**UNIVERSIDADE DE LISBOA**

**FACULDADE DE FARMÁCIA**


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Orientadora: Professora Doutora Ana Francisca de Campos Simão Bettencourt

Co-orientadores: Professor Doutor António José Leitão das Neves Almeida e

Professor Doutor Mário Augusto Pires Vaz

Tese especialmente elaborada para a obtenção do grau de Doutor em Farmácia na especialidade de Tecnologia Farmacêutica

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DEPARTAMENTO DE CIÊNCIAS TOXICOLÓGICAS E BROMATOLÓGICAS


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**2015**

*"… the dream of yesterday*
*is the hope of today*
*and the reality of tomorrow."*

Robert Goddard

*A meus filhos*

*Gonçalo, Beatriz, Nuno*

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# Agradecimentos

*"Science and everyday life*
*cannot and should not be separated"*
Rosalind Franklin

A realização de um trabalho conducente a um Doutoramento é um caminho árduo, longo e labiríntico. Um desafio intelectual que exige determinação e muita perseverança mas também a humildade de saber escutar. A concretização de mais esta etapa é para mim um momento de grande felicidade que não seria possível sem a colaboração de muitas outras pessoas, a quem eu gosto de chamar "equipa". Pretendo por isso partilhar este momento com todas elas.

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# Abstract

Antibiotic-loaded bone cement (ALABC), is the common designation for polymethylmethacrylate bone cement (BC), used as drug-delivery system to prevent or to treat bone related-infections. Although presenting some disadvantages, the use of ALABC is still considered the standard of care for patients with chronic bone and joint infection, providing local delivery of high levels of antibiotics for an extended period without exceeding systemic toxicity, while being a more cost-effective procedure when compared to cementless implants. Described and reported ALABCs drawbacks include the inadequate release of the loaded antibiotic, the lack of bioactivity and the poor diversity of antibiotics available in commercial premixed formulations.

The base-concept of this study was to develop a novel ALABC with improved antibiotic release through the inclusion of particulate drug delivery systems and a release modulator without hampering the antibacterial activity of antibiotics or the BC mechanical and biocompatibility properties.

Levofloxacin, a 3rd generation fluoroquinolone, and minocycline, a tetracycline, were the elected antibiotics to load into BC. The rationale behind this choice was related to their adequate microbiological and physicochemical characteristics. Both antibiotics present a broad-spectrum of activity against the main organisms responsible for bone and joint infections, namely *Staphylococcus* spp. Physicochemically, both are molecules with amphiphilic characteristics - greater for levofloxacin; soluble in acidic aqueous media; with high melting points (over 200ºC) and available in powder form; the latter two characteristics being restrictive when choosing for antibiotics to load into BC.

Two main strategies were explored for the inclusion of antibiotics into particulate systems previous to incorporation into BC: 1) by encapsulation into PMMA; 2) by adsorption into calcium-phosphate particles (CaPs).

To improve drug release from the matrix, a pharmaceutical excipient was used as release modulator, lactose, and loaded into the BC powder component.

A step-by-step approach was pursued:

1st. Assessment of antibiotics encapsulation into PMMA particles and of antibiotics *in vitro* release followed by loading antibiotic-loaded-particles into BC;

The PMMA biopolymer was chosen to prepare PMMA-particles (PMMAp) foreseeing a mechanical reinforcement of the final ALABC matrix, because PMMA is the base-polymer of both systems. Plain, levofloxacin- and minocycline-loaded particles were successfully prepared using the double-emulsion solvent evaporation method. Since only minocycline-PMMAp registered an interesting *in vitro* release profile, studies proceeded with these particles and 15% ($w_{particles}/w_{BC}$) were loaded into BC, which, on the other hand, hindered BC setting.

2nd. Effect of the inclusion of lactose into BC, monitoring antibiotics *in vitro* release, quasi-static mechanical properties and biocompatibility of the resultant matrices;

Each powdered antibiotic was directly loaded into BC, and lactose, was added to each formulation. The amount of antibiotic loaded corresponded to the low-dose currently used in commercial ALABCs formulations - 2.5% ($w/w_{BC}$) - in order to provide an effective antimicrobial activity and preserve the mechanical properties. As to lactose, 10% ($w_L/w_{BC}$) resulted in the optimised amount to be loaded into BC. This lactose-modified BC matrix allowed total release of the minocycline after a one-week period, and a 3.5-fold increase of levofloxacin release compared to control without lactose, over a 7-week period.

3rd. Inclusion of levofloxacin-adsorbed doped CaPs into BC and monitoring of the antibiotics *in vitro* release, quasi-static mechanical properties and biocompatibility of the resultant matrices;

Intending to improve antibiotic release and bioactivity calcium-phosphate particles (CaPs) were tested as drug delivery system. Mg- and Sr-doped CaPs were prepared as levofloxacin carriers and were loaded into the 10% ($w_L/w_{BC}$) lactose-modified acrylic BC at 2.5% ($w_{CaPs}/w_{BC}$). This novel BC composite revealed a sustained release of levofloxacin over an 8-week period, with concentrations over the *Staphylococcus* spp. minimum inhibitory concentration values after 48 h.

The novel 10% ($w_L/w_{BC}$) lactose-loaded ALABC, independently of the antibiotic or CaPs loaded, followed the same release mechanistic based on dissolution and subsequent diffusion of the antibiotic from the matrix. Both minocycline and levofloxacin maintained antibacterial activity against the *Staphylococcus* spp. after being released from ALABC matrix. Though this result suggest that polymerization setting did not affect these antibiotics, a novel *in silico* approach revealed the existence of covalent and non-covalent interactions between the levofloxacin and the BC matrix.

