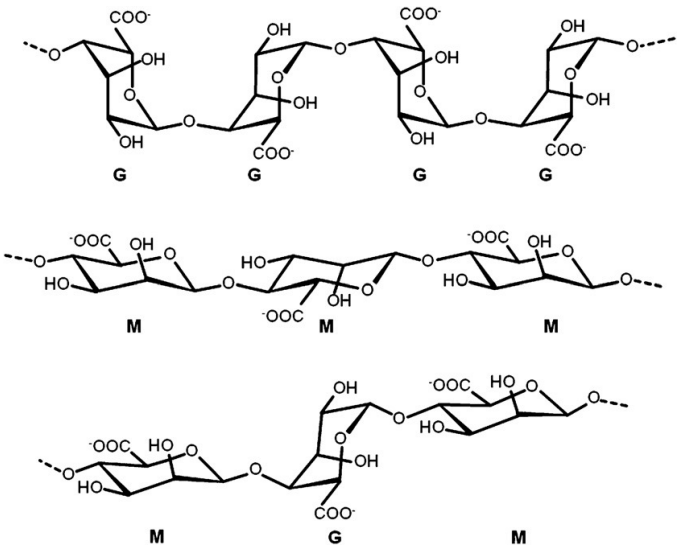

Figure 9 – Chemical structures of G-block, M-block, and alternating block in alginate (adapted from *Lee et al*., 2012).

1.5. Compliance of novel Bone Cement Formulations

Changes in bone cement formulation have to be carefully tested in terms of antibiotic release profiles and stability, surface and mechanical properties as well as antimicrobial activity and biocompatibility.
Those tests are of utmost importance once they will allow understanding the potential of novel formulations and discharging or accepting the worst/best ones, respectively.

1.5.1. *In vitro* drug release

In vitro release assays must be performed when testing novel BC formulations, in order to select the most promising in terms of amount and profiles of drug released. These assays aims to mimic physiologic conditions, namely physiologic pH 7 at 37 ºC with phosphate-buffered saline (PBS) solution with or without the presence of serum proteins like albumin. Also to evaluate the effect of pH at infected site (pH = 5) is of utmost importance.
The amount of released drug can be monitored and quantified by common analytical techniques like High Performance Liquid Chromatography (HPLC), during a defined period of time that usually goes for more than one month, and under standard conditions of temperature and agitation.

1.5.2. Surface Studies

Surface properties of the new formulations have also to be evaluated once new additives can promote for example changes on cement surface energy. Surface energy of a biomaterial is of major importance as it can influence protein adhesion, cell proliferation of osteoblasts, monocytes, fibroblasts, among others and also bacterial adhesion (*Anselme* 2000; *Redey et al*., 2000; *Vogler* 1998 and *Young et al.,* 2000).
The surface energy of a solid cannot be measured directly. It can be indirectly calculated trough the determination of contact angles of the surface of the solids with a liquid using for example the Wilhelmy plate technique that relies in the quantification of a force intensity when the solid is immersed in a liquid (*Garbassi et al.*, 1994).
The information obtained by the contact angle assay also provides information about the level of hydrophobicity and hydrophilicity of the surfaces.

1.5.3. Mechanical and structural Assays

There are various mechanical and structural tests that should and can be made namely compressive strength, bending strength and bending modulus, porosity and microstructure analysis (by FEG-SEM). In the present work compressive strength and FEG-SEM analysis were conducted. Compressive strength assay consists on applying a compressive force above and below the material until compressive strength limit is reached and fracture occurs (*Callister et al*., 2003). FEG-SEM is a type of electron microscope technique that scans the sample with a focused beam of electrons. Those electrons interact with the atoms in the sample producing signals that can be detected. The sample has to be previously prepared with a conductive material like gold, palladium or platinum (*Suzuki* 2002).

1.5.4. Microbiological Assays

Microbiological assays have to be performed in order to evaluate the stability of the antibiotic that is released from the cement and the antimicrobial efficacy of the novel BC formulations.
In these assays the selected bacteria strain (in this work *S. aureus* was tested) is cultured with all BC formulations plates in order to observe the susceptibility of bacteria to the released antibiotic from the cements giving in this way information about the microbiological activity of the antibiotic as well as the adherence of bacteria to the plates. Also, minimum inhibitory concentration (MIC) of the antibiotic can be determined, which corresponds to the lowest concentration of the antimicrobial agent that inhibits a given bacterial isolate from multiplying and producing visible growth in the test system, usually after overnight incubation (*Andrews*, 2001). Once the bacteria tested is a biofilm producer, minimum inhibitory concentration (MBIC) should also be determined, that corresponds to the minimum antibiotic concentration that inhibits biofilm formation. Biofilm quantification can be assessed through crystal violet staining technique that consists on the stain of the peptidoglycan layer, DNA, proteins, of the cells (Gram positive) and further absorbance measurement at 595 nm.

1.5.5. Biocompatibility

Finally, an important step to evaluate new bone cement formulations is to assess their biocompatibility. It is a fundamental requirement when developing new materials for medical devices and tissue engineering once the material is inside the patient *(Bruinink* and *Luginbuehl*, 2001; *Eloy*, 2012). ISO standards (*ISO 10993,* 2009) describe certain *in vitro* methods to assess the cytotoxicity of bone cement extracts and the sample itself. Those are realized in a mouse cell line of fibroblasts

(L929; NCTC) and others are recommended, like the use of a human osteoblast cell line (MG63; ATCC®CRL-1427). ISO proposes mainly three types of assays:

- extract tests;
- direct-contact tests;
- indirect-contact tests.

The *in vitro* effect of bone cement on cells viability is an important parameter of the biomaterial biocompatibility. Bone cement extracts evaluation gives quantitative information, i.e. the cell viability after being exposed to those extracts. Direct-contact assay can give both quantitative and qualitative information on materials biocompatibility. These assays can be performed with a dye – MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl- 2H-tetrazolium bromide) which is a yellow, water-soluble tetrazolium compound that is converted by viable cells to a water-insoluble, purple formazan. For quantitative assessment absorbance is measured at 570 nm. For the morphology assays, fluorescent dyes were used (rhodamine phalloidin) in order to stain actin and therefore observe the morphology of the extracellular matrix.

In sum, several studies (drug release, structural/mechanical, microbiological and biocompatibility) have to be conducted aiming to evaluate the potential for clinical application of novel bone cement formulations.

Chapter 2. Materials and Methods

2.1. Nanoparticles preparation

2.1.1. Materials

Chitosan (low, medium and high molecular weight, LMW, MMW and HMW, respectively), chloridric acid (37% w/w) and sodium hydroxide were obtained from Sigma-Aldrich (Spain); sacarose was obtained from Panreac Applichem (Germany); tripolyphosphate pentasodium (TPP) was purchased from Alfa Aesar, (Germany); acetic acid (100% w/w) was from Sigma-Aldrich (Spain); sodium alginate (40% G) and sodium alginate (60%G) Protanal LF10/60 from FMC BioPolymer (Norway); calcium chloride (99.5% p.a.) was from Merck (Germany).

2.1.2. Equipment

Analytical balance (Kern ALS 120-4, Germany); pH electrode probe (inoLab WTW Series, Germany); stirring plate (Variomag Multipoint Koret, UK); microplate reader (FLUOstar Omega, BMG Labtech, Germany); Size distribution and zeta potential reader (Zetasizer Nano-S and Nano-Z, Malvern Instruments, UK). The software associated with the equipments was: Omega for microplate reader; Zetasizer Nano-S for nanoparticles size distribution and zeta potential.

2.1.3. Methods

2.1.3.1. Preparation of Chitosan (Chi) nanoparticles

The CS nanoparticles were prepared using an ionic gelation technique previously optimized in our laboratory (*Figueiredo et al*., 2012).

The first step was to evaluate the appropriate formulation for scale up according to the following criteria:
1) highest values of absorbance that indicates high yield of production;
2) low values of size distribution (values must be in nano range);
3) zeta potential to evaluate the stability of nanoparticles (highest values, negative or positive, indicate highest stability).

The mentioned parameters of all samples that didn't present clusters formation were measured and the best formulation was chosen accordingly.

Chi solutions (2.5 and 1.25 mg/mL) were prepared by dissolving three types of chitosan polymer (LMW, MMW and HMW), in ultra-pure water with 1% (V/V) acetic acid solution and leaving it under stirring for 3h. The solutions were then separated into three and the pH was adjusted to 4, 5 and 6 for each type and concentration with a 0.5 N sodium hydroxide solution. A 1 mg/mL and 1.5 mg/mL of TPP solutions were prepared by dissolving TPP in ultra-pure water, and the pH was adjusted to 7.5, 8 and 9 for each concentration (Fig 10).

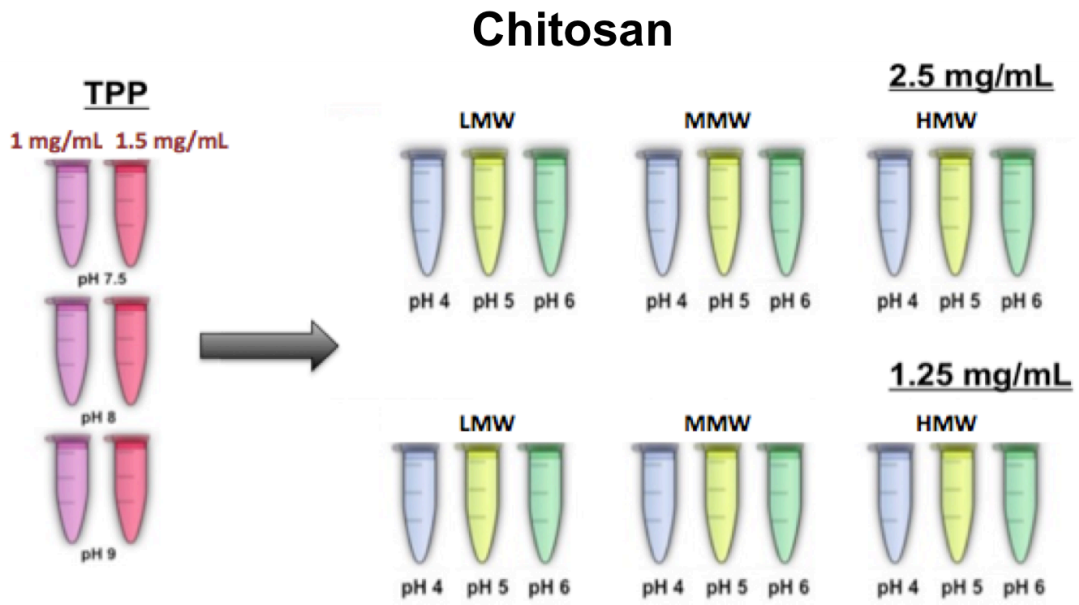

Figure 10 - Scheme of the different tested combinations of chitosan and TPP to obtain the appropriate nanoparticles formulation.

After analyzing all the mentioned criteria the best formulation was chosen to scale up, which consisted in 2.5 mg/mL LMW CS (pH 5) with 1.5 mg/mL TPP (pH 8).
For the scale up, the preparation of Chi and TPP was made following the procedure described above. The Chi solutions were divided in three and TPP was added slowly under vigorous magnetic stirring at room temperature for 15 min. Finally, cryoprotectant solutions of different concentrations were added (10%, 5% and 2.5% of sucrose) in order to evaluate their effect on the yield of preparation and as no significant variations were observed the lowest concentration (2.5%) was used. Samples were then freeze-dried (Christ Alpha 1-2, Martin Christ, Germany) for 48h before further use. Particle size distribution and zeta potential were measured before and after freeze-drying.

2.1.3.2. Preparation of Alginate nanoparticles

The alginate nanoparticles were prepared using also an ionic gelation technique (*Sarei et al*., 2013).
In brief, $CaCl_2$ solution was added to sodium alginate solution dropwise under a constant homogenization rate (1300 rpm) during 45 min at room temperature. Like for the CS nanoparticles also to alginate the best formulations for scale up were evaluated considering the same criteria of absorbance, size distribution and zeta potential of all samples that didn't present clusters.
Two types of sodium alginate were tested: 60% and 40% in G-content; 3% alginate solutions (pH 6) of each type were prepared for further use. Seven solutions of 0.3% sodium alginate of each type of alginate were prepared by diluting the initial 3% alginate solutions and then added $CaCl_2$ in three different proportions and five different concentrations (Fig. 11).

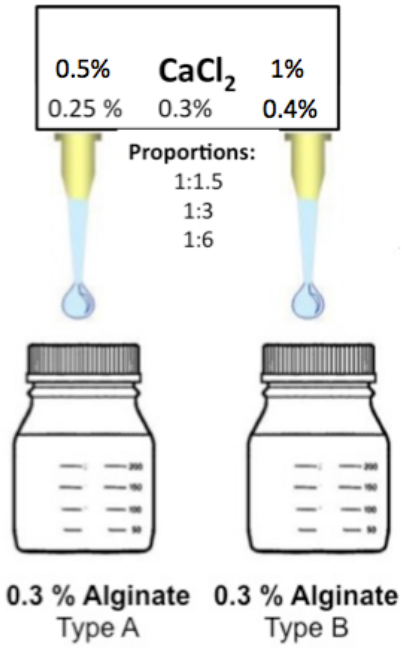

Figure 11 – Scheme of the different combinations of alginate (type A- 60% content of M and 40% content of G; type B- 10% content of M and 60% content of G) and $CaCl_2$ in different concentrations and proportions to obtain the appropriate nanoparticles formulation.

Then, 5 mL of $CaCl_2$ were added dropwise, using a syringe to maintain a constant flow, to 15 mL of sodium alginate. The suspension of NPs was maintained under constant homogenization (1300 rpm) for 45 min at room temperature.
All solutions were observed and analyzed through absorbance, particle size distribution, and zeta potential. The best formulation chosen to scale up was: 0.3% sodium alginate (40% G-content) with 0.5% $CaCl_2$ at 1:3 V/V proportion. Samples were then freeze-dried for 48 h before further use.

2.1.3.3. Characterization of nanoparticles

2.1.3.3.1. Particle size distribution

Mean particle size and polydispersity index (PDI) of nanoparticles were assessed by light scattering using a Zetasizer Nano-S.
Nanoparticles were dispersed in 0.22 μm filtered purified water and their distribution size was obtained from the average of three runs per sample. Results were expressed as mean ± standard deviation (S.D.).

The basis of this technique lays in the measurement of the autocorrelation function of the scattered light intensity, that is, the convolution of the intensity signal as a function of time with itself. The longer the correlation with time, the slower the movement of the particles through the solution, a property that can be quantified by means of diffusion coefficient. Variables, size and diffusion, are related. The strong point of this technique is its sensitivity both to the concentration and to the size of the particles, especially to the latter, allowing the detection of aggregation in an early stage.

2.1.3.3.2. Zeta-potential

Zeta potential was measured using a The Zetasizer Nano Z (Malvern Instruments, UK), using samples diluted with 0.22 μm filtered purified water. The equipment uses electrophoretic light scattering technology to measure zeta potential.

Zeta potential is a measure of the charge on particles in a system and it is known to correlate with coagulation performance. It's already used for quality control and to optimize chemical flocculent addition.

Zeta potential measures the magnitude of electrostatic or charge repulsion between particles at the boundary layer surrounding the particle, rather than on the particle itself. Zeta potential therefore quantifies the balance of repulsive and attractive forces that particles experience as they approach one another. At a zeta potential near zero, a system is unstable and highly prone to aggregation. A pronounced negative or positive zeta potential (+/-30 mV), on the other hand, is indicative of an electro-statically stable system that will resist particle aggregation.

2.2. Bone Cement preparation

2.2.1. Materials

The acrylic Bone Cement, CMW 1 and SmartSet (Table 2), were obtained from DePuy (Johnson & Johnson, UK); levofloxacin (98%), diclofenac sodium and chitosan were obtained from Sigma-Aldrich (Spain), and sodium alginate (40% G) was from FMC Biopolymer.

Table 2 – Qualitative and quantitative composition of the tested bone cements.

	SmartSet HV®	DePuy CMW 1®
Composition of Bone Cement Powder:		
Polymethylmethacrylate (% w/w)	-	84.73
Methylmethacrylate /Styrene Copolymer (% w/w)	-	-
Methylmethacrylate / Methylmethacrylate Copolymer (% w/w)	80.45	-
Benzoyl Peroxide (% w/w)	0.96	1.95
Barium Sulfate (% w/w)	-	9.10
Zirconium Dioxide (% w/w)	14.37	-
Composition of Bone Cement Liquid:		
Methylmethacrylate (% w/w)	97.50	98.50
N,N-Dimethyl-p-toluidine (% w/w)	≤ 2.50	≤ 1.50
Hydroquinone (ppm)	75	75

2.2.2. Equipment

Analytical balance from Kern ALS 120-4 and metallic molds.

2.2.3. Methods

2.2.3.1. Preparation of PMMA bone cement

Different BC matrices (plates or cylindrical) with varied compositions were prepared (Table 3). Powder components, using PMMA (2.5 g) were thoroughly but carefully mixed in a glass mortar and then the proportional quantity of liquid monomer methylmethacrylate (1.25 mL) was added. When it gets the desired consistency, BC dough was manually casted, into aluminum molds to get the BC specimens. Cure proceeds at room temperature for 1h. All specimens were then polished with a #1000 abrasive sand paper and measured, with a digital micrometer (Mitutoyo Digimatic, Painesville, OH, USA) with an accuracy of 0.01 mm and stored in a vacuum desiccator, at 23 ± 1°C for 24 ± 2 h before testing, according to ISO 5833 recommendations. (*Matos et al*., 2014)

Table 3 – Composition (% w/w) of the different BC formulations.

BC Specimen	Levofloxacin (%)	Diclofenac (%)	Chitosan NP's (%)	Alginate (%)
[BC]	0	0	0	0
Lev[BC]	2.5	0	0	0
Lev[BC]Diclo	2.5	2.5	0	0
Lev[BC]ChiNP	2.5	0	5	0
Lev[BC]ChiNP	2.5	0	7.5	0
Lev[BC]Alg	2.5	0	0	5
Lev[BC]Alg	2.5	0	0	7.5
Lev[BC]AlgNP	2.5	0	0	7.5
[BC]Diclo	0	2.5	0	0
Lev[BC]Diclo-Chi	2.5	2.5	7.5	0
Lev[BC]Diclo-Alg	2.5	2.5	0	7.5

Different BC specimens were prepared depending on the test. Thin plates were used for surface, microbiological and biocompatibility tests (Fig. 12). Cylinders were used for the release and mechanical studies (Fig. 13).

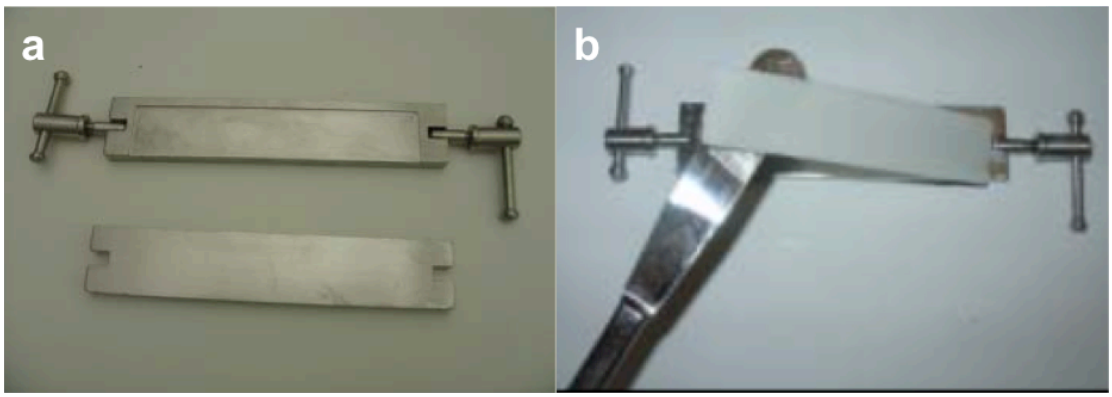

Figure 12 - Steel molds used for preparing the BC plates (a); example of a BC plate (b).