Evaluation of the antibiotic-lactose-modified BC matrices regarding the *quasi-static* mechanical properties according to standard ISO 5833, clearly demonstrated that the mechanical performance was not compromised.

Biocompatibility was also successfully evaluated following standard ISO 10993-5 with fibroblasts and osteoblasts cell lines incubated with extracts or in direct contact with BC composites, respectively. Results have shown that neither lactose nor the loaded antibiotics compromised the biocompatibility of the BC.

All considered, these features justify the potential of lactose-loaded BC as a valuable step forward on the development of novel BC composites, namely with lactose, as release modulator, and doped CaP particles, as antibiotic carriers, for the control of bone and joint infections.

**Keywords**: Acrylic bone cement; Antibiotic delivery systems; Calcium phosphate in bone cement; Mechanical properties; Biocompatibility

# Resumo

A designação de cimento ósseo acrílico com antibiótico incorporado (ALABC - acrónimo proveniente da designação inglesa), refere-se ao cimento ósseo (BC - acrónimo proveniente da designação inglesa) cujo polímero-base é o polimetilmetacrilato (PMMA), e que é vulgarmente utilizado como sistema de administração local de antibióticos para prevenir ou tratar infeções ósseas.

O uso de ALABC foi introduzido nos anos 1970 por *Buchholz* e *Engelbrecht* com um intuito profilático ao desenvolvimento de infeções ósseas e que permanece até hoje. Atualmente, na Europa a realização de artroplastia cimentada continua a ser preferencialmente realizada, e com menores custos do que a artroplastia não cimentada. A utilização do PMMA permitiu reunir a função estrutural do BC de fixação da prótese ao osso a uma função profilática ao desenvolvimento de infeções por conter antibióticos de largo espetro de ação incorporados. É também considerado o tratamento de eleição para pacientes com infeções crónicas das juntas ou do osso através da aplicação em procedimentos de revisão de infeção em artroplastia, na forma de esferas ou de espaçadores, permitindo uma veiculação *in situ* de elevadas concentrações de antibiótico durante um período de tempo prolongado sem, contudo, exceder a toxicidade sistémica nos tecidos circundantes. Existem formulações comerciais de BC contendo antibióticos de largo espectro de atuação contra os microorganismos responsáveis pelo aparecimento da infeção óssea. Contudo, os problemas associados à inadequada cinética de libertação do antibiótico a partir da matriz, tem permitido o desenvolvimento de formas de bactérias resistentes.

Outras desvantagens destes cimentos incluem a baixa bioatividade da matriz, que dificulta a osteointegração do tecido ósseo na superfície do BC podendo no limite conduzir à falência do implante, e a pouca diversidade de antibióticos disponíveis nas formulações comerciais do ALABC que se resumem à gentamicina e tobramicina (aminoglicosideos), incorporados individualmente para os casos de uso profilático, ou combinados com vancomicina (glicopeptideo) ou clindamicina (lincosamida) para situações de revisão da artroplastia diagnosticada com infeção.

A matriz do ALABC é considerada uma estrutura monolítica na qual a porosidade tem um papel primordial na libertação dos antibióticos. O número de poros disponíveis, o seu tamanho e a intercomunicação entre si, muitas vezes, tortuosa e sem vias de saída, influi no processo de penetração dos fluidos até ao interior da matriz assim como na difusão do antibiótico para o exterior da mesma, após a sua dissolução. Por sua vez, a entrada do fluido, de carácter aquoso, é dificultada pela hidrofobicidade destas matrizes que impede o seu avanço para o interior do biomaterial.

As propostas para melhorar estes problemas passam muitas vezes pela adição de componentes hidrofílicos e hidrossolúveis que, por um lado, diminuem a hidrofobicidade permitindo um melhor contacto entre o fluido e a matriz e, por outro, promovem o aumento da porosidade e consequentemente da libertação do antibiótico durante o movimento de saída do fluido do interior da matriz do biomaterial.

A grande desvantagem destas propostas reside no fato de a inclusão de novos constituintes ao alterarem a microestrutura base do BC poderem comprometer o seu desempenho, impedindo a sua utilização em artroplastia como profilaxia das infeções ósseas, na qual a função estrutural tem um papel preponderante e incontornável.

O conceito base deste estudo foi desenvolver uma matriz inovadora de ALABC com um melhor desempenho na libertação do antibiótico, através da inclusão de particulas como sistemas de libertação dos antibióticos, e de um excipiente com comprovada eficácia e segurança como modelador de libertação sem, contudo, prejudicar as suas propriedades mecânicas ou a biocompatibilidade.

Os antibióticos selecionados para incluir no BC foram a levofloxacina, uma fluoroquinolona de 3ª geração, e a minociclina, uma tetraciclina. O racional da escolha está relacionada com as características microbiológicas e físico-químicas destes antibióticos. Ambos apresentam largo-espectro de atividade contra os principais microorganismos responsáveis pelo desenvolvimento das infeções ósseas e articulares, nomeadamente as estirpes *Staphylococcus* spp. Físico-quimicamente estes antibióticos são moléculas pequenas; com características anfifílicas, mais acentuadas na levofloxacina; facilmente solúveis em meio aquoso a pH inferior a 6; com ponto de fusão acima dos 200ºC; disponíveis na forma de pó, sendo estas últimas características condicionantes na escolha de antibióticos para serem incorporados no BC.

Duas principais estratégias foram exploradas para incorporar estes antibióticos em partículas previamente à inclusão na matriz do BC: 1) por encapsulamento em partículas de PMMA; 2) por adsorção em partículas de fosfato de cálcio.

Para melhorar a libertação do antibiótico foi utilizado um excipiente como agente modulador de libertação, a lactose, incorporado diretamente na componente em pó do BC.

A metodologia seguida foi a seguinte:

1.º Caracterização do sistema de partículas de PMMA contendo os antibióticos encapsulados e estudo *in vitro* do perfil de libertação dos antibióticos antes de incluir as partículas na matriz do BC;

A escolha do polímero PMMA para a preparação das partículas para encapsular os antibióticos teve como principal motivo o fato de ser o mesmo biomaterial base do BC prevendo-se assim evitar o detrimento das propriedades mecânicas finais do novo ALABC. A inclusão de substâncias "estranhas" na matriz leva sempre a alterações e reajustes ao nível da microestrutura e consequentemente na resistência mecânica; sendo o mesmo material a incluir na matriz, esperava-se que esse efeito não fosse significativo.

O método da dupla-emulsão por evaporação de solvente foi utilizado com sucesso na preparação de lotes de partículas sem antibiótico e com os antibióticos encapsulados. Após o estudo *in vitro* da libertação dos antibióticos em meio aquoso, apenas as partículas de minociclina-PMMAp libertaram antibiótico, e consequentemente o estudo prosseguiu para a etapa seguinte tentando incorporar 15% ($w_{partículas}/w_{BC}$) no BC, que impediram a cura do cimento.

2.º Estudo do efeito da inclusão da lactose na matriz do BC, monitorizando a libertação *in vitro* dos antibióticos também introduzidos na matriz do BC, as propriedades mecânicas estáticas e as propriedades de biocompatibilidade das matrizes resultantes;

Cada antibiótico, em pó, foi incorporado diretamente no BC juntamente com a lactose também em pó. A quantidade de cada antibiótico incorporada correspondeu à dose-baixa usualmente utilizada nas formulações comerciais de ALABCs - 2,5% ($w/w_{BC}$) - a fim de proporcionar quer uma atividade antimicrobiana eficaz quer a preservação das propriedades mecânicas. Relativamente à lactose, o melhor valor a incorporar no BC resultou ser 10% ($w_L/w_{BC}$). Esta matriz de BC modificado com lactose permitiu a libertação total da minociclina após uma semana e um aumento de libertação de levofloxacina de 3,5 vezes o valor libertado de uma matriz controlo sem lactose, ao longo de 7 semanas.

3.º Inclusão no BC de particulas de fosfato de cálcio com levofloxacin adsorvida e monitorização da libertação *in vitro* do antibiótico, das propriedades mecânicas e da biocompatibilidade das matrizes resultantes;

Com a intenção de melhorar a bioatividade da matriz do BC modificada com lactose, foi testada a incorporação no BC o uso de partículas de fosfato de cálcio com duas funções, a de agente bioativo e a de sistema de libertação de fármacos, o que até ao momento nunca foi descrito. Assim, foram preparadas partículas de fosfato de cálcio dopado, quer com iões magnésio (Mg) quer com iões estrôncio (Sr), e com o antibiótico – a levofloxacina – adsorvida na sua superfície. A opção de utilizar fosfato de cálcio dopado com Mg e Sr deveu-se a estes catiões terem vindo a ser apontados como potenciadores do efeito de osteocondutividade dos fosfatos de cálcio, o que ia de encontro ao objetivo do trabalho. De seguida, 2,5% ($w_{CaP}/w_{BC}$) destas partículas (fosfato de

cálcio com levofloxacina adsorvida) foram incorporados, conjuntamente com 10% ($w_L/w_{BC}$) de lactose, no cimento ósseo. Como resultado, ocorreu uma libertação sustentada de levofloxacina durante 8 semanas, em que após 48 h as concentrações de levofloxacina estavam acima dos valores das concentrações mínima inibitória das *Staphylococcus* spp.

Este novo ALABC modificado com 10% ($w_L/w_{BC}$) lactose, independentemente do antibiótico ou dos fosfatos de cálcio incorporados, seguiu sempre o mesmo mecanismo de libertação baseado em duas etapas, a dissolução do antibiótico seguida da sua difusão através da matriz do ALABC. Tanto a minociclina como a levofloxacina mantiveram sempre a sua atividade antibacteriana contra as estirpes *Staphylococcus* spp. após a libertação da matriz do ALABC. Este resultado sugere que a reação de polimerização não afetou os antibióticos incorporados e que estes se libertaram da matriz. Um estudo inovador *in silico* revelou a formação de ligações químicas covalentes e não covalentes entre a levofloxacina e a matriz de ALABC durante a reação de polimerização, o que terá contribuído para a não libertação total da levofloxacina.

As matrizes dos ALABCs modificados foram avaliadas em relação as suas propriedades mecânicas *quasi-static* seguindo a norma ISO 5833 e o seu desempenho mecânico não foi comprometido devido à inclusão dos aditivos referidos. Foram ainda efetuados estudos de biocompatibilidade das matrizes por contato direto e de citotoxicidade dos extratos de libertação dos antibióticos seguindo as especificações da norma ISO 10993-5 em linhas celulares de osteoblastos e fibroblastos que demonstraram quer a biocompatibilidade quer a ausência de toxicidade dos antibióticos libertados e da lactose incorporada.