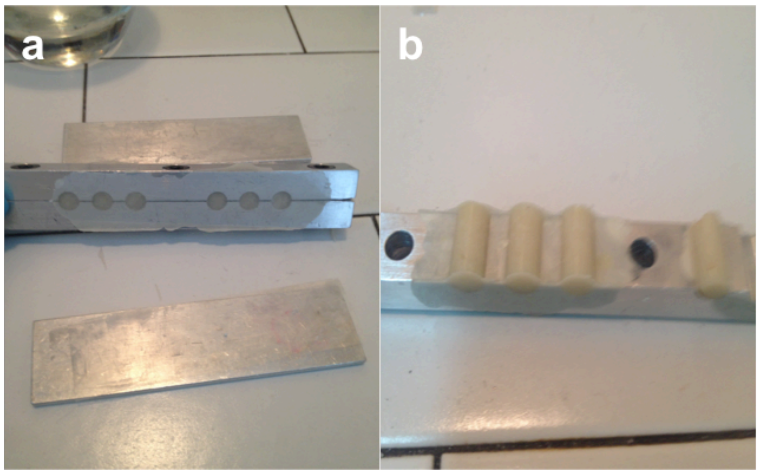

Figure 13 - Steel molds used for preparing the BC cylinders (a); example of BC cylinders (b).

2.3. *In Vitro* Release Assays

2.3.1. Materials

Chloridric acid (37%), orthophosphoric acid and sodium hydroxide were obtained from Sigma-Aldrich, (Spain); triethylamine (TEA) and polysorbate 20 (Tween20®) from Panreac, acetonitrile for HPLC was obtained from VWR Chemicals (Portugal); sodium chloride was obtained from Panreac Applichem (Germany). Deionized water was obtained from Millipore 83 analytical deionization system (F9KN225218).

2.3.2. Equipment

HPLC (UV spectrophotometry detector model SPD-SA; liquid chromatograph pump model Shimazdu LC-6A; autosampler model Waters 717 plus; communications bus module model Shimadzu CBM – 102); water bath from Memmert; vortex from Velp Scientifica; filters (0.45 µm) from Membrane Solutions; 5 µm column (LiChrospher® 100 RP-18, Merck KGaA, Darmstadt, Germany). The software associated with the HPLC equipment was GC Real Time Analysis.

2.3.3. Methods

In vitro levofloxacin and diclofenac release from cylindrical BC specimens (with average dimensions of 121 mm of length and 61 mm of diameter) was featured with each BC sample incubated in 2.5 mL of a solution of NaCl 0.9% (m/V) with 0.05% (V/V) Tween20®, in a shaking water-bath at a 37 °C. At predetermined intervals (0; 0.5h; 1h; 2h; 4h; 6h; 24h; 48h; 72h; and every week by then), throughout a 4-week period, aliquots of 250µL of the supernatant were collected and analyzed in triplicate. The withdrawn aliquots were then replaced with equal volumes of fresh release solution and sink conditions were guaranteed during the whole study.
Levofloxacin and diclofenac content were determined by HPLC.

Levofloxacin method was adapted from *Matos et al.,* (2015a). Diclofenac quantification method was adapted from the literature (*Emami et al*., 2007). In order to confirm the absorbance wavelength of diclofenac an absorbance spectrum was made from 220 to 500 nm and a peak at 276 nm was observed (Fig.- 14), differing this way, from the levofloxacin peak (284 nm). Optimized HPLC conditions are described in Table 4.

Table 4 - HPLC conditions for analysis of each drug. (*Emami et al.,* 2007 and *Matos et al.,* 2015a)

Drug	Mobile Phase	pH	Retention time (min)	Wavelenght (nm)	Injection Volume (µl)	Flow Rate (mL/min)
Levofloxacin	85% H_2O, 15% Acetonitrile, 0.60% TEA	3	3.50	284	20	1.20
Diclofenac	54.50% H_2O, 45% Acetonitrile, 5% Ortophosphoric acid	3.50	10	276	20	1.20

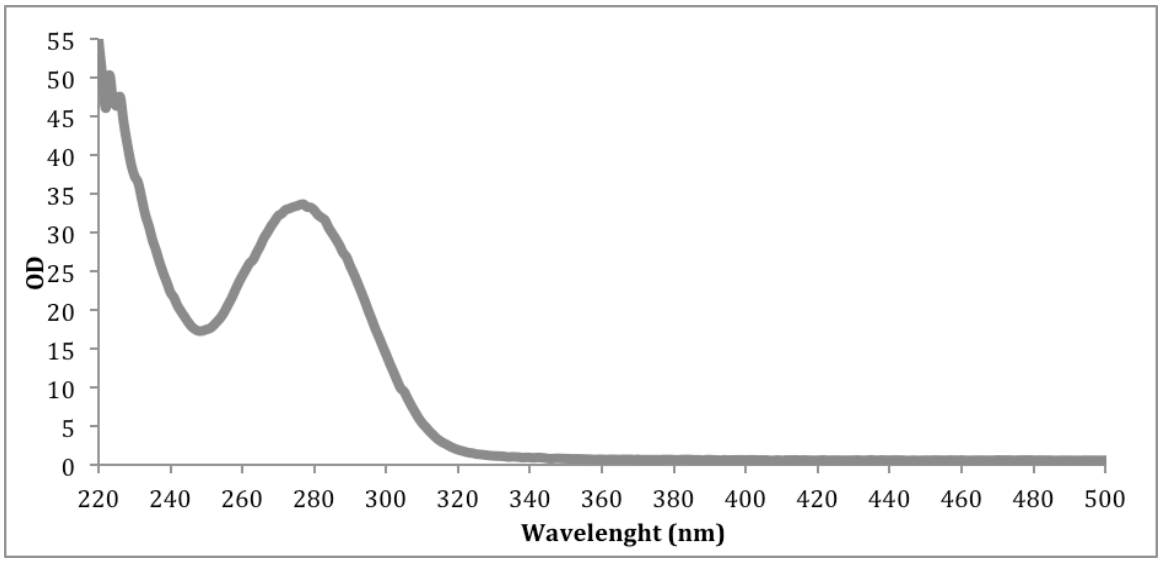

Figure 14 - Diclofenac absorbance spectrum.

Calibration curves for Levofloxacin and Diclofenac quantification

The Calibration curves to quantify levofloxacin and diclofenac were conducted daily before any assay. Examples of the curves are shown on Figures 15 and 16.

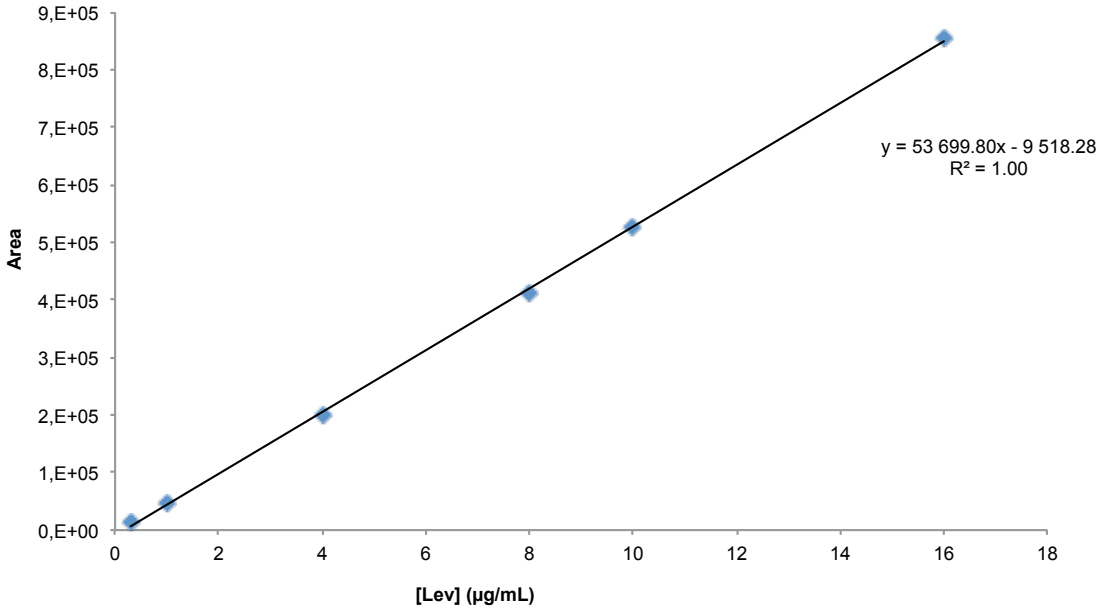

Figure 15 - Calibration curve for Levofloxacin (23rd October, 2014).

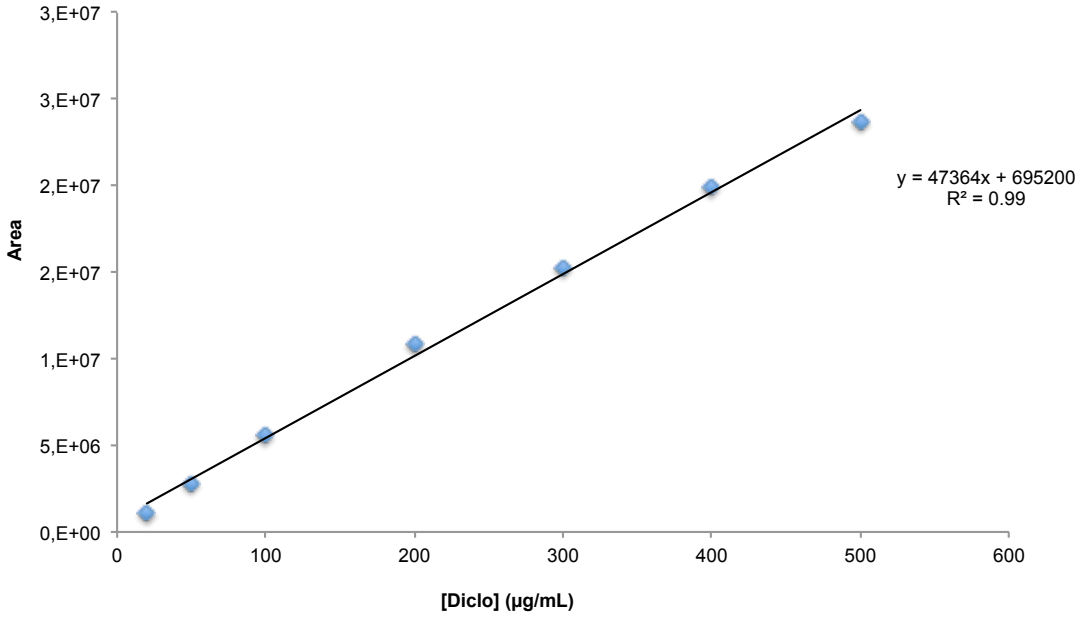

Figure 16 - Calibration curve for Diclofenac (20th November 2014).

2.3. Bone Cement Characterization

3.3.2. Contact angle and surface energy determination

For contact angle measurements, BC matrices were cut in thin plates (apx 1.2 ×25×15 mm). Assays were performed with a Kruss K12 tensiometer (Kruss GMBH, Hamburg, Germany) using the Wilhelmy Plate method, by immersing the plates 5 mm into the test liquids (purified water and 1,2- propanediol from Merck) at a speed of 3 mm/min, at 25.0 ± 0.1 °C. Advancing contact angles were used for surface energy (γ) estimation of all BC matrices, as well as its dispersive (γ_d) and polar components (γ_p) based on the harmonic mean method proposed by *Wu* (1971) and expressed by Eq. (1), where γ_{12} is the interfacial tension between phases 1 and 2, which each have a surface tension consisting of a polar and dispersive component. Three replicates were carried out for each plate. Equations for surface tension estimation were solved using the equation handling Kruss-software program: contact angle measuring system K100 (version 2.05) (*Matos et al*., 2014).

$$\gamma_{12} = \gamma_1 + \gamma_2 - \left(\frac{4\gamma_1^d\gamma_2^d}{\gamma_1^d + \gamma_2^d}\right) - \left(\frac{4\gamma_1^p\gamma_2^p}{\gamma_1^p + \gamma_2^p}\right) \quad \text{(Eq. 1)}$$

3.3.3. Surface Structure (SEM) and Energy Dispersive Spectroscopy analysis

The inner matrix of the BC specimens was analyzed and photographed through a thermal field emission scanning electron microscopy, FEG-SEM, model JSM7001F (JEOL, Japan) operated at 5 kV. Briefly, slices of 3 mm of a selected group of representative specimens were cut, using a cut-off machine (Struers Accutom-5®, Struers, Denmark) provided with a diamond wafering blade (Buehler 11-4285 series 10LC Diamond®, Buehler LTD, US), and were mounted onto aluminium stubs and their surface was coated with a gold–palladium film (thickness of 30 nm) under vacuum in an argon atmosphere (Quorum Technologies, Polaron E5100). Images were made using a backscattered electron detector. Back-scattered emission (BSE) was applied to improve the surface contrast photographs and ease element analysis through the atomic weight. The elemental chemical composition of the samples was determined by energy dispersion spectroscopy (EDS) with an Oxford Inca Energy 250 spectrometer (*Matos et al*., 2015a).

2.3.3. Mechanical Tests

Compressive Strength

Compressive strength tests were performed on cylindrical specimens (average dimensions 6 × 12 mm), placed between a flat non-compressible surface and the platen of the testing machine. Applied crosshead rate was 20 mm/min until reaching the upper yield point or the cylinder fracture. Tests were performed at room temperature in a servo-hydraulic universal machine (TIRAtest® 2705) (*Matos et al*., 2014).

2.4. Microbiological Assays

2.4.1. Chemicals

The following chemicals and solvents were used: Mueller-Hinton agar, agar bacteriologique type E and Mueller-Hinton Broth from Biokar Diagnostics (France); tryptic soy broth and brain-heart infusion (BHI) broth from Liofilchem Diagnostics (Italy); D-glucose monohydrate 99.0% from Fluka Analytical (Germany); acetic acid glacial from Scharlau (Australia); ethanol absolute 99.5% from Emparta (Germany), sulphuric acid 95-97% and barium chloride from Merck (Darmstadt, Germany); crystal violet from Sigma-Aldrich (St. Louis, USA).

2.4.2. Microorganisms and culture conditions

The *Staphylococcus aureus* ATCC 25923 strain was obtained from the American Type Culture Collection (ATCC), stored at -80ºC and maintained on tryptic soya agar (TSA) slants. A calibration curve (Fig.17) with a standard barium chloride standards prepared in an aqueous solution of sulfuric acid (1%) was made to adjust the absorbance of inoculate that corresponds to 0.5 McFarland units. The standards were prepared by mixing different volumes of barium chloride (1%) and sulfuric acid (1%) to obtain different turbidity solutions. The absorbance of the different McFarland standards (0.5, 1, 2, 3, 4 and 5) was measured and cell densities were assigned in colony forming unit per mL (CFU/mL).
In all biofilm preformed assays *S. aureus* was cultured overnight in BHI, adjusted to a density of 0.5 McFarland units and then diluted 1:200 (V/V) (to 0.5×10^5 CFU/mL) in BHI with 1% (w/V) of glucose medium.

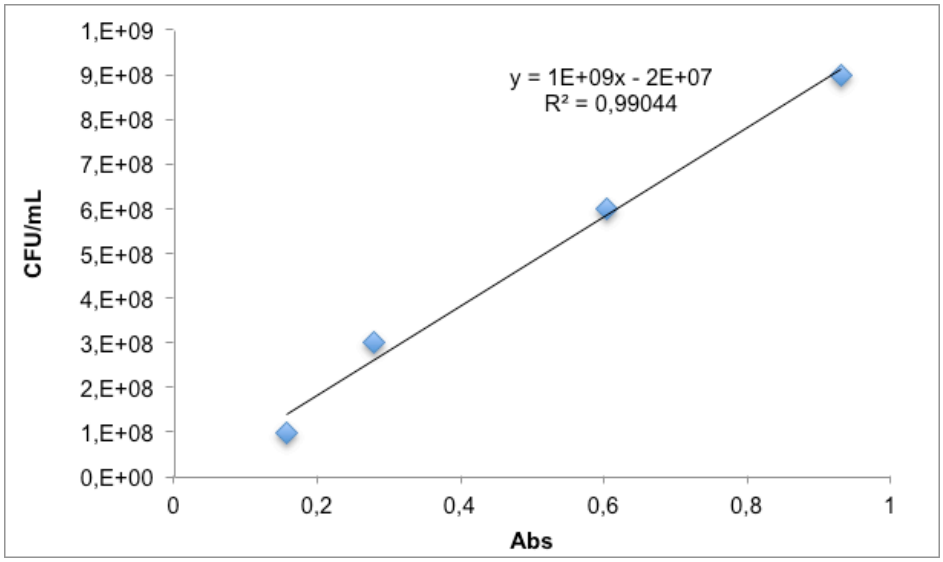

Figure 17 - Calibration curve of McFarland Units correlating absorbance measured with CFU units.

2.4.3. Bone Cement Preparation

Bone cement plates (8×8×0.2 mm) were prepared according to previously described procedure (2.2.3.1).

2.4.4. Antimicrobial activity evaluation

2.4.4.1. Plain bone cement

The effect of plain [BC] on levofloxacin microbiological activity was tested by minimum inhibitory (MIC) and minimum inhibitory biofilm (MIBC) concentrations. Furthermore, the possible synergetic effect of diclofenac and levofloxacin on preventing biofilm adhesion onto BC was also evaluated.

2.4.4.1.1. *Minimum inhibitory concentration*

Determination of MIC was conducted according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI). Plates of BC were glued in 24-well microtiter plates (MTP) and six different concentrations of levofloxacin in the BHI medium were tested (4, 2, 1, 0.5, 0.25 and 0.125 μg/mL). Plates were then inoculated with *S. aureus* for 24h at 37 ºC (Revco Ultima). The bacterial growth was then evaluated in each well.

2.4.4.1.2. *Effect of levofloxacin on Biofilm*

The MBIC biofilm quantification assay followed the conditions described above and was assessed by the crystal violet (CV) staining method (Ribeiro *et al.*, 2014). In brief, attached cells were washed with PBS, fixed with 96% (V/V) ethanol, stained with 0.1% (w/V) CV solution and washed with purified water. After drying, each BC plate was removed to an eppendorf tube, the attached dye was solubilized in 1% (V/V) acetic acid solution and absorbance was measured at 595 nm in a microplate reader (Anthos, Zenyth 3100).

2.4.4.1.3. *Antimicrobial Activity of diclofenac*

In addition, a possible antimicrobial effect of diclofenac and levofloxacin was tested.
For antimicrobial activity of diclofenac, plain bone cement plates ([BC]) were glued in the wells of a MTP and four conditions were established: 1) BHI medium, 2) BHI medium with a concentration of 0.3 μg/mL of levofloxacin, 3) BHI medium with a concentration of 0.3 μg/mL of levofloxacin and 10 μg/mL of diclofenac and 4) BHI medium with a concentration of 0.3 μg/mL of levofloxacin and 100 μg/mL of diclofenac. Then all 24-wells were inoculated with *S. aureus* (0.5×10^5 CFU/mL) and incubated at 37º C, 240 rpm. Biofilm quantification was assessed at 24h by the CV method described above (2.4.4.1.2).