No global, os resultados obtidos justificam o potencial do BC modificado com lactose como um valioso passo em frente no desenvolvimento de novos compósitos bioativos de BC, nomeadamente com lactose, como agente modulador da libertação de antibióticos, e com partículas de fosfato de cálcio, como portadores de antibióticos, para o controle de infeções ósseas e articulares.

**Keywords**: Cimento ósseo acrílico; Sistemas de libertação de antibióticos; Fosfatos de cálcio no cimento ósseo; Propriedades mecânicas; Biocompatibilidade

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# Abbreviations

| | |
|---|---|
| ALABC | Antibiotic-loaded acrylic bone cement |
| ANOVA | One-way analysis of variance |
| ASTM | American society for testing and materials |
| ATCC | American type culture collection |
| ATR-FTIR | Attenuated total reflectance Fourier transform infrared spectroscopy |
| BC | Bone cement |
| BIC | Biofilm inhibitory concentration |
| BSAC | British Society for Antimicrobial Chemotherapy |
| BSE | Back scattering electrons |
| ca. | Approximately |
| CaP | Calcium phosphate |
| CLSI | Clinical and Laboratory Standards Institute |
| CMW1 | Bone cement commercial brand from Depuy Synthes (Johnson & Johnson) |
| DCM | Dichloromethane |
| DE | Dissolution efficiency |
| DESE | Double-emulsion solvent evaporation |
| DFT | Density functional theory |
| DL | Drug loading for formulations |
| DMSO | Dimethylsulfoxide |
| DSC | Differential scanning calorimetry |
| *e.g.* | For example |
| EDS | Energy Dispersive Spectroscopy |
| EE | Encapsulation efficiency |
| EMA | European Medicines Agency |
| EUCAST | European Committee on Antimicrobial Susceptibility Testing |

| | |
|---|---|
| FDA | Food and Drug Administration |
| FEG-SEM | Field emission gun scanning electron microscopy |
| FTIR | Fourier Transform Infrared spectroscopy |
| HA | Hydroxyapatite |
| HPLC | High-performance liquid chromatography |
| ICH | International Conference on Harmonisation |
| ISO | International Standard Organisation |
| L | Lactose |
| L929 | Mouse fibroblasts cell line |
| Lev | Levofloxacin |
| M | Minocycline in Chapter 3 – Section 1 |
| MG63 | Human osteoblasts cell line |
| Mg-HA | Magnesium-Hydroxyapatite |
| MIC | Minimum inhibitory concentration |
| Mino | Minocycline in Chapter 2 |
| MMA | Methylmethacrylate |
| MRSA | Methicillin-resistant *Staphylococcus aureus* |
| MSSA | Methicillin-susceptible *Staphylococcus aureus* |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| $M_w$ | Molecular weight |
| NCTC | National Collection of Type Cultures |
| PBS | Phosphate-buffered saline |
| PLGA | Poly(lactic-co-glycolic acid) |
| PMMA | Polymethylmethacrylate |
| PMMAp | Polymethylmethacrylate particles |
| PTFE | Polytetrafluoroethylene |
| PVA | Poly(vinyl alcohol) |
| SD | Standard deviation |
| SDS | Sodium dodecyl sulphate |
| SEM | Scanning electron microscopy |
| Sr-HA | Strontium-Hydroxyapatite |
| TCP | Tricalciumphosphate |

| | |
|---|---|
| UV | Ultraviolet |
| VRE | Vancomycin resistant *enterococci* |
| VWM | Volume weighted mean |
| wt% | Weight percent mass per mass |
| XRD | X-ray diffraction |
| ZP | Zeta potential |

# Aims and Organisation of the Thesis

The base-concept of this work was to develop a novel ALABC by including particulate antibiotic delivery system, and a pharmaceutical excipient, lactose, into commercial bone cement (BC) to attain an improved antibiotic release profile without hampering antibacterial activity of antibiotics or the mechanical and biocompatibility properties of the BC.

Different approaches were explored to load the antibiotics into BC and improve drug release from the matrix, each carried out through a sequence of steps described as follows:

**1.** Antibiotic encapsulation into PMMA particulate systems.

- *Characterisation of the PMMA particles*

  PMMAp were produced using the double-emulsion solvent evaporation method and characterised regarding morphology, size distribution, surface charge, encapsulation efficiency and drug loading.

- *Assessment of antibiotic stability after particle formulation*

  PMMAp preparation involves harsh conditions including high-shear agitation, contact with organic solvents, as well as temperature and pressure variations during freeze-drying. Therefore, antibiotics were evaluated for physicochemical integrity and stability using standard techniques (*e.g.* FTIR). Antimicrobial activity was studied using standard microbiology assays with relevant bacteria strains, such as *Staphylococcus aureus* and *Escherichia coli*.

- *Antibiotic release from PMMA particles*

  *In vitro* antibiotic release experiments were performed in sink conditions according to standard methodology. Different kinetic models were applied to study the release profile of antibiotics from the different antibiotic-loaded PMMAp formulations.

- *Loading of PMMA particles into BC*

  An amount of particles with microbiological significance was thoroughly mixed with the BC powder component before adding the monomer to proceed with polymerisation according to the product specifications. The BC curing process was then monitored.