2.4.4.2. Modified bone cement formulations

2.4.4.2.1. *Biofilm inhibitory activity of bone cement*

After the analysis of plain BC, modified BC formulations were evaluated, namely the inhibitory biofilm effect of the different BC matrices.
Initially, the assay was conducted in some selected matrices: Lev[BC]; Lev[BC]Alg; Lev[BC]Diclo-Alg and Lev[BC]Diclo-Chi in order to adjust the experimental conditions as the time of the assay. It was observed that bacterial growth occurred in the majority of the tested matrices after 72h, consequently it was the elected time point to proceed in the subsequent assays.

Biofilm inhibition was then evaluated on all BC matrices plates:Lev[BC]; Lev[BC]Alg; Lev[BC]Chi; Lev[BC]Diclo; Lev[BC]Diclo-Alg; Lev[BC]Diclo-Chi.

Biofilm inhibition assay was based on the method described by Minelli and colleagues (*Minelli et al.,* 2011) and Kwasny and Opperman (2010). *S. aureus* was cultured overnight in BHI, adjusted to a density of 0.5 McFarland units and then diluted 1:200 (V/V) (to 0.5×10^5 CFU/mL) in BHI medium with 1% (w/V) of glucose. This diluted suspension (1 mL) was used to inoculate each well of the 24 well-microtiter plate containing a previously glued cement plate. Negative and positive controls were also evaluated, As negative controls the correspondent BC matrices plates were used without being inoculated, and as positive controls BC plates without drugs: [BC], [BC]Alg and [BC]Chi. Biofilm quantification was performed by the CV method as described above (2.4.4.1.2), after 72h incubation at 37 ºC and 240 rpm.

2.4.4.2.2. *Levofloxacin released quantification*

Simultaneously to biofilm evaluation, levofloxacin released from the different BC matrices was quantified through time. This was a parallel assay that followed all conditions of biofilm quantification test but without bacteria inoculation and had 120h of duration.
Levofloxacin calibration curves (Figure 18) were prepared in BHI medium with 11 standards (*i.e*. 0.25; 0,5; 1; 2; 4; 8; 16; 32; 64; 128 and 256 μg/mL) and then measured with an excitation wavelength of 280-290 nm and an emission wavelength of 460-470nm in a microplate reader (FLUOstar Omega, BMGLabtech, Germany); BHI were also measured to be used as blank (BHI medium and samples were diluted 10 times in order to reduce background fluorescence intensity).

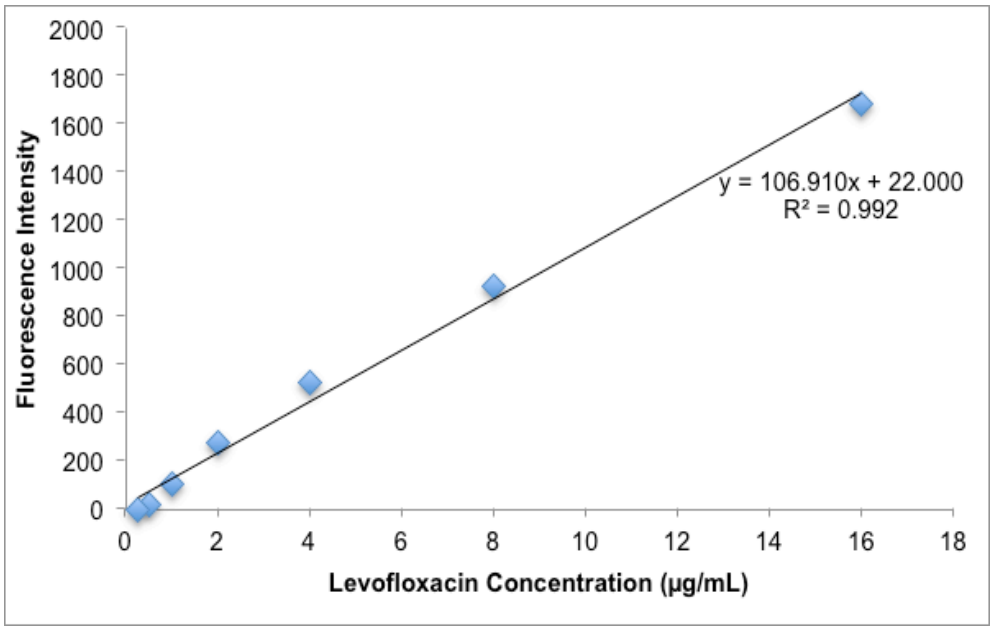

Figure 18 – Example of calibration curve for levofloxacin in BHI medium.

2.5. Biocompatibility – *In vitro* cellular Assays

2.5.1. Cell lines

The two cell lines L929 Mouse fibroblast cell line (ATCC® CCL-1™) and MG63 human osteoblast cell line (ATCC CRL-1427™) were used.

2.5.2. Culture media and reagents

Fetal Serum Bovine (FSB), RPMI 1640 culture medium, penicillin G (sodium salt), streptomycin sulfate, L-glutamine, phalloidin- TRITC and DAPI were obtained from Life Technologies (UK); cell-dissociation enzyme (TrypLETMExpress) was from ThermoFisher Scientific (USA); Sodium Dodecyl Sulphate (SDS) and dimethylsulfoxide (DMSO) were from Merck, (Germany), MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl- 2H-tetrazolium bromide from Sigma-Aldrich (UK), 10mM Phosphate Buffer Solution at pH 7.4 (PBS) was from Applichem (Germany).

2.5.3. Cell viability studies

2.5.3.1. Cell growth and maintenance

Each cell line was kept at optimal conditions in RPMI 1640 culture medium, supplemented with 10% Fetal serum bovine, 100 units/mL of penicillin G, 100 µg/mL of streptomycin sulfate, and 2mM L-glutamine, at 37 ºC with 5% CO_2 (Nuaire NU4750E, UK), until confluence levels reached at least 75%. At this point cell lines were dislodged from surface with 1 mL of enzyme and subsequently transferred to new T-flasks at a tenth of its original volume.

2.5.3.2. Cytotoxicity evaluation of the BC extracts

The cytotoxicity was assessed using general cell viability endpoint MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl- 2H-tetrazolium bromide) (*Mosmann*, 1987). MTT is a yellow, water-soluble tetrazolium dye that is converted by viable cells to a water-insoluble, purple formazan.
Cell viability was assessed after 24h and, 48h of incubation with: a) BC extracts from release assays corresponding to 2 release time points: 24h and 3-week, and b) the standard drug solutions in water: levofloxacin (567 µg/mL), diclofenac (647 µg/mL) and a mixture of both maintaining the same concentrations. All samples were previously diluted twice with culture medium containing 20% Fetal Serum Bovine.
The day before experiment the two cell lines L929 and Mg63 were seeded in sterile flat bottom 96 wells tissue culture plates (Greiner, Germany), at a cell density of $2x10^5$ cells/mL and $1x10^5$ cells/mL 100 µL per well, respectively. Cells were incubated at 37ºC and 5% CO_2.
On the next day, culture medium was replaced by fresh medium containing the different samples to be analyzed; each concentration was tested in six wells per plate. Cells were incubated for 24h and 48h. The negative control was the release medium and positive control was sodium dodecyl sulfate (SDS) at 1 mg/mL. After each time of exposition, medium was replace by medium containing 0.5 mg/mL MTT. The cells were further incubated for 3h. The media was removed and the intracellular formazan crystals were solubilized and extracted with 100 µL DMSO. After 15 min at room temperature the absorbance was measured at 570 nm in Microplate Reader (FLUOstar Omega, BMGLabtech, Germany).
The relative cell viability (%) in comparison to negative control was calculated by the equation: $[\text{Absorvance } 570\text{nm}]_{\text{sample}}/[\text{Absorvance } 570\text{nm}]_{\text{negative control}} \times 100$.

2.5.3.3. Direct contact with BC composites

Cell response in direct contact with the different BC matrices was evaluated by the observation of the cell culture proliferation by phase contrast after MTT reduction and by fluorescence microscopy.

2.5.3.3.1. Phase-contrast microscopy

Bone cement plates (8 × 8 × 0.2 mm) prepared according to previously described procedure (section 2.2.3.1.), were placed in 24 well tissue culture plates (Greiner, Germany) and MTT assay was performed. Cell viability was assessed after 24h of incubation of the two cell lines (L929 and MG63) in all BC plate matrices. The procedure was performed as described above (section 5.1.1). Cells were cultured at a density of $2.5x10^5$ cells cm^{-2} and $1.0x10^5$ cells cm^{-2}, respectively in direct contact with the BC composite matrices. Glass slides were used as negative controls (non-toxic material). After 24h, MTT solution was added to a final concentration of 0.5 $mg.mL^{-1}$ and incubated for 3h at 37 ºC. Following incubation, culture plates were observed under an inverted microscope (MEIJI TC5400, Japan) coupled with a digital camera (Canon A650 IS G4, Japan) and photos were taken of the interface between matrices and plate.

2.5.3.3.2. Fluorescence microscopy

Cells (L929 and MG63) were grown in sterile glass slides (control material) or tested matrices into 24 wells tissue culture plates (Greiner, Germany) for immunocytochemistry assays. After incubation, cells were rinsed three times with 10mM PBS containing 20 mM glycine at pH 7.4, before and after being fixed for 15 min (at room temperature in dark) with paraformaldehyde 4% (w/V) in PBS. After cell fixation, and for actin staining with rhodamine phalloidin, cells were permeabilized with 0.1% (V/V) Triton X-100 for 4min and then rinsed the same way as described above. The 6.6 mM phalloidin-TRITC solution in 10 mM PBS was added to the cells for 30 min at room temperature. Then, and after rinsing, cell slides were mounted in fluorescent mounting medium ProLong Gold antifade reagent with DAPI their fluorescence was observed and recorded on an Axioskop 40 fluorescence microscope (Carl Zeiss, Germany) equipped with an AxioCam HRc (Carl Zeiss, Germany) camera. Images were processed with the software Axiovision Rel. 4.8.1. (Carl Zeiss, Germany).

2.6. Statistical Data Analysis

All data are expressed as mean and standard deviation (mean ± SD) of independent experiments (minimum n=3, depending on the assay). Statistical evaluation of data was performed using one-way analysis of variance (ANOVA). Pos-hoc Tukey´s multiple comparison test (GraphPad PRISM 5 software, USA), was used to compare the significance of the difference between the groups. The level of statistical difference was defined at a $p<0.05$.

Chapter 3. Results and Discussion

3.1. Chitosan and alginate nanoparticles preparation and characterization

In order to choose the best formulation to scale up and produce a large amount of nanoparticles the parameters, absorbance, size distribution and zeta potential of all samples that didn't present clusters formation, were measured and the best two formulations were selected.

Table 5 – Example of parameters obtained for different chitosan formulations.

Formulation	Absorvance	Z-Ave (d.nm) ± SD	PDI ± SD	ZP (mV)
1	0.53	366.43±14.47	0.77±0.04	+28.23±0.70
2	0.51	6493.00±1032.42	0.41±0.28	+33.30±0.55
3	0.32	8125.33±1443.12	0.66±0.40	+0.63±1.39
4	0.82	631.47±25.78	0.48±0.02	+15.00±1.51

Note: Z-Ave, average size; PDI – polydispersive index; ZP, zeta potential.

Different formulations, varying reagents concentrations and pH are represented in table 5 as numbers to simplify reading. Formulation nº3 was immediately excluded as formed agglomerated of nanoparticles.
The selected formulation for scale-up was Formulation nº1 as it presented the lowest size distribution and PDI values. The obtained values for size and zeta potential are in concordance with those obtained by *Figueiredo et al*., (2012), demonstrating the reproducibility of the method.

In what concern alginate nanoparticles, some combinations were immediately discarded by the formation of a gel solution (that was visually observed); the ones that didn't form a gel were analyzed, like de chitosan nanoparticles and the formulation chosen to scale up was: 0.3% sodium alginate (40% G-content) with 0.5% $CaCl_2$ at 1:3 proportion which presented 600±34.16 nm, +0.50±0.11 of PDI and -23.73±1.93 mV of zeta potential. *Sarei et al.,* (2013) obtained smaller and more negative particles (around 100nm and -47 mV), but for the purpose of the work the particles obtained are good as well.

3.2. *In vitro* Release Assays

Release studies were assessed aiming to evaluate different variables on levofloxacin release profile namely:

i) Bone cement composition;
ii) Loading of biodegradable polymers as permeability enhancers;
iii) Loading of a second drug;
iv) Release media composition.

Furthermore, the effect of permeability enhancers (polymeric nanoparticles) and release media composition was also evaluated on diclofenac release profiles.

3.2.1. Part I – *Effect of bone cement composition and additives loading on levofloxacin release*

First assays were conducted with two bone cements (BC) with different compositions: CMW 1 and SmartSet. The aim was to evaluate the impact of different physicochemical BC properties on levofloxacin release profiles.

BC matrix loaded only with levofloxacin ([BC]Lev) was used as a control. The biodegradable polymers, chitosan and alginate, were also loaded into BC to evaluate the effect of their inclusion on levofloxacin release (Fig. 19 - 22). The assays were conducted during a period of 3 weeks in NaCl 0.9% (w/V) medium, which presents an acidic pH (5), simulating the common pH value of inflammated areas (*Lardner* 2001 and *Rajamäki et al*., 2013). It should also be noted that two different loading percentages of both polymers were tested (5% and 7.5% w/w). Also, different forms of alginate, namely powder or nanoparticles (NPs) were evaluated. In respect to chitosan, only NPs were tested as it is already referred in literature that chitosan in powder form diminished drug (gentamicin) release and didn't prevent biofilm adherence (*Tunney et al.,* 2008). On the other hand other studies revealed that chitosan NPs presented higher antimicrobial activity without compromising BC mechanical integrity (*Shi et al.,* 2006).

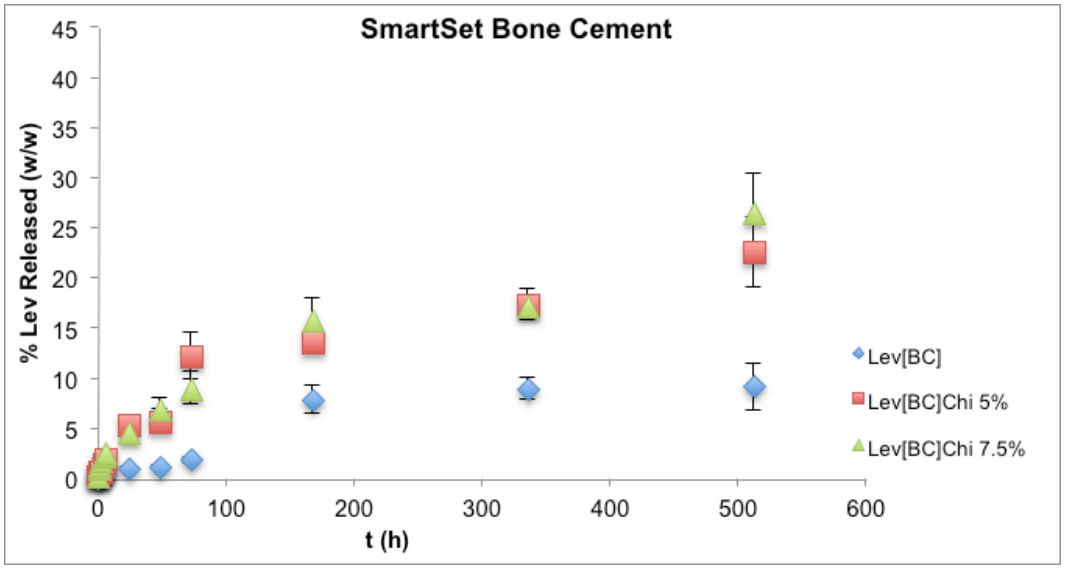

Figure 19 – Effect of chitosan (Chi) loading on the release profiles of levofloxacin during a 3-week period. Polymer % refers to the loading of nanoparticles.

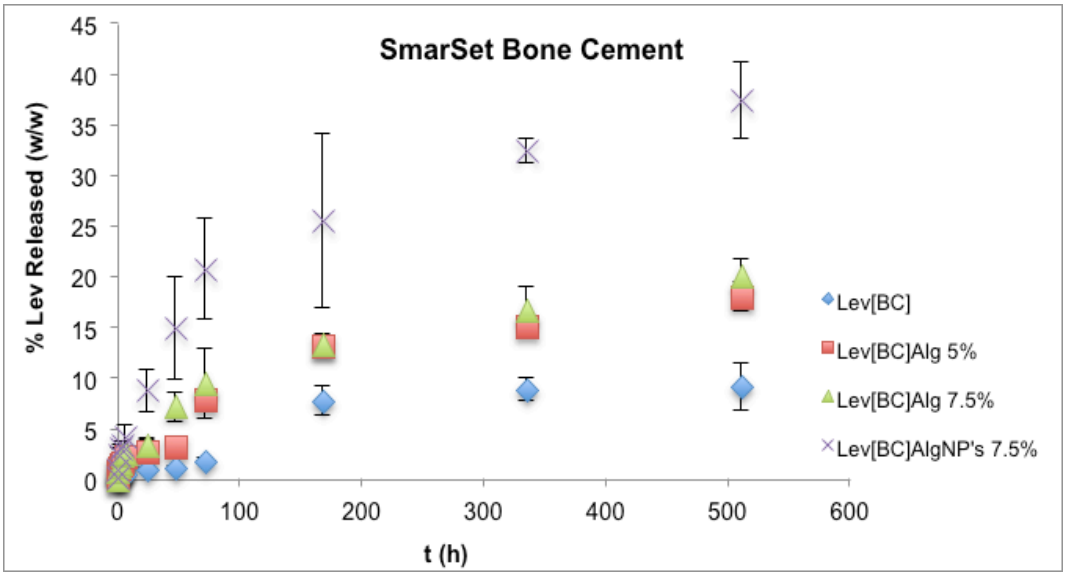

Figure 20 – Effect of alginate (Alg) loading on the release profiles of levofloxacin during a 3-week period. Polymer % refers to the loading of powder form and nanoparticles (NPs).

Levofloxacin release profiles from SmartSet showed that control matrix (Lev[BC]) presents lower amounts of released drug when compared to BC matrices loaded with the polymers (Fig. 19 and 20). It can also be observed that the insertion of chitosan NPs in two different amounts does not significantly alters the release when compared to alginate in powder form, whoever alginate NPs considerably increases the drug release.

Thereafter CMW 1 was tested (Fig. 21 and 22).

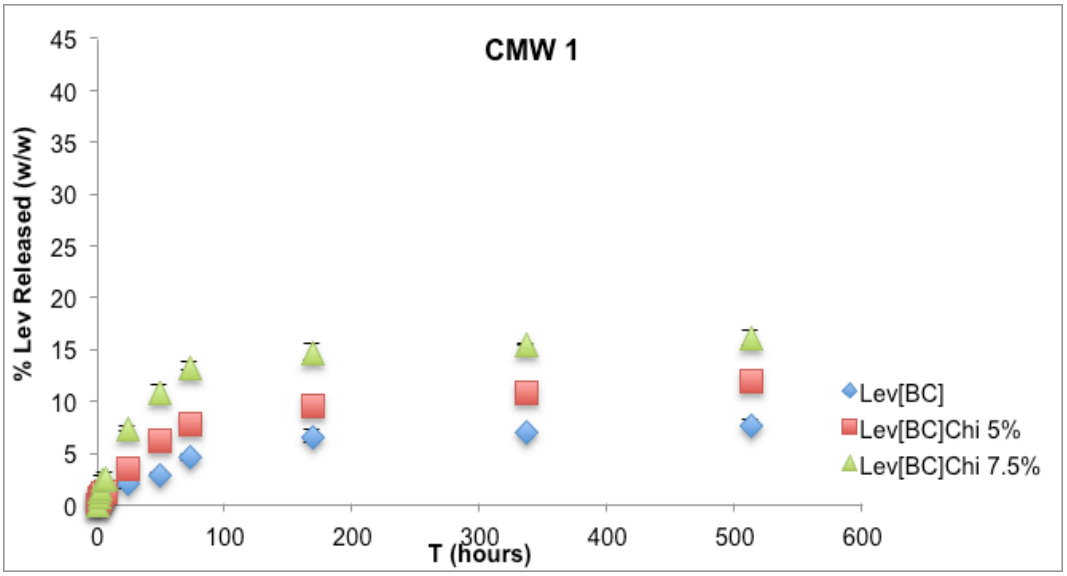

Figure 21 - Effect of chitosan (Chi) loading on the release profiles of levofloxacin during a 3-week period. Polymer % refers to the loading of nanoparticles.