**2.** Use of lactose as a release modulator of the BC matrix.

- *Assessment of the bulk structure after antibiotics and lactose loading*

  In the presence of foreign-molecules the PMMA polymer suffers changes and readjustments resulting in increased porosity. SEM analysis, FTIR, XRD and porosity evaluation were performed to assess these structure changes.

- *Release profile of antibiotics from ALABC*

  *In vitro* release experiments were performed in sink conditions using different matrices of ALABCs. Standard methodology and a relevant release medium were used. Different kinetic models were applied to better understand the mechanistic profile of antibiotic release from the different matrices.

- *Assessment of antibiotic stability after loading into BC*

  The occurrence of a free radical vinyl polymerisation reaction of the monomer methylmethacrylate (MMA) occurs during BC setting, which is an exothermic reaction. Antibiotics were evaluated for physicochemical integrity and stability using FTIR and XRD. Also, antimicrobial activity after antibiotic release was assessed against relevant bacteria strains (*S. aureus, S. epidermidis* and *E. coli*) using standard microbiological assays.

- *Assessment of mechanical properties and biocompatibility*

  The incorporation of “foreign-molecules”, like the antibiotics and lactose, always induces matrix structure changes. *Quasi-static* mechanical properties and biocompatibility compliance of the novel ALABC were evaluated according to international guidelines, ISO 5833 and ISO 10993-5, respectively.

**3.** Loading doped-CaP particles, as levofloxacin delivery systems, into BC.

- *Assessment of the bulk structure after CaPs loading*

  Eventual structural change of the modified-BC composite was monitored through SEM, XRD and surface energy analysis.

- *Release profile of antibiotic from the CaPs-loaded BC*

  *In vitro* release experiments were performed following the same methodology refered above. Mathematical kinetic modelling was applied to describe the mechanistic profile of levofloxacin release from this BC composite.

- *Assessment of antibiotic stability after release from the CaPs-loaded BC*

  For the same reason referred above, antimicrobial activity of the levofloxacin released from BC-composites was assessed against relevant bacteria strains *(S. aureus, S. epidermidis and E. coli)* using standard microbiological assays.

- *Assessment of mechanical properties and biocompatibility of the CaPs-loaded BC*

  For the same reason explained earlier in 2., q*uasi-static* mechanical properties and biocompatibility compliance of the novel CaPs-loaded BC composite were evaluated according to international guidelines, ISO 5833 and ISO 10993-5, respectively.

Therefore, this thesis is organized as follows:

**Chapter 1** provides a brief overview of BC historical and chemical background, and of the development of related infections and causative microorganisms, including the concept of ALABC as a breakthrough in prophylaxis and treatment of related-bone infections. A summary of the kinetic models for drug release from the biomaterial matrix is also given, as well as the role of different additives and their effect on the mechanical performance.

**Chapter 2** describes the preparation of PMMA particulate systems for the encapsulation of levofloxacin and minocycline, using the double-emulsion solvent evaporation method, full characterisation of particles, *in vitro* release studies, microbiological assessment of the released antibiotics and loading of the selected particle formulation into BC.

**Chapter 3** presents the assessment of the main properties of the ALABC prepared by direct loading of antibiotics into a lactose-modified BC. Is divided in two sections:

**Section 1** describes the loading of minocycline into lactose-modified acrylic BC, solid-state characterisation, *in vitro* release profile, antimicrobial stability, biomechanical properties, and *in vitro* biocompatibility studies. An optimised lactose-loaded BC matrix is proposed. It is published as a full paper.

**Section 2** describes the loading of levofloxacin into the previously optimised lactose-loaded acrylic BC. Studies include preparation, solid-state characterisation, *in vitro* release profile, antimicrobial stability, *in silico* evaluation of levofloxacin-BC chemical bonding, biomechanical properties, and *in vitro* biocompatibility tests. It is published as a full paper.

**Chapter 4** describes the loading of Mg- and Sr-doped calcium-phosphate particles (CaPs), as bioactive agents and levofloxacin carriers, into the optimised lactose-loaded acrylic BC. Preparation and inner structure characterisation, *in vitro* release profile, antimicrobial stability, biomechanical properties, and *in vitro* biocompatibility tests are presented. It is published as a full paper.

**Chapter 5** presents a general discussion of the obtained results, conclusions and near-future perspectives.

**Annex** describes the HPLC-UV method validation developed to quantify the levofloxacin resultant from all *in vitro* release studies.

# Chapter 1

## GENERAL INTRODUCTION

# Foreword

The will of helping people live longer and healthier lives is leading breakthrough research and innovative medicines.

Fighting against serious diseases and bringing cure hope to patients in suffer is the commitment of worldwide researchers, clinicians and pharmaceutical companies. All engaged in finding new solutions – from early discovery to market access – that may help prevent and/or treat diseases.

One of those breakthroughs was the concept of drug targeting introduced by Paul Ehrlich in the mid-1950s (Kreuter, 2007).

Drug targeting, in opposition to traditional systemic treatments, has the ability to achieve rapidly the site of illness and control the drug release over a specified period of the treatment, ensuring high drug efficiency with minimal systemic toxicity (Meani and Romanó, 2007). This was a tremendous and promising challenge that conducted the drug-delivery domain from being a simple part of the pharmaceutical production process to become a driving force for innovation and benefit to the patients, with improved compliance and medical outcomes (Thassu et al., 2007).