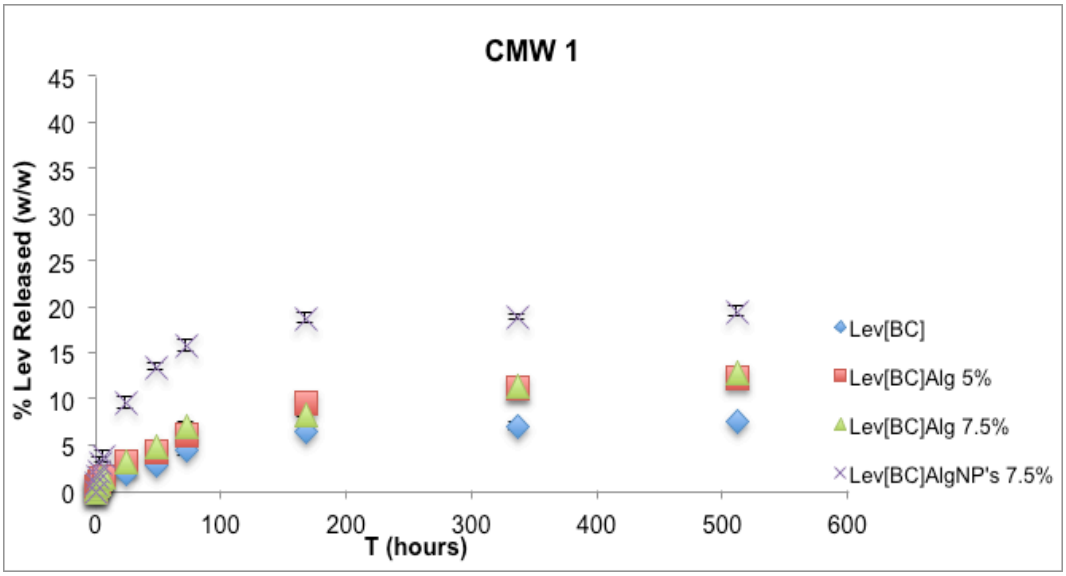

Figure 22 - Effect of alginate (Alg) loading on the release profiles of levofloxacin during a 3-week period. Polymer % refers to the loading of powder form and nanoparticles (NPs).

Levofloxacin release profiles from CMW 1 were similar to the ones observed for SmartSet matrices. Higher amount of chitosan NPs increased drug release. Alginate NPs loading produced a higher release of drug in comparison to the powder form.

Overall, all BC matrices loaded with the evaluated polymers have showed higher drug release when compared to control Lev[BC], which proves that the insertion of any tested additives increases drug liberation (in both cements). Usually similar results are obtained when these and other polymers or compounds are included into BC. In *Matos et al*., (2014), the addition of 10% (w/w) lactose in a bone cement matrix, increased significantly the minocycline release; in *Matos et al*., (2015a) a new BC composite, containing levofloxacin- adsorbed CaP particles loaded into a lactose-modified acrylic BC, was tested obtaining 6% of drug release after 8 weeks and released values superior to *S. aureus* MIC after only 6 hours; also in *Matos et al.,* (2015b) the insertion of only lactose in BC increased levofloxacin released in 20% when compared to BC control (loaded only with the drug). Interestingly some authors refer to a decrease of drug release due to the insertion of additives into BC. For example, *Tunney et al.,* (2008) concluded that the insertion of chitosan powder 1-5% (w/w) in PMMA bone cement decreased gentamicin release and also decreased significantly the mechanical properties of the cement.

To date several studies have been made using these polymers (chitosan and alginate) as carriers (beads, nano- and microparticles) for drug release; those studies show that there was no interaction polymers/drug *(Amit et al.,* 2012; *Balaji et al.,* 2015 and *Fernandez-Hervas et al.,* 1998), and all drug release was a pH dependent phenomena. It is shown that chitosan is more soluble in acidic media, and on the contrary alginate at very low pH values don't swell, being insoluble *(Fernandez-Hervas et al.,* 1998). As our studies were conducted at pH 5, that is slightly acidic, it is reasonable to assume that although having some influence, pH is not the main factor influencing drug release. Another factor that may influence drug release will be polymer-NPs size as it may affects BC porosity. In the context of our results the particles size may then be the most relevant factor *i.e*., at the initial times the amount of drug released to the media is the drug at the bone cement surface allowing inner porous being wetted and allowing the release of other drug particles, with the insertion of biodegradable polymers,

and once they are soluble, along the time more porous will be formed allowing a major amount of drug to be released. At pH 5 both chitosan and alginate are soluble, so or this value is more suitable for one that for another or NPs size that influences the release. Chitosan nanoparticles have an average size distribution of 366 nm and alginate nanoparticles of 600 nm, which is almost twice, as such when polymer is solubilizing, alginate NPs will make bigger porous allowing more drug to be release, explaining this way the results obtained until now.

As previously described, not all compound loading into BC have a positive effect in antibiotics release. In this context, once studies concerning the release of levofloxacin from PMMA bone cements loaded with chitosan have not been made to date, and studies with alginate have not been realized at all, it is imperative that *in vitro* release studies are conducted before other tests are realized.

Comparing the two types of cements after 1h and a 3-week period (Table 6), it can be observed that the composition of cement highly influences the release of levofloxacin. All SmartSet matrices showed higher levofloxacin release than CMW1. This could be due to cement composition, SmartSet presents 80% (w/w) of methylmethacrylate copolymer whereas CMW 1 presents 84.7% (w/w) of poly(methylmethacrylate) copolymer, being this last with higher viscosity, which could result in less porosity in BC formulation after curing. This fact could explain the lower amounts of antibiotic released when compared to SmartSet as less pores are available for drug dissolution and diffusion.

It was also verified for both cements that the matrix that presented higher values of levofloxacin release was BC with alginate NPs, comparing to control Lev[BC] (Table 6).

Table 6 – Comparison between different types of bone cements (mean ± SD; n=3), values in percentage of drug released (%).

Matrices			
Time = 1h	**CMW 1**	**SmartSet**	**Significance**
Lev[BC]	0.99±0.11	0.27±0.03	P < 0.01
Lev[BC]Chi NPs 5%	0.72±0.03	0.75±0.02	NS
Lev[BC]Chi NPs 7.5%	1.28±0.05	1.39±0.06	NS
Lev[BC]Alg 5%	0.79±0.09	1.20±0.03	P < 0.01
Lev[BC]Alg 7.5%	0.73±0.05	1.38±0.04	P < 0.01
Lev[BC]Alg NPs 7.5%	1.69±0.17	2.45±0.29	NS
Time = 3 weeks			
Lev[BC]	7.69±0.16	11.68±0.71	P < 0.01
Lev[BC]Chi NPs 5%	12.01±0.07	23.60±1.41	P < 0.01
Lev[BC]Chi NPs 7.5%	16.07±0.26	29.00±1.60	P < 0.01
Lev[BC]Alg 5%	12.44±0.20	18.05±0.59	P < 0.01
Lev[BC]Alg 7.5%	13.01±0.27	20.12±0.69	P < 0.01
Lev[BC]Alg NPs 7.5%	19.54±0.21	37.44±1.52	P < 0.01

NS = no statistical significant difference, p >0.05

Studies proceeded with CMW 1 as it is the cement already under in investigation in the research group where the present work has been conducted.
Among different CMW 1 formulations, BC loaded with polymeric nanoparticles (7.5 % w/w) were selected to be further evaluated as are the ones that show high levofloxacin release.

3.2.2. Part II – *Effect of diclofenac and polymer loading on drug release*

a) *Release studies in NaCl*

Studies continue evaluating the effect of loading a second drug, diclofenac, into BC.

When implanted into living tissue, PMMA bone cement initiates an inflammatory response around the implant for being a foreign body and due to material erosion processes that release wear particles from the cement into the tissues. Due to stimulation of a local inflammatory response, granulomas may be formed leading to aseptic loosening (*Wooley et al*., 2004), implant failure and joint revision surgery (*Bettencourt et al.,* 2007).

Therefore, the rational to include diclofenac, an anti-inflammatory drug, was to reduce the inflammation associated with implant loosening.

Another limitation of PMMA cement is its lack of adhesion to the bone leading to the formation of a fibrous tissue around the cement that prevents the bone growth towards the surface. So, to achieve better biocompatible results a bioactive PMMA cement that develops a direct bond with the living bone is desirable, and some authors propose that non-steroidal anti-inflammatory drugs may have this effect being enhancers of bone tissue (*Lopes et al.,* 2013; *Konstantinidis et al*., 2013). However, the effect of these drugs on bone metabolism is still controversial. Some studies have reported that anti-inflammatory drugs improve bone mineral density and bone level as well as bone formation and decrease bone adsorption (*Bauer et al.,* 1996; *Carbone et al.,* 2003; Jee 1998; *Kasukawa et al.,* 2007), but the contrary was also observed by other authors (*Dimmem et al.,* 2009; *Kohrt et al.,* 2010; *Persson et al.,* 2005). Further research on this matter is necessary.

Furthermore, diclofenac could play a role in decreasing biofilm adhesion. *Reslinski et al.,* (2013) described a decrease in biofilm formation in the surface of propylene mesh when diclofenac (1 µg/mL) and ibuprofen (20 µg/mL) were used. The mechanism is not fully understood once that it would depend on the bacterial strain and with the drug used. For example to *E. coli* it was demonstrated that changes in bacteria hydrophobicity could be one explanation when ibuprofen is used. In *Riordan et al.,* (2011) it was determined that sub-inhibitory concentrations of diclofenac alter the expression of hundreds of genes associated with resistance to antimicrobials, by altering the expression of regulatory and structural genes associated with cell wall biosynthesis/turnover and transport, increasing the susceptibility of *S. aureus* to the fluoroquinolone antibiotics ciprofloxacin, norfloxacin and ofloxacin, so being of utmost interest to add to bone cement.

Results showed that diclofenac loading into BC did not have a significant effect on levofloxacin release (Fig. 23).

When polymers as additives were also added to BC the effect of alginate is not so evident as previously observed (Fig. 20 and 22). On the contrary, chitosan has a high effect on levofloxacin release.

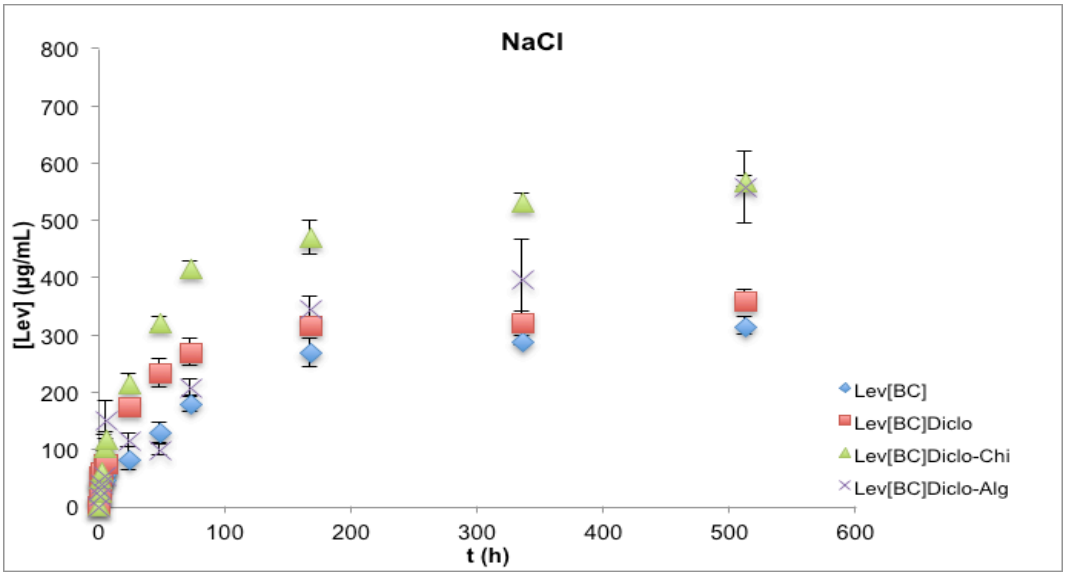

Figure 23 – Levofloxacin release profiles in a 3-week period. Lev[BC] was used as a control.

Diclofenac release profiles were also evaluated (Fig 24). It was observed that the presence of levofloxacin decrease diclofenac release (Fig. 24). It was also verified that adding chitosan NPs didn't improve drug release. On the contrary alginate seems to increase the quantity of released diclofenac. *Muhammad et al.,* (2015) reported physical-chemical interactions between levofloxacin and diclofenac. It was demonstrated that at pH 6.8 levofloxacin availability in the presence of diclofenac sodium suffer a tremendous rise, however the availability of diclofenac sodium in the presence of levofloxacin was observed to be low, which may be associated with the formation of charge-transfer complex, due to rearrangement of electrons. The described interaction between drugs could explain the obtained results, that are diclofenac does not affect levofloxacin release whereas diclofenac release was significantly affected by levofloxacin presence in the BC.

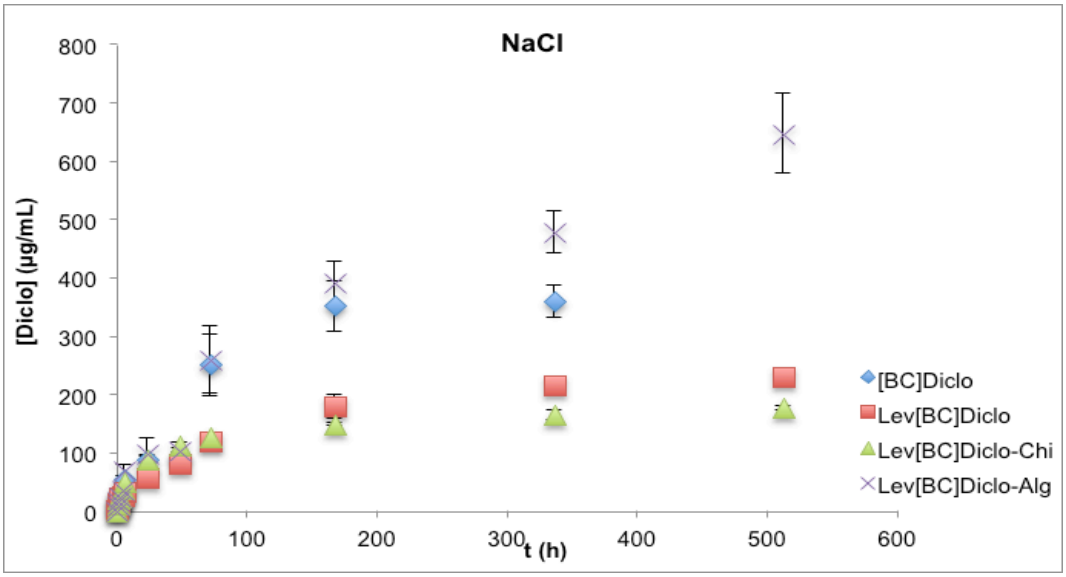

Figure 24 - Release profiles of diclofenac in a 3-week period (except for Lev[BC]Diclo, stopped at 2-week due to fungi contamination).

b) *Release studies in PBS*

Release studies in PBS (physiological pH = 7) were then conducted with the intention to evaluate the effect of pH on drug release profiles.
Studies showed that PBS medium influenced both drugs release (Fig. 25 and 26).
For levofloxacin (Fig. 25), it was verified that release has changed in comparison to pH=5, maintaining chitosan the matrix that releases more antibiotic (Fig. 21). Alginate did not affect the release in comparison to the control (Lev[BC]Diclo).

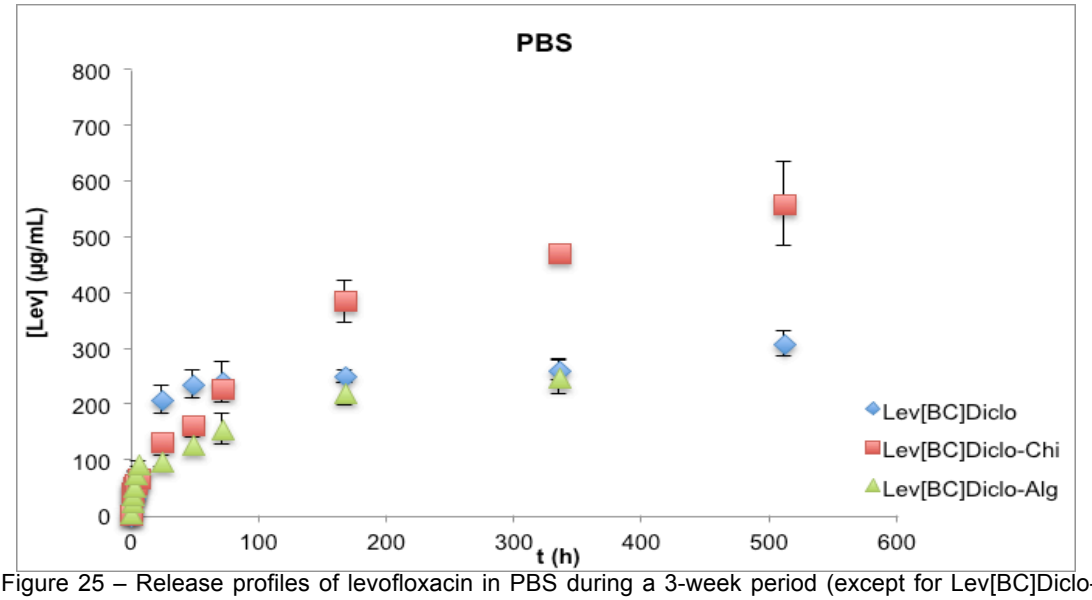

Figure 25 – Release profiles of levofloxacin in PBS during a 3-week period (except for Lev[BC]Diclo-Alg, stopped at 2 week due to fungi contamination).

In respect to diclofenac (Fig. 26), it was observed that Lev[BC]Diclo and Lev[BC]Diclo-Chi keep the same release behaviour. However, for alginate matrix, PBS medium seems to significantly affect diclofenac release. Whereas in NaCl (pH=5), alginate matrix shows high diclofenac release (Fig. 22), in PBS (pH=7) it shows the lower values (Fig. 26), which means that PBS medium has a major influence in alginate matrix.

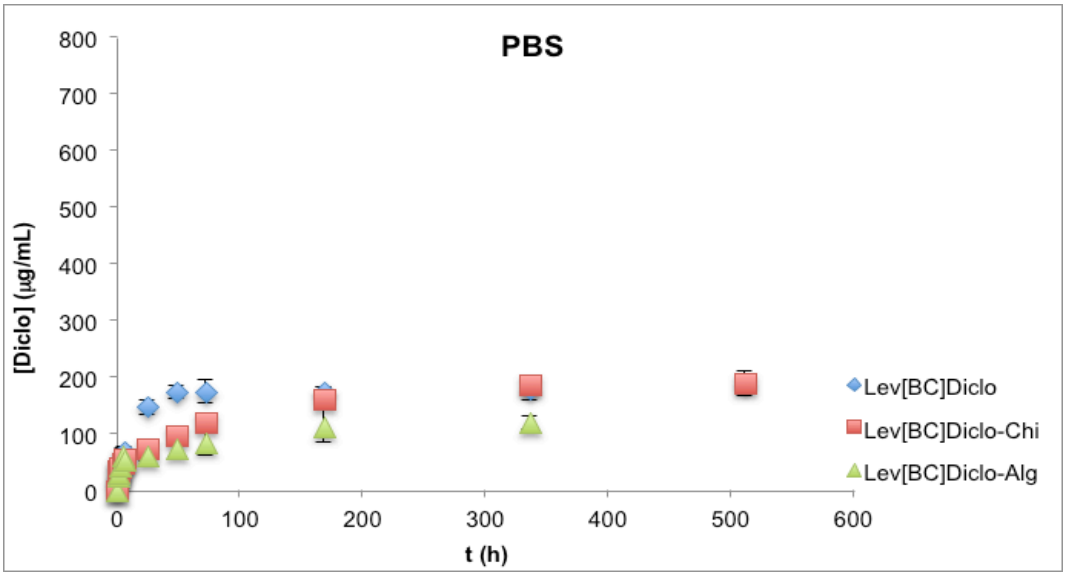

Figure 26 – Release profiles of diclofenac in PBS during a 3-week period (except for Lev[BC]Diclo-Alg, stopped at 2 week to fungi contamination).

According to *Fernandez-Hervas et al.,* (1998), it was expected that alginate polymer in PBS pH 7 released drug more easily that chitosan, but that was not observed. This could be due to the phenomena described by *Fernandez-Hervas et al.,* (1998) in which alginate swells at higher pH's. At pH = 7 it could be possible that the swelling causes bone cement pores clogging impeding this way the release of both drugs, as observed in the results.

c) *Release studies in albumin solution*

Finally, release assays were conducted in albumin solution in PBS (5%). Albumin is the most abundant protein in human blood plasma and acts as a transporting protein (*Peters* 2012). Therefore, the aim of the study was to evaluate the effect of albumin on drug release.

For levofloxacin (Fig. 27), the release due to polymeric nanoparticles loading is increased in the presence of albumin.

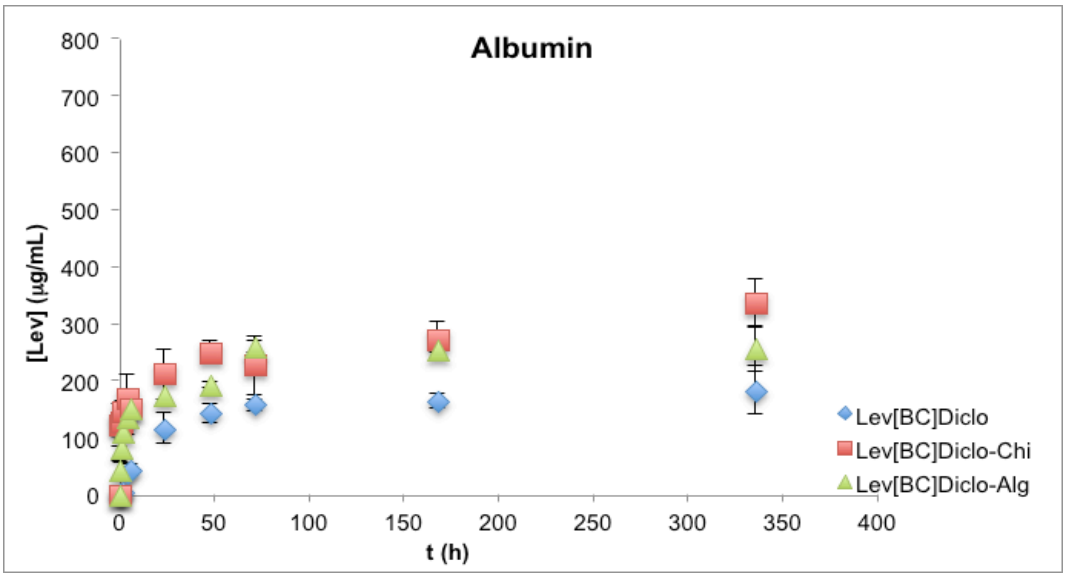

Figure 27 – Release profiles of levofloxacin in albumin solution during a 2-week period.

In respect to diclofenac (Fig. 28), release is also increased in bone cement loaded with polymers, in particularly for chitosan.

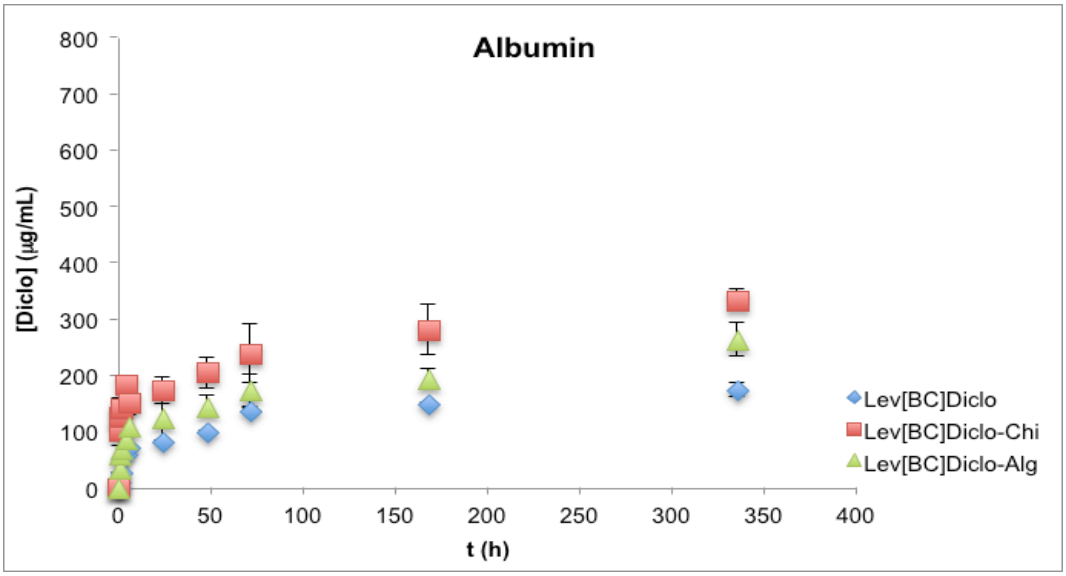

Figure 28 – Release profiles of diclofenac in albumin solution during a 2-week period.

Table 7 compares the maximum values of release for both compounds in two media (PBS or albumin solution).
Chitosan increases levofloxacin release in both media in comparison with control matrix (Lev[BC]Diclo) and alginate loaded BC. Chitosan also increases diclofenac release in albumin media. Alginate decreases diclofenac release in PBS but increases its release in albumin media.
Levofloxacin release from chitosan loaded BC is higher in PBS in comparison to albumin. On the contrary, diclofenac release is higher in albumin.
Thus, results suggest that albumin seems to bond preferentialy to levofloxacin rather than to diclofenac. *Seedher et al.,* (2013) describes that albumin has two binding sites being site II the only for diclofenac and site I and II for levofloxacin. As such, it is possible that levofloxacin binds to both sites of albumin explaining this way its decrease of release in albumin medium. As levofloxacin is being captured by albumin, less quantity of the antibiotic interacts with the anti-inflammatory drug allowing diclofenac to be released in higher quantities.

Table 7 – Maximum concentration values (µg/mL) of levofloxacin and diclofenac released during a 2 week period, in the different tested matrices (mean ± SD; n=3).

	PBS Media		PBS and Albumin Media	
Matrix	Concentration of Levofloxacin (µg/mL)	Concentration of Diclofenac (µg/mL)	Concentration of Levofloxacin (µg/mL)	Concentration of Diclofenac (µg/mL)
Lev[BC]Diclo	261.23 ± 18.82	179.51 ± 19.15	183.75 ± 41.56	173.42 ± 12.37
Lev[BC]Diclo-Chi	471.14 ± 12.37	188.18 ± 16.19	335.86 ± 42.19	334.05 ± 18.69
Lev[BC]Diclo-Alg	248.89 ± 29.65	119.94 ± 10.28	257.33 ± 38.90	264.82 ± 29.71

Overall, release studies provide important information about drug behaviour in different media and what to expect for further studies. These type of assays are of utmost importance because they allow to choose the best and most suitable formulations to proceed and discard the unsuitable ones.

Along the reported studies several conclusions were taken:

i) the composition of BC highly influences the drug release due to several factors as for example different viscosities;

ii) the loading of alginate nanoparticles (7.5 %) improved levofloxacin release in comparison to the powder form;

iii) diclofenac loading into BC didn't affect levofloxacin release but the inverse occurred;

iv) the effect of loading polymeric nanoparticles on drugs release was highly influenced by release media composition (pH and albumin). In most cases chitosan NPs had a higher effect on levofloxacin and diclofenac release than alginate.

3.3. Bone Cement Characterization

It is of major importance to analyze the effects that additive loading has on bone cement inner structure and possible alterations on its surface and mechanical properties. Therefore, several assays were conducted in order to observe possible changes, namely, determination of surface energy by measurement of contact angles, evaluation of surface structure change by Scanning Electron Microscopy (SEM), and biomechanical test (compressive strength).

3.3.1. Surface Energy

Adding additives to bone cement may cause changes on surface energy depending on additives own physico-chemical properties. It is of utmost importance to determine those differences in order to predict the future behavior of cement when in contact with proteins and cells. Surface energy of a material influences protein adhesion and proliferation of cells like osteoblasts, monocytes and endothelial (*Marques et al.,* 2002). Once it is not possible to determine surface energy directly, it was necessary to determine contact angles between BC plates and two liquids, one polar (water) and another less polar (1,2-propanodiol), results are shown in Fig. 29 and 30.

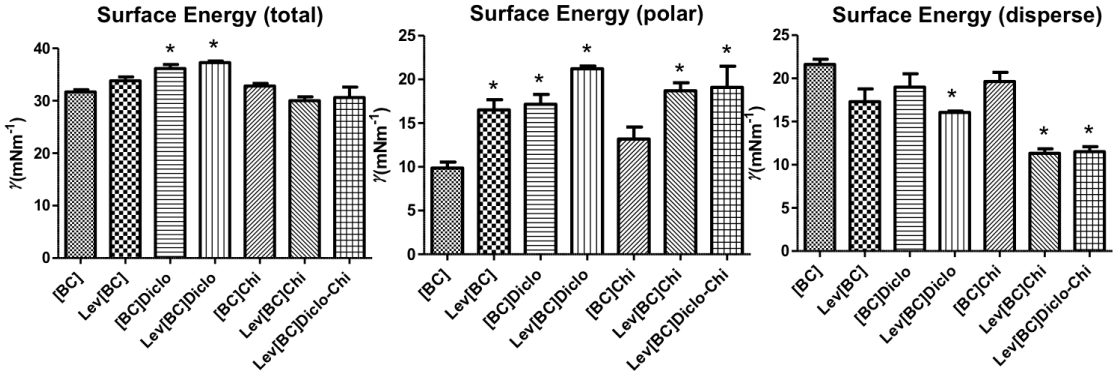

Figure 29 - BC experimental values for total, polar and dispersive surface energy (mean ± SD; n=3) for chitosan polymer matrices. The * means a significant difference to [BC] matrix, $p < 0.05$.

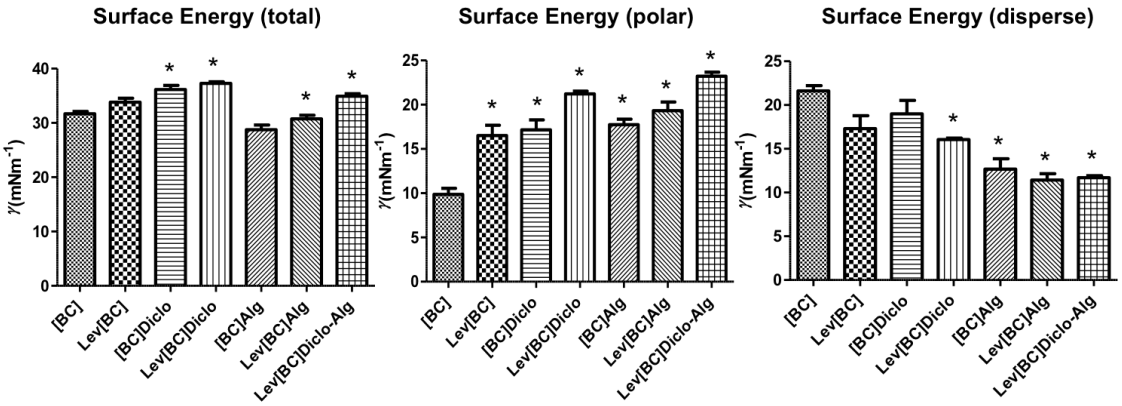

Figure 30 - BC experimental values for total, polar and dispersive surface energy (mean ± SD; n=3) for alginate polymer matrices. The * means a significant difference to [BC] matrix, $p < 0.05$.

Results show that all matrices maintained or increased total surface energy. Furthermore, all BC matrices, excepting [BC]Chi, increased the polar component becoming more hydrophilic. This fact could be due to the insertion of more polar functional groups present in the compounds added to polymer. In *Matos et. al.,* (2014 and 2015a and b) the inclusion of minocycline and levofloxacin did not have also influenced the total surface energy of BC., In the present study although levofloxacin did not affect the total surface energy, diclofenac presented major influence on total surface energy (Fig. 29 and 30).

The next assays (surface structure and compressive strength) were only conducted with the more complex matrices, that is loaded with both drugs and the biopolymers (Lev[BC]Diclo-Chi and Lev[BC]Diclo-Alg), as these matrices will be the ones with major changes.The matrix Lev[BC]Diclo was used as a control.

3.3.2. Surface Structure (Scanning Electron Microscopy - SEM)

SEM analysis was conducted to evaluate the effect of loading of the various additives on BC structure (Fig. 31). Cut-off slices of the compression specimens were used to locate and evaluate the dispersion of the polymeric nanoparticles on the BC composites matrices.

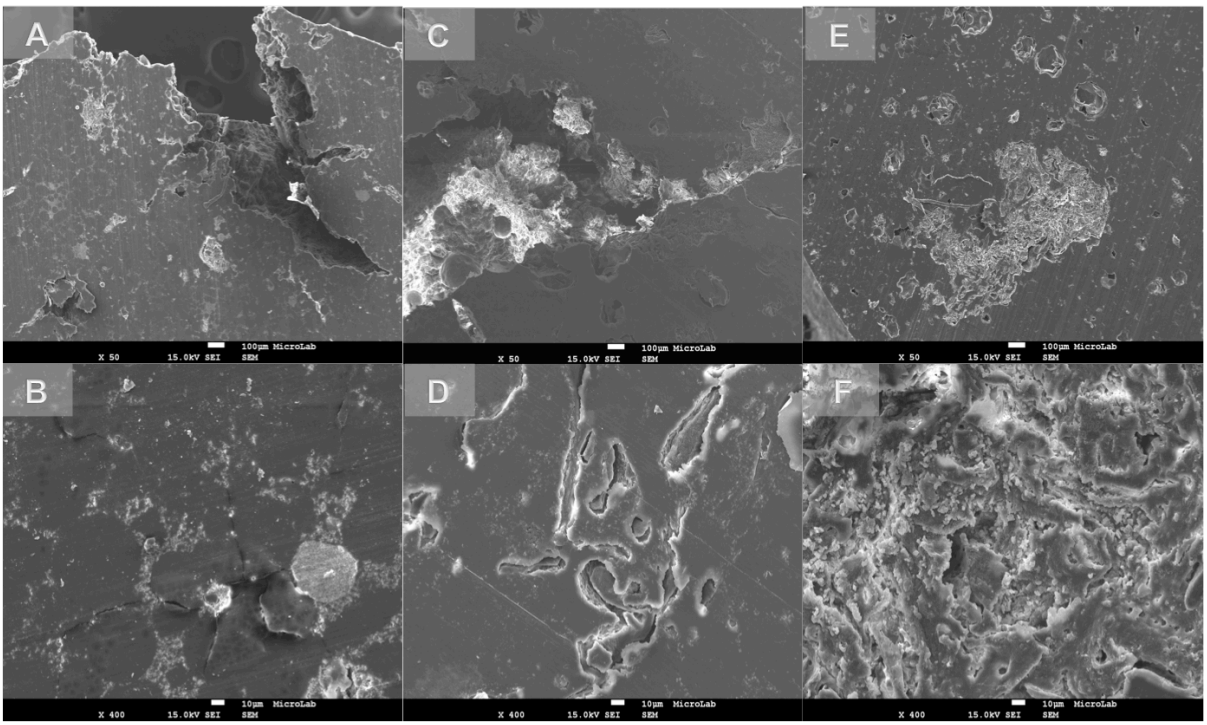

Figure 31 - SEM images of cylindrical surfaces of different BC formulations. A and B – Lev[BC]Diclo, ampliation 50x and 400x respectively; C and D – Lev[BC]Diclo-Chi, ampliation 50x and 400x respectively; E and F – Lev[BC]Diclo-Alg, ampliation 50x and 400x respectively.

SEM images (Fig. 31) revealed a porous surface with cracks and pores distributed along the matrix core, predicting the existence of inner channels. It is also visible that in matrices with nanoparticles (C/D and E/F) those are very evident, and increased porosity comparing to Lev[BC]Diclo (A/B). In particularly alginate matrices presents high porosity. This is of major importance once that corroborates the result of the nanoparticle incorporated matrices releases higher amounts of drugs, as it is possible to perceive that are more pathways to drugs to be released. Similar results were obtained

by *Matos et al.,* (2014a and 2015b), concerning lactose loading in bone cements. The inclusion of lactose also increased cement porosity when compared to the unloaded BC.

It is predictable that after release those pores and cracks should be more visible especially in matrices with alginate and chitosan nanoparticles, once that polymer through time dissolves and leave wider holes. To observe that effect it would be interesting to have also SEM images of those BC specimens, (obtained after the release process has been concluded).

Complementary EDS analysis was also conducted (Fig. 32). Data confirmed the presence of characteristics elements of some of the additives loaded in the cements formulations, namely the chlorine from diclofenac and sodium from diclofenac salt and alginate. Fluorine from levofloxacin does not appear in EDS analysis, probably because its concentration is below the EDS detection limit. Levofloxacin has only one atom of fluorine (Fig. 32, A). Diclofenac has two atoms of chlorine (Fig. 32, B) and the detection is very low, except for Lev[BC]Diclo-Alg (Fig. 32, F) that has higher peaks suggesting higher amounts of diclofenac on the cement surface due to higher pores and cracks as observed in (Fig. 32, image F).