Clearly, this strong development closely related to materials science burst development, specifically influenced biomaterials science research and ultimately led to an adjustment in its updated definition: *"A biomaterial is a substance that has been engineered to take a form which, alone or as part of a complex system, is used to direct, by control of interactions with components of living systems, the course of any therapeutic or diagnostic procedure, in human or veterinary medicine"*, which was introduced by D.F. Williams (2009) in his leading opinion paper.

This was of particular importance in the history of biomaterial-related infections where different methods for antibiotic encapsulation intended for local delivery without invasive procedures have been under investigation (Kreuter, 2007). In fact, after Sir John Charnley in the 1960s succeeded in anchoring femoral head prostheses using acrylic bone cement (BC) and established its use for orthopaedic purposes, Buchholz and Engelbrecht in the late 1970s used the acrylic BC as a drug delivery system for gentamicin, a broad-spectrum antibiotic, aiming prophylaxis and treatment of prosthetic joint infection (Buchholz and Engelbrecht, 1970).

Subsequently, the use of antibiotic-loaded acrylic bone cement (ALABC) has been considered the standard of care for patients with chronic bone and joint infection, providing local delivery of high levels of antibiotics for an extended period without exceeding systemic toxicity, while being a cost-effective procedure when compared to cementless implants (Jameson et al., 2015; Zilberman and Elsner 2008).

Herein a comprehensive insight on the ALABC as a well-established procedure in cemented arthroplasties and its importance for prophylaxis and treatment of prosthetic joint infection will be presented. Also, the attempts on the development of customisable acrylic BC, almost on demand for each particular case, will be described.

# 1. The Bone Cement

## 1.1. History and Biological Backgrounds of Bone Cement

The core material of BC is the synthetic polymer polymethylmethacrylate, commonly referred to as PMMA. Its history dates back to 1901 when the chemist Otto Röhm wrote a dissertation entitled “Polymerization Products of Acrylic Acids” (Kuhn, 2005). But it was not until 30 years later that it found commercial use first in the automobile market and then as a structural material through the well-known Plexiglas®, a glass-like hard material. This material presented formidable properties such as toughness, highly transparency with excellent resistance to ultraviolet radiation and weathering. Moreover, it could be coloured, moulded, cut, drilled, and formed. PMMA belongs to the first generation of biomaterials, according to Hench’s classification, characterised for being bioinert (Hench, 1980).

Currently, in the biomedical domain, PMMA is ineluctable in areas such as ophthalmology (contact and intraocular lenses), nephrology (dialysis membranes), dentistry (dental crowns and bridges) and on orthopaedics. In pharmaceutical technology it has been proposed for the microencapsulation of vaccines and coatings for oral drug delivery (Kreuter, 1992).

PMMA is a macromolecular polymer structure obtained by a free radical vinyl polymerisation reaction of the monomer methylmethacrylate (MMA) (Fig.1).

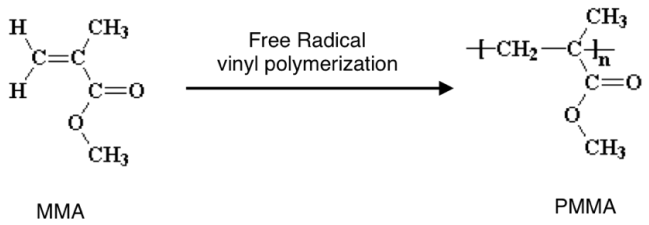



**Fig. 1** Polymerization reaction leading to a PMMA chain with *n* monomers.

Due to its remarkable rigidity properties PMMA was coveted to clinical use in orthopaedics. The first attempt was made with the polymer resultant from a polymerisation at 100°C by mixing ground PMMA powder to a liquid monomer MMA and benzoyl peroxide. This polymer was used as an attempt to close cranial defects in monkeys in 1938 and later for closing cranial defects in humans by producing plates in laboratory to adjust the hardened material on place. Then, the important discovery by Degussa and Kulzer (1943), of the self-polymerization of MMA at room temperature when added a tertiary amine as co-initiator, was considered the hour of birth of PMMA-bone cements, in a process still valid until today (Kuhn, 2005). From that moment on, PMMA attracted interest in the field of orthopaedics. In the middle 1940s Jean and Robert Judet reported the development of acrylic femoral hemiarthroplasties and in the early 1950s, Kiaer and Haboush reported the femoral fixation implants. Also, the occurrence of the II World War and the

thousands of resulting injuries with amputated limbs and serious skull damages bursted the biomedical use of PMMA in orthopaedics (reviewed by Kuhn, 2005).

However, modern success and recognition of PMMA in orthopaedics is attributed to Sir John Charnley, a British orthopaedic surgeon who pioneered the hip replacement operation when in 1960s he succeeded in anchoring femoral head prostheses using PMMA as BC (Fig. 2). Charnley's early clinical accomplishments established a foundation for the continued use of PMMA in orthopaedics (reviewed by Kuhn, 2005) and is recognised as one of the medical breakthroughs of the 20$^{th}$ century (Fig. 2).


**Fig. 2** Sir George Charnley and a stamp tributing in 2010 "Total hip replacement operation pioneered by Sir John Charnley 1962" as a Medical Breakthrough (images adapted from: http://www.nw-hip-knee-clinic.com/total-hip-replacement.html and http://www.collectgbstamps.co.uk/explore/issues/?issue=22580)

PMMA-based BC, simply called as BC, is an inevitable biomaterial across the orthopaedic domain, being currently used in arthroplasties (Lewis, 2015), vertebroplasthy, kyphoplasty (Meng et al., 2013; Papanastassiou et al., 2014; Robinson et al., 2011), cranioplasty (Aydin et al., 2011), and craniomaxillofacial reconstruction surgery (Fernandes da Silva et al., 2014). Its main indications are in joint rebuild due to osteoarthritis, rheumatoid arthritis, traumatic arthritis, vascular necrosis, osteoporosis and severe secondary joint destruction after trauma and review of previous techniques of arthroplasty as well as definitive skull implants. In spite of its non-adhesive properties, BC provides a good mechanical anchorage between the irregular bone surface and the prosthesis. By filling the free space between the implant and bone tissue, the BC allows an even distribution of weight, and other forces, between the various components and bone (Fig. 3).

Through the years, however, the "bone cement" designation became also indifferently used for the second generation of biomaterials that gradually introduced requirements as bioactivity and osteoinduction into their design, thus promoters of bone regeneration. Namely, vertebroplasty and kyphoplasty domains adopted injectable BCs introducing calcium phosphates, calcium sulphates and composites along with the acrylic BCs. These materials, now commercialized under a plethora of compositions and different brands, present the upside of being resorbable at different rates, depending on composition, but the downside of the poor mechanical performance making them unsuitable for load-bearing applications (Kuhn, 2005; Lewis, 2006; Spierings, 2005).

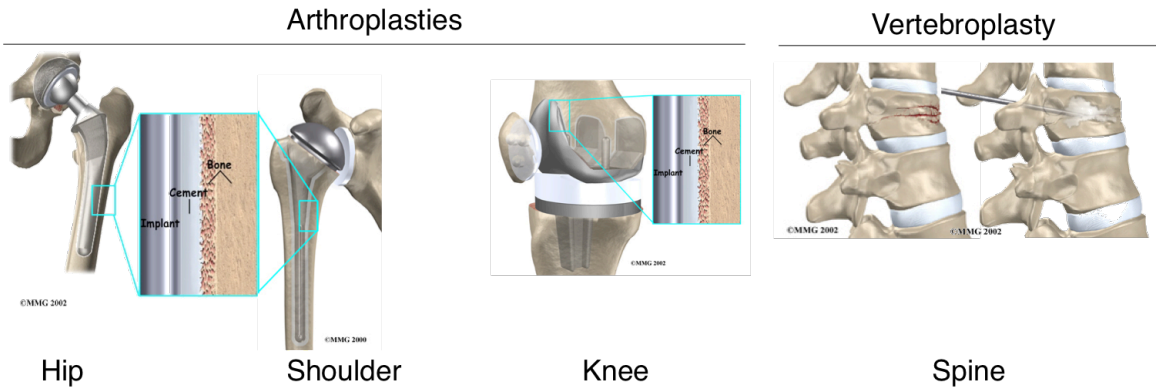



**Fig. 3** Schematic illustration of BC use in arthroplasties (hip, shoulder and knee) and vertebroplasty. Images were adapted from http://www.eorthopod.com/content/.

## 1.2. Chemical Background of Bone Cement

The BC base-composition has not greatly changed and is commercially presented as a sterilised two-component system - a powder and a liquid - that is allowed to polymerize inside the patient. The powder component consists of pre-formed PMMA beads-shaped and/or methacrylate copolymers with a radiopacifier included in the chains and benzoyl peroxide included in the polymer beads or simply admixed to the PMMA powder (Table 1). The liquid component contains the monomer stabilised with hydroquinone and also a tertiary amine (N,N-dimethyl-p-toluidine) as the reaction activator. Both components may also have other constituents such as colouring agents, viscosity and rigidity modifiers or antibiotics (Kuhn, 2005; Lewis, 2009).

When both components are joined at room temperature, the activator from liquid component reacts with the initiator from the powder component and radicals are formed as shown on Table 2. These very reactive species, promote chain growth from reaction with other MMA molecules as well as with the pre-formed PMMA chains by adding to the reactive C=C doubled bond, conducting to polymer chains reaching molecular weights of 100,000 to 1,000,000 g/mol. During reaction the viscosity of the mixture will increase and a dough is formed. As viscosity increases monomer molecules decreases mobility and chain recombination takes place conducting to the end of polymerization. Surgeons have to monitor time very carefully because dough-handling time is short, after which it gets impossible to work with. The overall setting time of BC depends on its composition. However, according to commercial available information, it never exceeds 15 minutes (Depuy, 2004).

In the first years two factors were often considered adverse to the use of acrylic BC. The temperature achieved during polymerization and the residual monomer-leaching were frequently related to BC failure. PMMA polymerization is an exothermic chemical reaction (with 57 kJ per mole MMA), so dough temperature increases during hardening. As this occurs inside the patient

this fact was mentioned to cause necrosis in the implant boundary bone tissue and, in turn, the implant aseptic-loosening.

**Table 1.** BC components constitution and information about its identification and function (based on Kuhn, 2005 and Lewis, 2009).