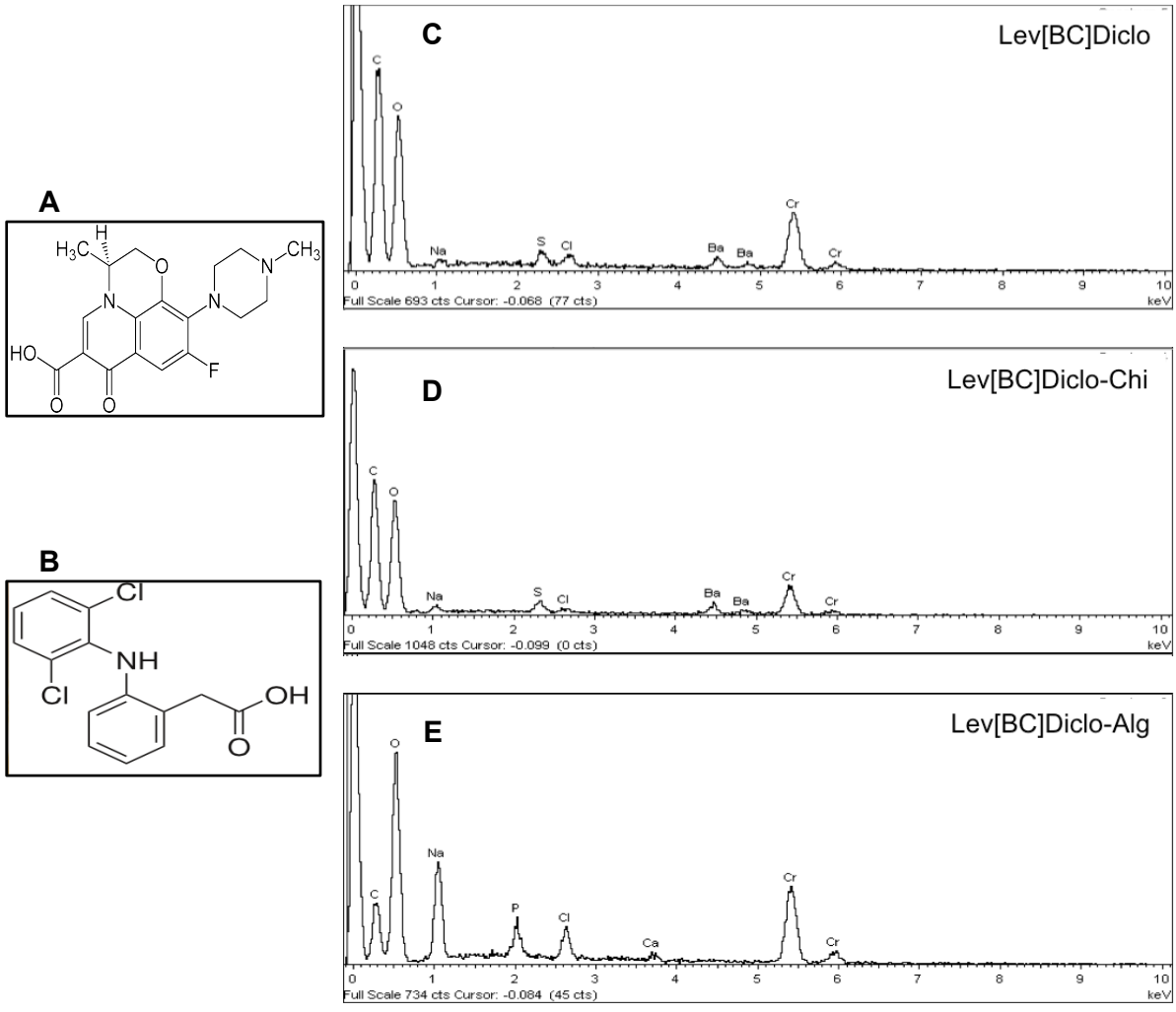

Figure 32 - Levofloxacin (A) and Diclofenac (B) structures; Energy Dispersion Spectroscopy graphics of three different BC formulations (C-E) respectively.

3.3.3. Biomechanical Tests – Compressive Strength

Finally, mechanical tests were conducted to better understand the structural effects due to the addition of the compounds. In this field several tests are realized and results must be in concordance with ISO 5833 required values. The compressive strength test results are shown below (Table 8).

Table 8 – Compressive Strength (MPa) of three types of BC matrices (mean ± SD; n=3).

Matrix	Compressive Strength, Mpa
Lev[BC]Diclo	114.69 ± 5.93
Lev[BC]Diclo-Chi	101.82 ± 3.38
Lev[BC]Diclo-Alg	105.85 ± 4.59

Normative ISO 5833 requires that for compressive strength, BC has to show values higher than 70 MPa to be accepted, and as table 8 shows all tested matrices presented higher values. *Matos et al.,* (2015a) obtained the same results when incorporated calcium-phosphate particles and levofloxacin in BC, it was demonstrated that mechanical properties were not decreased.

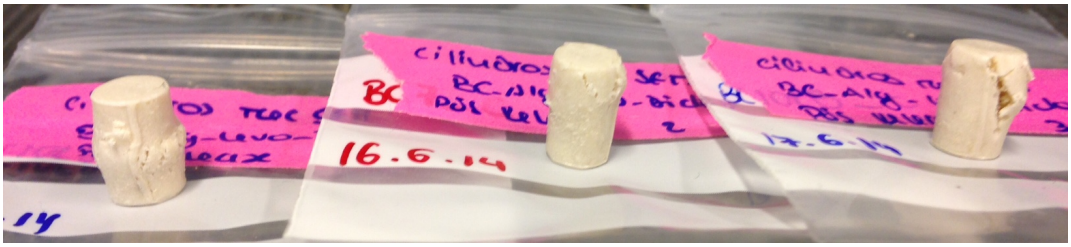

Figure 33 – Pictures of bone cement specimens after mechanical assay of compressive strength.

Figure 33 demonstrates the effect of compressive tests in bone cement matrices.

Further studies should be conducted in order to better understand the inner BC structure and outer surface, like X-ray diffraction studies, flexural strength and flexural modulus among others.

3.4. Microbiological Assays

In order to verify the antimicrobiological effect of the different BC formulations, several assays were conducted.

3.4.1. Antimicrobial activity evaluation

First assays served as controls to confirm that levofloxacin did not lose activity during BC setting, namely during the polymerization process, where high temperatures are reached and reactive species are produced (Matos *et al.,* 2014).

3.4.1.1. Plain bone cement

Standard levofloxacin was tested in order to evaluate if the drug keep its antimicrobial activity in the presence of BC plates (if for example does not interact with any BC surface component). Plain BC plates were exposed to different levofloxacin solutions in BHI and *S. aureus* was then inoculated. After 24h bacterial growth was observed to determine MIC (minimum inhibitory concentration) and MBIC (minimum biofilm inhibitory concentration).

3.4.1.1.1. *Minimum inhibitory concentration*

The MIC value was 0.25 µg/mL, since it was the lowest concentration corresponding to no observable bacterial growth.

This value is in accordance to what is described in literature, in *Matos et al.* (2015a) and *Schmitz et al.,* (1998) as the MIC value for *S. aureus* is described in those studies to be equal or superior to 0.25 µg/mL.

3.4.1.1.2. *Minimum biofilm inhibitory concentration*

To determine MBIC, the biofilm formation in BC plates after exposure to different BC plates was evaluated (Table 9):

Table 9 – Concentrations of levofloxacin and the correspondent biofilm (measured in absorbance).

[Lev] (µg/mL)	4	2	1	0.5	0.25	0.125
Biofilm (abs)	0.46	0.60	0.89	1.11	1.35	3.08

As observed, biofilm was formed in all plates in all levofloxacin tested concentrations, so the MBIC value determined, under this assay conditions, is ≥ 4 µg/mL.

As observed in MIC and MBIC assays, levofloxacin maintained its activity in BHI medium and against the tested strain of *S. aureus* in the presence of plain [BC] plates.

3.4.1.1.3. *Antimicrobial Activity of diclofenac*

The aim of the assay was to evaluate a potential synergetic effect of diclofenac on levofloxacin microbiological activity.

Some studies point to the fact that diclofenac solutions may have antibacterial properties once it limits biofilm adhesion (*Reslinski et al.,* 2013). It could be a major benefit to include diclofenac into BC formulation once it could act at the same time as an anti-inflammatory drug, diminishing the associated BC tissue inflammation, and as antibacterial agent reinforcing levofloxacin action and consequently inhibiting biofilm formation.

For that purpose an assay was conducted where plain [BC] plates were exposed to an inoculated solution of levofloxacin ([LEV]=0.3μg/mL, *i.e*. around MIC) and diclofenac.

Contrary to what it was expected it is clear that with increasing concentrations of diclofenac also an increase on biofilm formation was observed (Fig. 34). The obtained contrasting results could be due to various reasons, as in the mentioned study different experimental protocols were conducted: strains of *S. aureus*, (clinical strains) and methods for biofilm quantification are also very different (a qualitative method based in the reduction of colorless 2,3,5-triphenyl tetrazolium chloride (TTC) to red formazan and a quantitative method based on assessment of colony number previously incubated in agar).

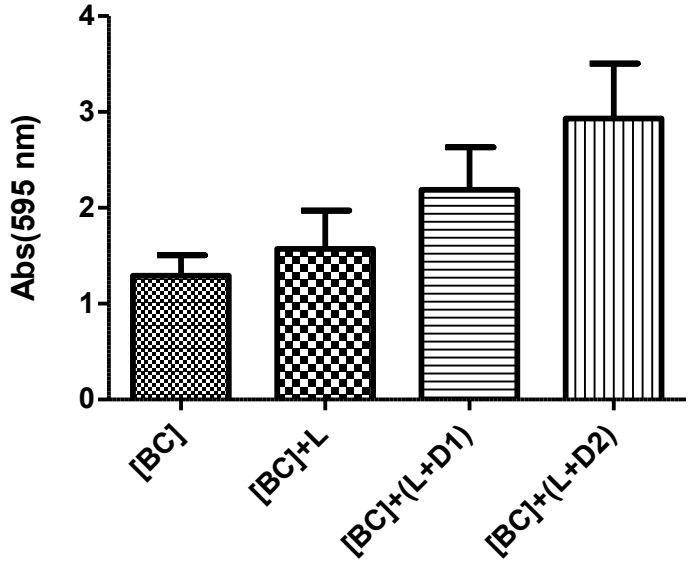

Figure 34 - Biofilm produced in BC matrices incubated in different conditions in a 24 h period. Results are presented as mean±SD; L means [Lev]=0.3 μg/mL; D1 and D2 means [Diclofenac] = 10 μg/mL and D2 = 100 μg/mL, respectively.

In sum, this assay showed that, in the tested conditions, diclofenac does not show any antibacterial function, on the contrary it seems to potentiate biofilm growth.

The possible diclofenac effect on biofilm inhibition is rather controversial.

Several studies have been made in order to analyse the effect of diclofenac on different organisms. Many of these studies are realized in aquatic organisms once that are ecological studies to evaluate

diclofenac toxicity. The studies in *Lawrence* (2007), shows that diclofenac (100 g/L) appeared to have a significant increase in bacterial biomass and increased thickness of biofilm in Eubacteria, Cytophaga-Flavobacterium and Proteobacteria. In *Ferrari et al.,* (2004) several organisms like cyanobacteria, chlorophyta and diatomacea also showed an increase in growth. On the contrary in more complex organisms like small crustaceans and some Pisces diclofenac seemed to have a toxic effect leading to mortality (*Haap et al.,* 2008). Other studies like *Riordan et al*., (2011) revealed that diclofenac increases susceptibility of *S. aureus* to some antibiotics namely some fluorquinolones (ciprofloxacin, ofloxacin and norfloxacin): however in the same study it was also demonstrated that diclofenac can have an adverse effect, reducing susceptibility (inducing phenotypic resistance) to some antibiotics (oxacillin and vancomycin), that could be due to the different bacteria strains. In our assays, when diclofenac was tested with levofloxacin, a reduced susceptibility of *S. aureus* to the antibiotic could have happened.

3.4.1.2. Modified bone cement formulations

The antimicrobial activities of different BC formulations were evaluated by their activity against *S. aureus* biofilm formation. In addition the release of levofloxacin in the BHI medium was also assessed.

3.4.1.2.1. Biofilm inhibitory activity of bone cement

The aim of the assays was to evaluate the effect of different BC formulations on biofilm formation.

The assay took 72h, even if no bacterial growth was observed biofilm was quantified. Results are show in Figure 35.

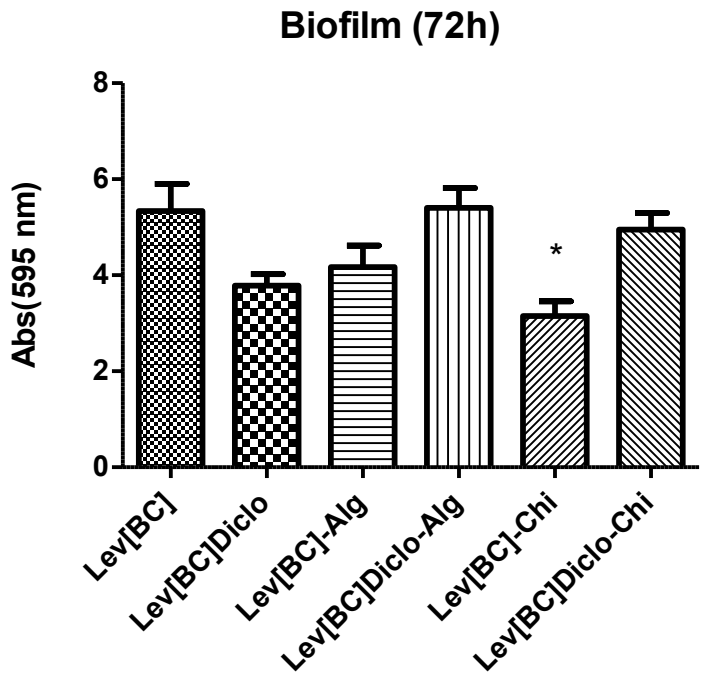

Figure 35 – Quantification of biofilm formed in different BC matrices during 72 h. *means a significant difference to Lev[BC] matrix, $p<0.05$.

Table 10 – Percentage of biofilm decreased, assuming Lev[BC] matrix as 100% of biofilm growth.

Matrix	Biofilm's Percentage (%)
Lev[BC]	0
Lev[BC]Alg	26.25
Lev[BC]Chi	50.09
Lev[BC]Diclo	31.87
Lev[BC]Diclo-Alg	2.64
Lev[BC]Diclo-Chi	19.28

The Lev[BC]Chi matrix has a statistically significant difference from Lev[BC] matrix in decreasing biofilm formation. It can also be seen that matrices with both, polymers and diclofenac, had a slight or even null decrease of biofilm. Table 10 shows in terms of percentage the decrease of biofilm growth in each tested matrix.

The obtained results can be due to several factors. To date, numerous studies have been conducted in order to understand the mechanism of bacterial adherence to various types of substrate surfaces. It is a very complex process, not fully understood yet and with many variables, being the type and behavior of bacteria and type and characteristics (physicochemical mainly) of surfaces, the most relevant ones.
Some studies refer to hydrophobicity and charge as the main factors of bacteria adhesion. *Gottenbos et al.,* (2001) and *Rzhepisheuska et al.,* (2013) concluded that for Gram-negative bacteria, the substrate surface charge has a major influence on adhesion. In *Rzhepishevska et al.,* (2013) negatively charged surfaces prevent bacterial formation of *P. aeruginosa. Gottenbos et al.,* (2001) demonstrated that negatively charged Gram-negative bacteria binds firstly to positively charged surfaces due to electrostatic interactions between negative bacteria and positive surface but after adhesion no growth is observable due to strong electrostatic interactions that don't allow cells to expand, and that negative surfaces reduce adhesion preventing biofilm formation.

In order to understand the adhesion process in Gram-positive bacteria other studies were conducted. *Gross et al.,* (2001) described that *S. aureus* could adhere to hydrophobic surfaces and slightly negative charged surfaces, by van der Waals forces, that are mainly attractive, and interionic forces that could be attractive and repulsive. They also add that even if bacteria and substrate surface are charged alike, van der Waals forces can overtake repulsion leading to bacteria adhesion. *Rawlinson et al.,* (2011) reinforce those ideas, suggesting that *S. aureus* is slightly negative (zeta potential -10mV) and with a hydrophobic character.

Zmantar et al., (2011) introduced another determinant factor that could help to explain the adhesion, namely acid/base interactions and the involvement of electron donor/electron acceptor properties. These studies were of major importance once revealed that bacterial adhesion to surfaces depended on environmental factors like the pH value. It was proved that at different pH values *S. aureus* presented different hydrophobicity and different electron donor/electron acceptor behavior. In this study it was demonstrated that when *S. aureus* (negative charge) interacts with surfaces that don't create repulsive interactions or that those are weak, hydrophobic interactions play a more important role. They concluded that even if free energy of the process is positive (not favorable to occur) the adherence occurs anyway, due to *S. aureus* capacity of reaction according to environmental factors, so *S. aureus* has the ability to adhere to hydrophilic and hydrophobic surfaces, being stronger in hydrophobic ones.

This behavior makes bacterial adhesion a dynamic and complex process, being difficult to explain when all the information is not determined.

From all the tested matrices, only Lev[BC]Chi had significant biofilm decrease around 50% in relation to Lev[BC] matrix, that is in agreement with the literature as chitosan is considered an antimicrobial agent specially in the form of nanoparticles (*Arora et al*., 2013; *Shi et al*., 2006).

An initial aim of the work was to evaluate if diclofenac would have a synergetic effect with levofloxacin in decreasing biofilm formation. However as previously reported diclofenac (3.4.1.1.3) does not enhances levofloxacin activity against biofilm.

The matrix with both chitosan and diclofenac (Lev[BC]Diclo-Chi) decreased biofilm formation in only 19%, what could be due to the diclofenac effect suppressing the chitosan microbiological activity. Interestingly, Lev[BC]Diclo showed a higher decrease in biofilm inhibition (32%). Differences could be partly explained by the influence of different surface properties on the adhesion of *S. aureus*. Results of surface energy showed that Lev[BC]Diclo has higher total and disperse energy in comparison to Lev[BC]Diclo-Chi (Fig 29-30). Another factor could be BC surface charge that will be worth to measure.

Concerning Lev[BC]Alg and Lev[BC]Diclo-Alg, very low biofilm decrease was observed (26% and 6% comparing to Lev[BC], respectively). An explanation could be the negative charge of alginate that creates repulsive interactions leading to adhesion.

Besides the physicochemical characteristics of the substrate – hydrophobicity and charge – it is also described that is necessary to know the exact state of hydrophobicity and electron donor/electron acceptor properties of bacteria in order to know all the interactions and better understand the biofilm appearance (*Zmantar et al.,* 2011). It is then necessary to further investigate on this field and in particularly on the matrices characterization – e.g. to determine surface charges – to create a BC that suits the medical needs of preventing biofilm formation.

3.4.1.2.2. Levofloxacin released quantification

To correlate biofilm appearance with levofloxacin concentration in the media, antibiotic was quantified by fluorescence as referred in section (section 2.4.4.2.2.) during 120 h (Fig 36).

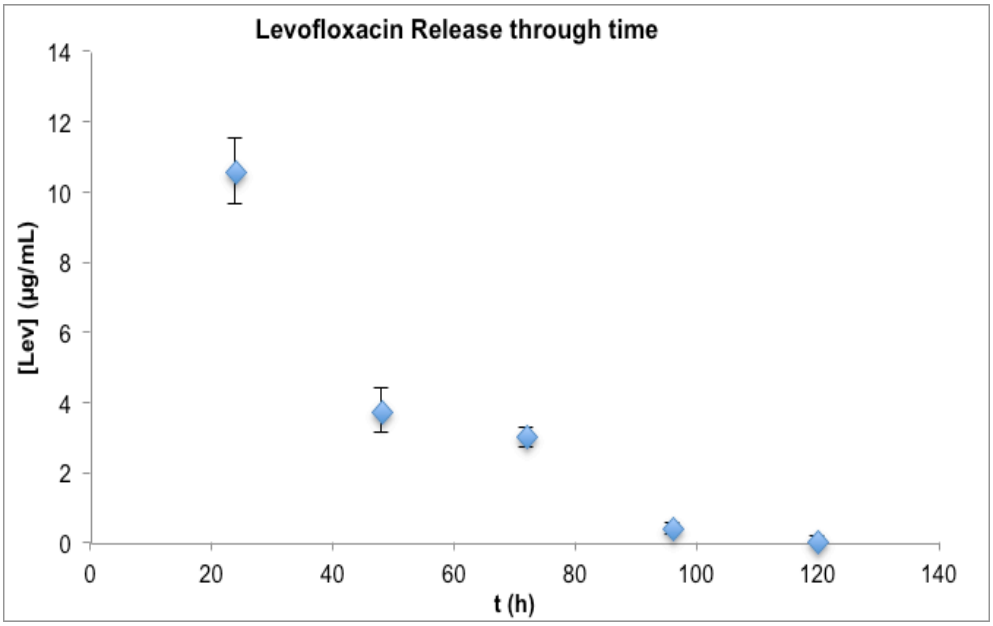

Figure 36 – Example of the release profile of levofloxacin in BHI medium from Lev[BC]Diclo-Chi.

Figure 36 represents an example of the release assays in BHI medium. The same profiles were observed for all the matrices.

These results allowed correlating biofilm formation with the quantity of levofloxacin released to the medium. After 72 h, data shows that matrices Lev[BC]Chi, Lev[BC]Diclo and Lev[BC]Diclo-Chi released significant higher levofloxacin concentrations than the control Lev[BC] (Table 11).

Table 11 – Concentration of levofloxacin in aliquots of BHI medium after 72 h.

Matrix	[Levo] (µg/mL) ± SD
Lev[BC]	1.22 ± 0.17
Lev[BC]Alg	1.40 ± 0.31
Lev[BC]Chi	2.69 ± 0.31
Lev[BC]Diclo	3.05 ± 0.25
Lev[BC]Diclo-Alg	0.40 ± 0.09
Lev[BC]Diclo-Chi	3.01 ± 0.26

As expected for all the matrices the levofloxacin concentrations are lower than MBIC value (4 µg/mL) previously assessed (section 3.4.1.1.2).

Alginate BC matrices released lower amounts of levofloxacin than chitosan (Table 11), as such the low effect on biofilm decrease previously obtained (Table 10) was expected. Levofloxacin release from all the chitosan BC matrices was quite similar (Table 11). The fact that the formulation which showed higher activity against biofilm formation was Lev[BC]Chi suggests that diclofenac suppresses the potential antimicrobial activity of chitosan.

In fact, as already concluded in the "*Antimicrobial Activity of diclofenac"* test *(*4.1.1.3), diclofenac seems to potentiate biofilm formation. The reason for that could be various as the adsorption of diclofenac at BC surface can cause changes (polarity, acid/basic characteristics, charge, etc) in BC surface favoring bacteria adhesion, so further investigation on this field is essential. Also, the method used for biofilm quantification – crystal violet – present some disadvantages once the compound can be adsorbed by the peptidoglycan layer of all cells present, being dead or alive, so the quantification has the inherent error of dead cells (*Chiba et al.,* 1998*)*, for that reason complementary techniques as live/dead assay should be of major interest once that show the amount of live and dead cells given this way a more accurate result, also this assay can be complemented with SEM analysis in order to visually observe the differences in the biofilm formed.

3.5. Biocompatibility – *In vitro* cellular Assays

In vitro cellular studies are of utmost importance in assessing the potential benefit and safety of new bone cement for human use. It is important to evaluate the interaction of the matrices with the cells and what biological/toxic effects can result from that contact. When bone cement formulation is changed, biocompatibility evaluation in particularly cytotoxicity studies is mandatory. In the present studies *in vitro* biocompatibility assays were conducted using two different cell lines, namely mouse fibroblasts (L929) once it is a cell line often used as a model in biocompatibility studies of biomaterials and it is recommended by the international guidelines ISO 10993-5. Also human osteoblasts (MG63) once the cements are to be used in contact with bone forming cells.

3.5.1. Cell viability studies

Several tests are commonly used to evaluate the biological effects of bone cements. In the present work the tests were:

a) cytotoxic evaluation of bone cement release extracts,

b) direct contact studies to analyze the interface of the cell layer with outer contact areas of all matrices and quantification of cell proliferation on surface,

c) staining actin protein assay to evaluate cell morphology structure after adhesion and proliferation of both cell lines in bone cement surface.

3.5.1.1. Cytotoxicity evaluation of the BC extracts

The MTT assay was performed to evaluate BC extracts toxicity on fibroblasts and osteoblasts. MTT is a method based on the conversion of a yellow tetrazolium salt into purple formazan crystals by the living cell's NAD(P)H-dependent mitochondrial dehydrogenases, hence giving the information of the amount of living cells (*Mosmann*, 1983). This method allowed the evaluation of cytotoxicity by cell exposure to the bone cement extracts collected after of 24h release and at the end (3 weeks). Release medium alone was used as a negative control and SDS as positive control. Solutions of free drugs in RPMI were also used as controls.

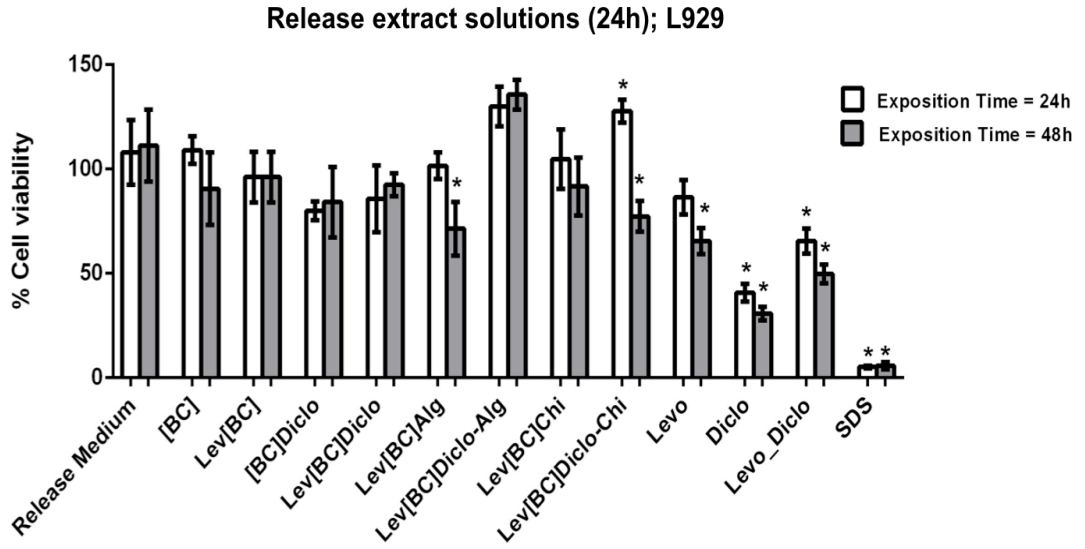

Figure 37 - Cell (L929) viability of the BC extracts (3 weeks) after 24h and 48h of incubation (mean±SD; n=6). Comparison is conducted at the same cell exposition time; *means a significant difference to [BC] extract solution $p < 0.05$.

In figure 37 cytotoxicity results for 24h release extracts and standard drug solutions are shown. It is visible that all BC matrices extracts didn't presented significant decrease in cytotoxicity relatively to [BC] matrix for the first 24h of assay. For 48h of assay only Lev[BC]Alg and Lev[BC]Diclo-Chi presented significant decrease in comparison to [BC] cement. For the control drugs, both presented significant cytotoxicity at both times, being the diclofenac the most toxic.

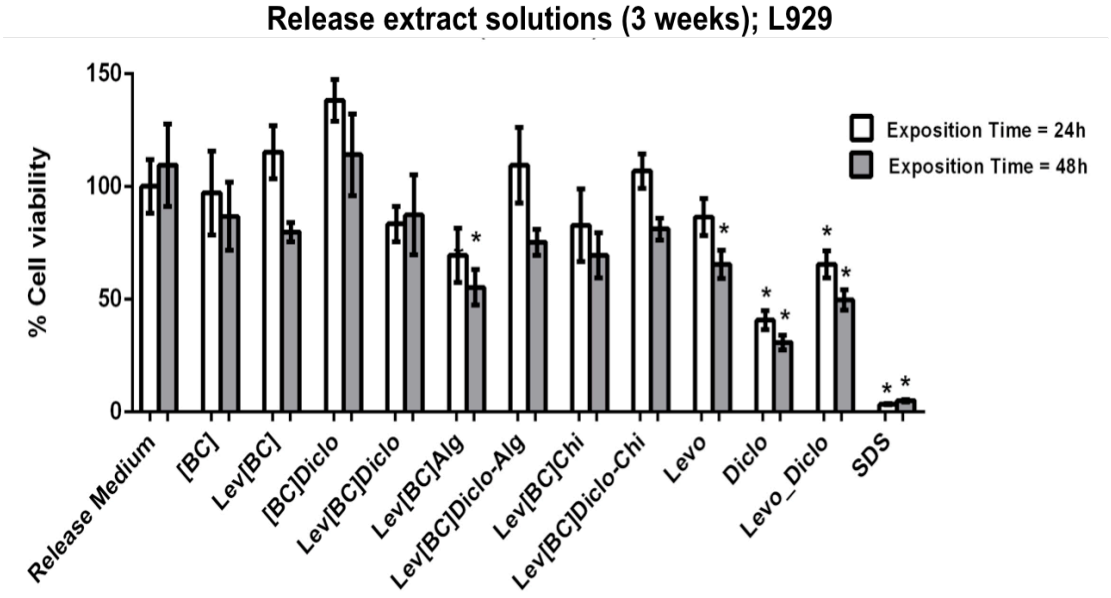

Figure 38 - Cell (L929) viability of the BC extracts (24h) after 24h and 48h of incubation (mean±SD; n=6). Comparison is conducted at the same cell exposition time; *means a significant difference to [BC] extract solution $p < 0.05$.

Similar results were obtained to 3-week extracts (Fig. 38), all matrices had no significant decrease in cell viability for 24h exposition time, and for 48h exposition time only Lev[BC]Alg matrix showed decreased cell viability. For the control-drugs the same behavior was observed as previously reported for 24h BC extracts (Fig. 37).

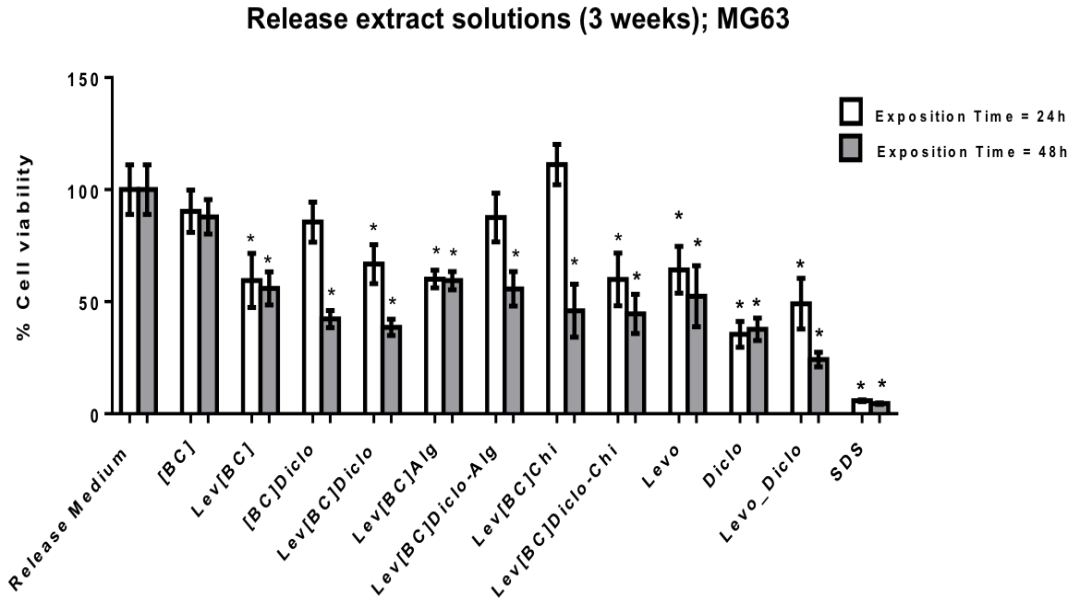

Figure 39 - Cell (MG63) viability of the BC extracts (3 weeks) after 24h and 48h of incubation (mean±SD; n=6). Comparison is conducted at the same cell exposition time; *means a significant difference to [BC] extract solution $p < 0.05$.

In what concern MG63 cells, experiments were conducted only with the 3-week extracts once that 24 hours extract samples volume did not was sufficient to made the assay with this cell line. For this cell line results were very different from those obtained with L929 cell line. Almost all matrices showed increased toxicity when compared to [BC], except for [BC]Diclo, Lev[BC]Diclo-Alg and Lev[BC]Chi matrices for the first 24 h (Fig. 39). In what concerned 48 h of incubation, all matrices showed increased toxicity. Control-drugs behaviour seems to be similar to results obtained with L929 cells (Fig. 37).

It is important to evaluate the cytotoxicity of the extracts obtained from novel BC formulations once there are several new additives in BC interacting with each other's and being released to the medium. For L929 cell line (Fig. 37 and 38), it was observed that all BC extracts presented low levels of cytotoxicity for both exposition times, being the Lev[BC]Alg and Lev[BC]Diclo-Alg extracts that presented lower cell viability after 48 h of exposition (when compared to [BC]). For MG63 cell line (Fig. 39), it could be observed that all matrices extracts except [BC]Diclo, Lev[BC]Diclo-Alg and Lev[BC]Chi (in the first 24h) presented cytotoxicity.

The obtained results in this work can be explained by the drugs released from the matrices. There are several studies showing that diclofenac and levofloxacin present toxicity to animal cells on the range of concentrations found in the released medium. *Krischak et al.,* (2007) observed that in a concentration of 250.0 ± 47.7 µg/mL, diclofenac inhibits the number of osteoblasts within a cancellous bone defect in rats at a very early stage; *Kaspar et al.,* (2005) incubated an osteoblast-like cell line with 50 µg/mL diclofenac for 24h and both osteoblast proliferation and expression of type I collagen were significantly reduced after 48h; *Chouhan et al.,* (2012) also concluded that diclofenac treatment over a continuous period of 30 days seems to be a matter of clinical concern being an interfering agent with osseous metabolism affecting osteoblastogenesis, and collagen fiber organization. This effect was also evident in fibroblasts. *Nguyen* (1993) demonstrated that diclofenac was the most effective agent in inhibiting fibroblast proliferation, with inhibition occurring at a range of concentrations above 10^{-3}-10^{-4} mM.

For levofloxacin, cytotoxic effects are also described. *Holtom et al.,* (2000) reported that administration of 80 µg/mL of levofloxacin has inhibitory effects on fibroblast cell-like and therefore was shown to be able to affect cell viability. *Tan Y. et al.,* (2012) demonstrated that levofloxacin when used on cell monolayers at concentrations of 14–224 µM presented cytotoxicity in fibroblasts-like cells.

The toxicity of free drugs was also observed in our studies as tested concentrations (levofloxacin - 567 µg/mL, diclofenac - 647 µg/mL) are far higher than those referred in literature.

3.5.1.2. Direct contact with BC composites

The response of cells (MG63 and L929) in direct contact with the BC composites was evaluated by the observation of the cell culture proliferation by phase contrast after MTT reduction and by fluorescence microscopy.

3.5.2.2.1. Phase-contrast microscopy

This assay was assessed in order to evaluate the adhesion and proliferation of both cell lines directly on bone cement plates (Fig. 40). Glass was used as control (non-toxic material) (Fig. 41).

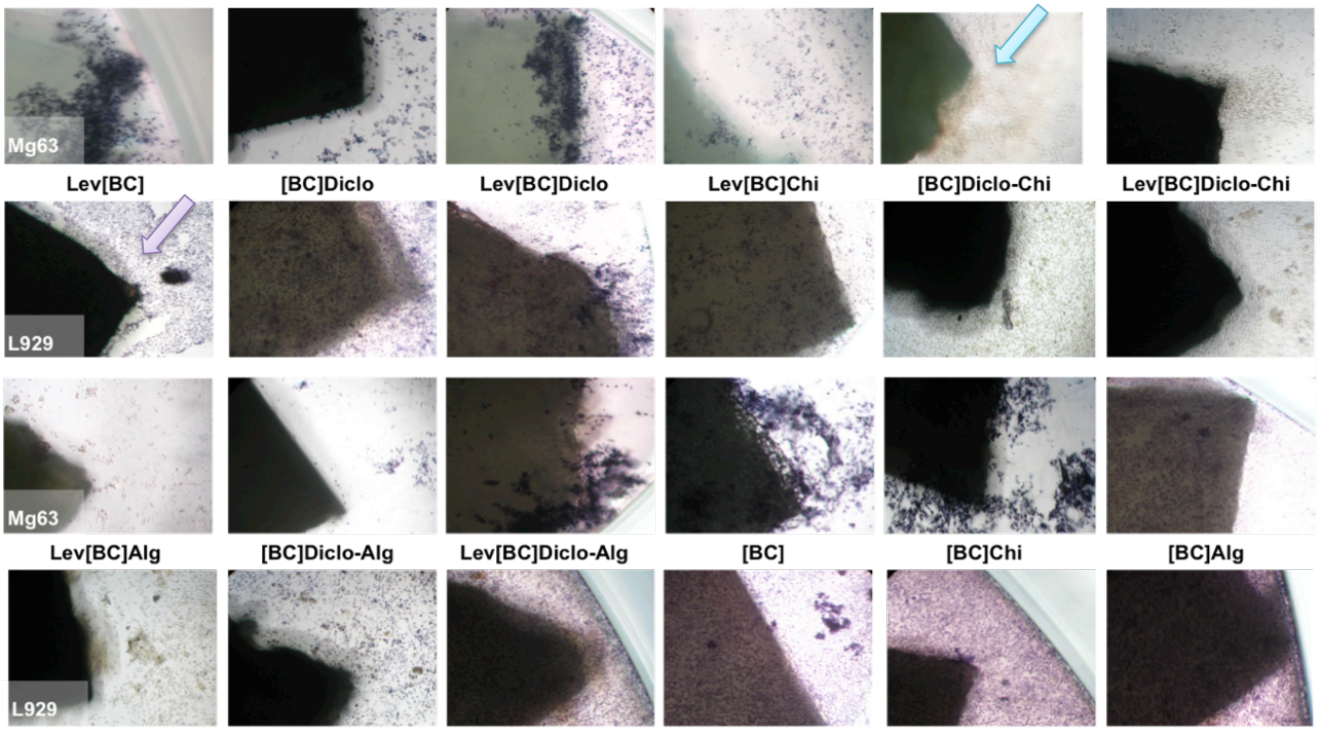

Figure 40 - Phase-contrast micrographs of the interface of the cell layer, L929 and MG63, with outer contact areas of the different BC composites.

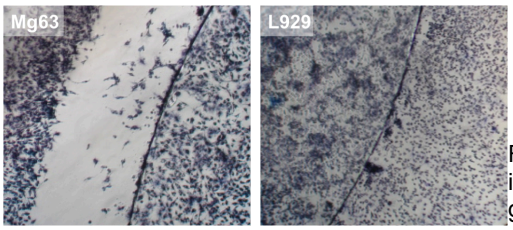

Figure 41 - Phase-contrast micrographs of the interface of the cell layer, L929 and MG63, with glass controls (positive controls).

Through this assay (Fig. 40) it is possible to visually the monolayer formation on some cement surfaces (indicated by the purple arrow) and cell death in others (indicated by the blue arrow).
Also, quantitative quantification of cell viability was assessed for both cell lines L929 and MG63, results are shown bellow (Fig. 42 and 43).

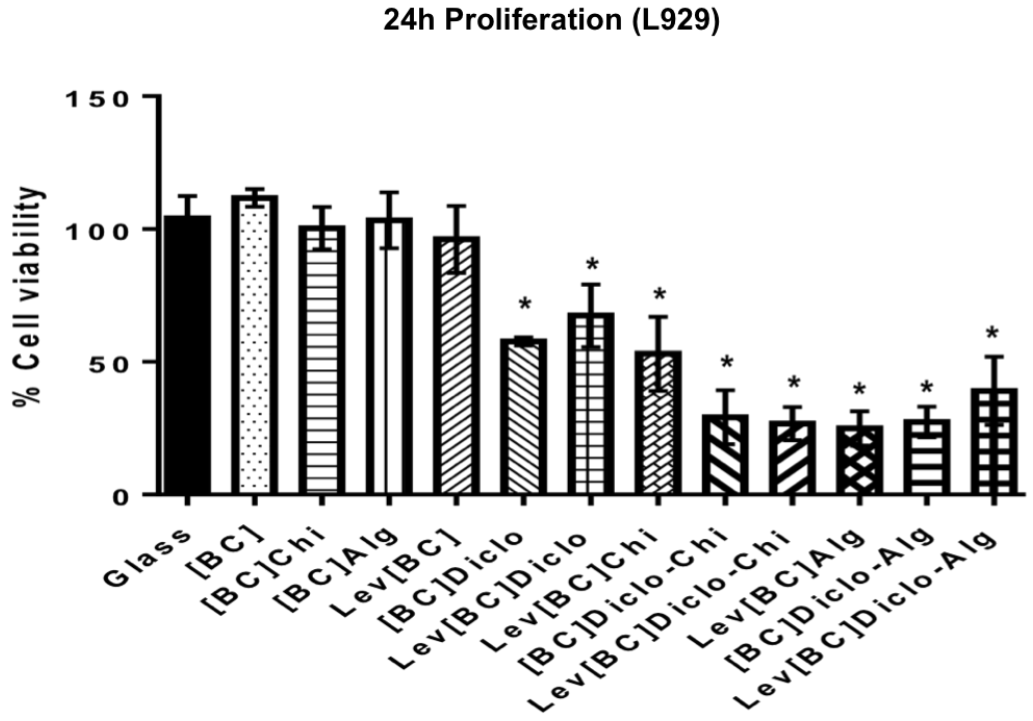

Figure 42 – Cell (L929) proliferation on the BC composites during 24h (mean±SD, n=6). *statistically different from [BC], $p<0.05$.

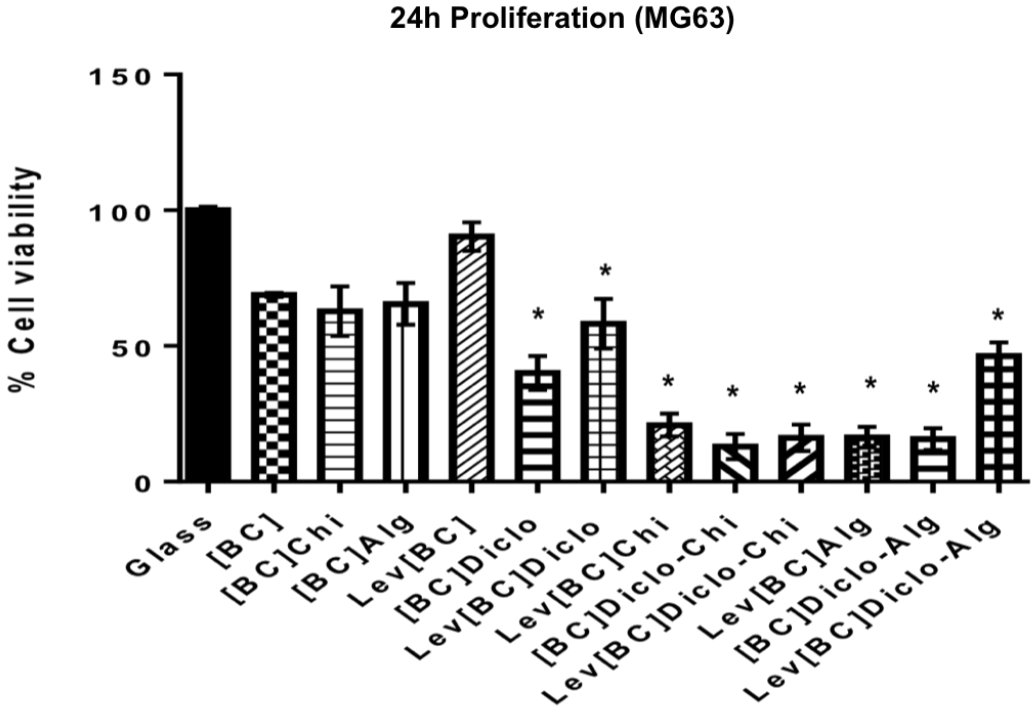

Figure 43 - Cell (MG63) proliferation on the BC composites during 24h (mean±SD, n=6). *statistically different from [BC], $p<0.05$.

For both cell lines results show that control matrices [BC], [BC]Alg and [BC]Chi have similar cell viability as the glass, meaning that the matrices with alginate and chitosan alone are biocompatible as already mentioned in other studies (*Cecoltan et al.,* (2015); *Lee et al.,* (2012); *VandeVord et al.,* (2001)). For all the other matrices a decrease in cell viability was observed. This fact could be due to the high amount of drugs released at the interface within 24 h, that could be toxic to the cells as previously mentioned. *Matos et al.,* (2015a) demonstrated that no cytotoxic effects for levofloxacin were observable after 48h assay but the amount of drug release was around 1 µg/mL. It is expected for matrices with polymers that levofloxacin released amount would be higher (according to the release studies realized previously – section 3.2). If the assay was elongated and cells were daily added/replaced to the wells it could be possible to find higher cell viability.

This result has been reported by *Krischak et al.,* (2007) who found that for higher exposition times (10 days) cell viability increases and toxic effects of drugs are diminished. Also as the media will have to be changed every 48h the amount of drug in contact with the cells would be less once there was a continuous decrease of drug released from the bone cement matrix. The differences observed between matrices are probably related with bone cement characteristics and consequently amount of released drug, that in some matrices was less and others was higher.

Furthermore *Marques et al.,* (2002) describes several reasons that can alter cell adhesion, namely polymer surface characteristics like wettability, surface charge, surface free energy and topography. It has been reported that moderate hydrophilic surfaces allows higher absorption of serum and cell adhesion proteins than in extremely hydrophobic or hydrophilic.

Furthermore biomaterials surface properties as may influence cell topography as more roughened surfaces promote an irregular cell adhesion. Also other factors as chemical properties are involved as the amount of carboxyl and hydroxyl groups that was reported to have a major influence (*Marques et al.,* 2002).

3.5.2.2.2. Fluorescence microscopy

This assay was also assessed to evaluate the morphology of cell adhesion and proliferation of both cell lines directly on bone cement plates and mainly to evaluate the changes in cell structure through actin staining (Fig. 46). Glass was used as a control (non-toxic material) (Fig. 45).

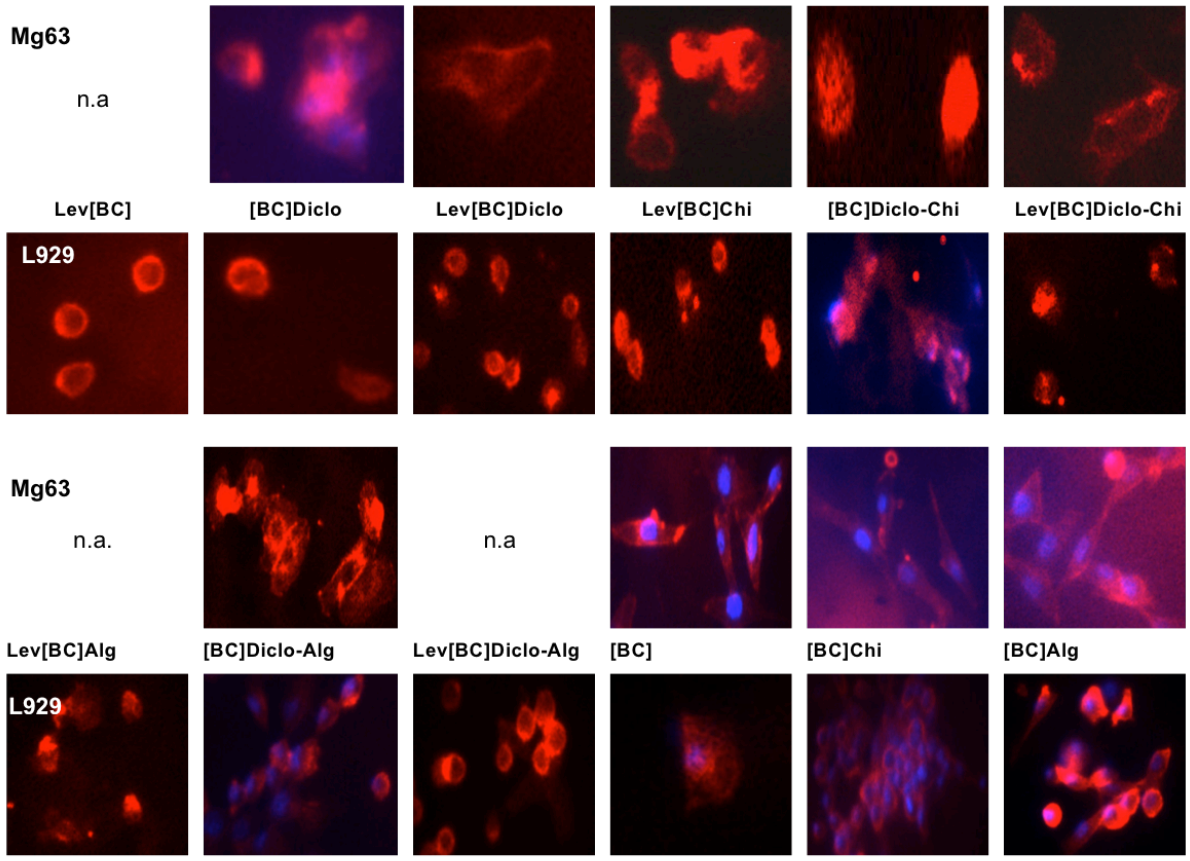

Figure 44 - Fluorescence images of MG63 and L929 cell lines on surface of BC composites (blue staining of nucleus and red staining of actin in cells cultured on the surface of the materials; 40x magnification). n.a.- not applicable, once in these composites MG63 stained cells were not found.

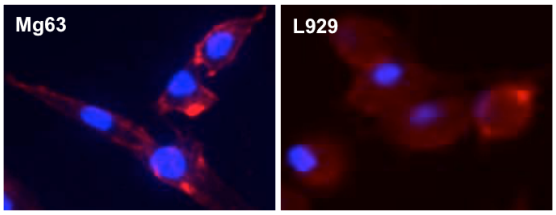

Figure 45 – Fluorescence images of MG63 and L929 cell lines on glass (positive controls, 40x magnification).

Fluorescence microscopy results show that MG63 cell line only maintained its cellular morphology in [BC], [BC]Alg and [BC]Chi, that is in accordance with the phase-contrast microscopy results previously described (Fig 40). Cell shape of MG63 is normally elongated and in contact with drug-loaded BC matrices it is possible to observe a rounder shape. Release of high levels of diclofenac and levofloxacin could be responsible for the toxicity of those BC matrices.
All matrices seem to be less toxic to L929 cells as already found in previously assays namely the BC extract tests. This cell line appeared to better maintain its morphology when in contact with all matrices, a rounder shape similar to that obtained with glass plates.

Once again in *Matos et al.*, (2015a) both cell lines maintained its cell morphology in all tested BC matrices, indicating that the compounds and the levofloxacin present in bone cements did not affect cell adhesion and proliferation. As previously mentioned levofloxacin loaded BC matrices tested by *Matos et al.,* (2015a) released lower drug amounts that the ones tested in the present study.

Overall, biocompatibility evaluation is of major importance once that it provides crucial information about the novel bone cement formulations. It was possible to conclude that most matrices release cytotoxic amounts of drugs to the cells in the first 48h of release, osteoblast is a more sensitive cell type than fibroblasts and the cell adhesion phenomena is very complex involving several surface characteristics.
Phase-contrast and fluorescence microscopy assays complete each other providing important data to guide the selection of suitable BC matrix to proceed in further studies. Nevertheless, one must always keep in mind that *in vivo* the turnover of drugs at the interface cell/biomaterial will be much higher in comparison with the *in vitro* situation. As such, *in vitro* cytotoxicity data will not definitively exclude BC matrices which show important microbiological activity.
Further *in vitro* and *in vivo* studies are then necessary to fully evaluate the BC biocompatibility, namely osteoblasts density through calcium deposits detection assay, osteoblasts proliferation through alkaline phosphatase detection assay, osteoblasts differentiation through immunoblotting assays, among others (*Stein et al.,* 1993).

Chapter 4. Conclusions and Future Work

The general aim of the thesis was the development of a novel biocompatible antibiotic-loaded bone cement with an improved drug release profile. The selected antibiotic to be loaded was levofloxacin, a fluoroquinolone with adequate bacterial spectrum for bone infections, and to the present scarcely addressed for the intended application. The increase on drug release was planned to be obtained through an increase in cements porosity by the addition of biopolymers (chitosan and alginate). Furthermore an anti-inflammatory drug – diclofenac - was also loaded into BC. The association of an antibiotic with an anti-inflammatory consists in a great novelty and it was expected to improve BC properties as it will combine in the same formulation antimicrobial and anti-inflammatory activity.

For that purpose, thesis work started by producing and characterizing chitosan and alginate nanoparticles. As planned particles in nano scale range were obtained with the predicted zeta potential (chitosan NPs = 366 ± 14 nm; ZP of +28 ± 0.7 mV and alginate NPs = 600 ± 34 nm; ZP of -23 ± 1.9 mV). Different bone cement formulations were then obtained, loaded either with chitosan (NPs) or alginate (in the form of polymer and NPs). The effect of those poragens on drugs release profiles in different biomimetic media was evaluated. Results clearly showed that the polymers in the form of nanoparticles clearly improved both levofloxacin and diclofenac release. Chitosan loaded BC formulations were the ones that released higher amounts of the drugs in the majority of the cases. It was also demonstrated that the media composition and pH value had major influence on drug release. It was shown for example that pH value changes could affect biopolymers swelling. Also the presence of albumin due to its different affinity to levofloxacin and diclofenac has a high influence on drugs release profiles.

Mechanical and structural assays demonstrated that the insertion of the additives did not compromised BC mechanical properties and as expected increased biomaterial porosity. Contact angle experiments provided very important information concerning changes on bone cement surface properties namely surface energy of the new BC formulations. All novel BC matrices revealed no significant alterations on the total surface energy. In contrast, it was observed an increase of the polar component in most matrices meaning that the incorporation of the additives substantially increased the BC hydrophilicity.

The antimicrobial activity of the new formulations was also assessed. Furthermore, levofloxacin MIC and MBIC values for *S. aureus* in the presence of unloaded BC samples were determined. Obtained MIC value is in concordance with the ones referred in literature being 0.25 μg/mL and MBIC value was ≥ 4 μg/mL. Due to the controversial results described in literature, diclofenac antimicrobial activity was also assessed. It was demonstrated that diclofenac did not decreased bacterial growth. In fact, it increased biofilm formation. Among all the tested modified bone cements, Lev[BC]Chi was the one that presented significant biofilm inhibitory activity.
Alginate and diclofenac BC loaded matrices did not had an inhibitory biofilm effect. The presence of diclofenac as well as charge/surface effects may explain the obtained results. These microbiological assays also served to demonstrate the chemical stability of levofloxacin in bone cements meaning that it did not lose its microbiological activity during cement preparation and polymerization.

All BC matrices were also tested in relation to biocompatibility by *in vitro* cellular assays. Two cell lines were tested (L929 fibroblasts and MG63 osteoblasts). Assays included BC extracts as well as direct contact assays between BC and cells.
Bone cement extracts significantly decreased L929 cell viability after 48h of exposure, whereas in MG63 cell line a higher decreased on cell viability was obtained after 24h. Results showed that the amount of drugs (levofloxacin and diclofenac) released to the medium at the time of experiments was

very high leading to cytotoxic effects on the cells in particularly with osteoblasts. Direct contact assays also demonstrated that effect, for MG63 some matrices presented cell death (significant decrease in cell viability) and alterations in morphology. L929 cell line seemed to be more resistant to drugs.

In sum, it can be concluded that the addition of alginate or chitosan nanoparticles increased drug release without affecting BC mechanical integrity. Taking into account both the microbiological and biocompatible assays, Lev[BC]Chi is the formulation which shows the most promising results to proceed for further evaluation. This matrix has chitosan biopolymer that increased antibiotic release in comparison to the control (Lev[BC]). It was the only matrix that presented a significant decrease in biofilm and did not present a meaningful loss in cell viability. It is then the matrix that revealed more potential for clinical application.

Further studies need to be assessed to better understand the interaction of the new bone cement formulations with the biological environment and ensure a safe biomedical application and a successful antimicrobial activity of these cements.

Due to a variety of reasons, lack of time, not in the ambit of the thesis, equipment or reagents unavailable, among others, it was impossible to obtain all the information needed for the characterization of all the BC matrices. It will be of major importance to realize many other assays in order not only to confirm certain results here presented but also to obtain more information on this subject.

Concerning mechanical and surface studies it would be important to realize more tests in order to better understand the effect of the additives in the inner structure and outer surface, namely X-ray diffraction, flexural strength and flexural modulus and porosity among others. It would be of major importance to determine surface charge of the cements once that is a factor that is related mostly with bacterial adhesion.

About microbiologic assays, another tests should be made like live/dead and calorimetry assays to better quantify the amount of bacterial growth, SEM analysis of the surfaces after bacterial growth are also important to analyze biofilm layer formed on the surface of the cement. Even the assays that were realized during this thesis could be repeated and assay conditions improved to more suitable ones.

Although cell viability tests demonstrated some toxic effects of some matrices in both cell lines, further information is necessary both *in vitro* as *in vivo*. It is necessary to determine osteoblasts proliferation, density and differentiation through several assays like alkaline phosphatase and calcium deposits detection and immunobloting assays as well as evaluate the inflammatory response to assess the effect of diclofenac loaded BC matrices.

Besides the measures proposed it is also important to analyze the current methods, have a critical sense and adapt the methodologies used in the assays to better *in vivo* mimic the effect. The formulation proposed to be further tested was Lev[BC]Chi, but as was observable this matrix presents some toxicity, that is correlated with the amount of drug released. So it is important to have a solution that can lower that toxic effect, but continue to have the antimicrobial activity or if possible to enhance it. This could be made by adjusting the amount of loaded antibiotic and/or nanoparticle size. Also diclofenac was seen to be a bad choice to incorporate in bone cements, but the use of another anti-inflammatory drug should not be discharged. The same goes for the use of alginate biopolymer. Further studies in different conditions could reveal other potential effects of alginate, and use of others biopolymers are also another good approach for the future.

Overall, the present study suggests that from all the evaluated bone cements there is one that stands out and reveals potential to be a safe and advantageous matrix to proceed. It is important then, to improve this matrix concerning drug release with an appropriated kinetic profile, microbiological activity and toxicological effects without compromising the mechanical structure, in order to have a novel biocompatible antibiotic-loaded bone cement that could be implanted in humans in a near future.

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