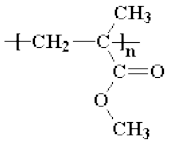
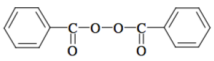
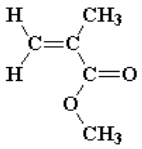
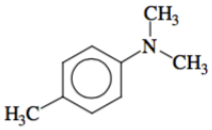
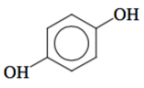
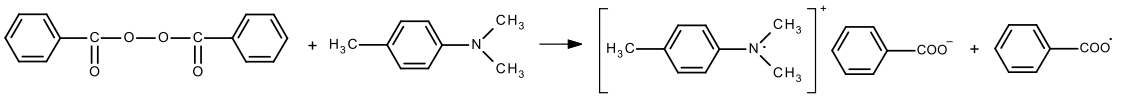
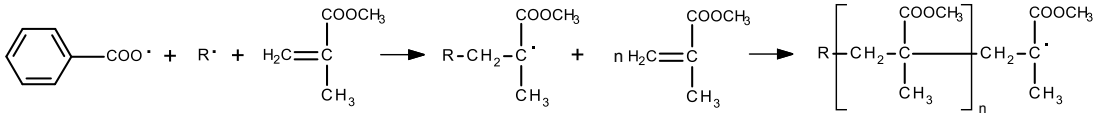
| BC Component | Constituent | Name | Chemical Structure | Function |
|---|---|---|---|---|
| **Powder** | Polymer | PMMA or MMA-copolymers (varied molecular weights) |  | Pre-formed polymer chains as bead-shape microparticles diameters (generally 40 μm) that will grow in the presence of the activated monomer. |
| | Initiator | Benzoyl Peroxide |  | Initiator of the radical polymerization creating radicals that will promote chain growth. |
| | Radiopacifier | Barium Sulphate<br>Zirconium dioxide | $BaSO_4$<br>$ZnO_2$ | Allows x-rays-monitoring and eventually BC failure. |
| | Additives | Antibiotic(s)<br>Dye<br>Plasticizer<br>Stiffer | | Prevent or treat infection; Improve distinction from body tissues during surgery;<br>Increase viscosity;<br>Increase resistance. |
| **Liquid** | Monomer | MMA (varied molecular weights) |  | Base-unit of the PMMA that will be activated in the presence of free radicals. It will promote chain growth.. |
| | Activator | N,N-dimethyl-p-toluidine |  | Enables cold curing of the polymer by activating benzoyl peroxide to start radical formation. |
| | Inhibitor | Hydroquinone |  | Avoids premature polymerization during storage. |
| | Additives | Dye | | Improve distinction from body tissues during surgery. |

**Table 2.** Principal reactions occurring during BC setting (adapted from Bettencourt, 2006 and Kuhn, 2005).

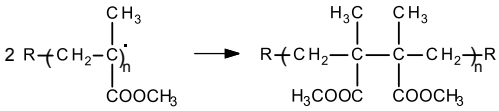
**1st step – Radical formation**



**2nd step – Chain Growth**



**3rd step – Chain recombination (end of polymerisation)**



Yet those assumptions were based on the *in vitro* measurements of temperature during BC hardening (Spierings, 2005), which however do not correspond to those actually reached *in vivo*, varying between 40 and 46ºC at the bone tissue-BC interface (Kuhn, 2005). *In vitro* BC assayed specimens were at least two-fold thicker than that encountered *in vivo*, 3 to 5 mm (Fig. 4). Also, *in vivo*, blood circulation and metallic implant are important paths for heat dissipation.

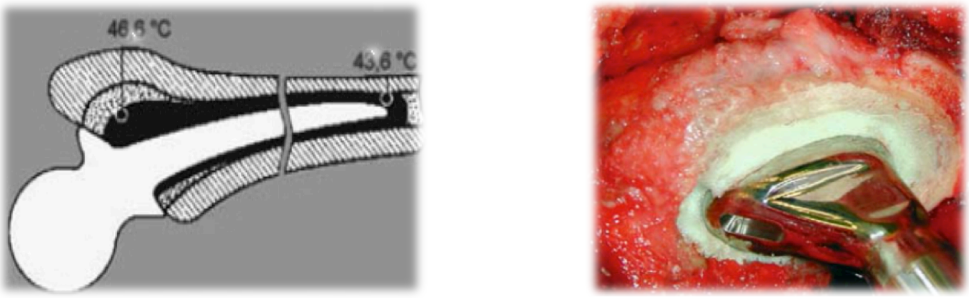



**Fig. 4** Images of the BC thickness, in black at the schematic illustration (left) and in whitish at the photo of a real surgery procedure (right).

As temperature peaks are strongly dependent on the cement composition, manufactures have found ways of changing constituents in order to diminish this problem – *e.g.* by decreasing the amount of monomer added and PMMA pre-polymer weight (Spierings, 2005). This action also led to less residual monomer leaching. It is now currently accepted that this effect is negligible on causing failure of bone cement (Bettencourt et al., 2000; Hendricks et al., 2004).

# 2. Bone cement related infection

Arthroplasties are, throughout the world, the most common surgical procedure using BC to anchor implants to the bone (Lewis, 2015). With the sustainable aging of the population, the number of elderly and trauma patients requiring joint replacement or internal fixation devices is steadily increasing. The surgery eliminates pain and patients dramatically regain mobility and functionality of the joint, therefore, quality of life (Malchau and Breusch, 2005).

Although those surgical procedures are generally highly successful, with satisfactory longevity and clinical results, they still experience numerous failures, like aseptic loosening, as a result of wear debris of the materials used in procedure, or infection, mainly related to the risk of inclusion of a foreign-material along with an exposed incision during surgery. Infections associated with prosthetic joints occur less frequently than aseptic failures, but represent the most devastating complication with high morbidity and substantial cost (Trampuz and Widmer, 2006). Depending on the number of bacteria introduced during surgery, on the virulence of the bacteria and on the condition of the host's defence, eventually deep surgical infection appears (Frommelt, 2005).

The actual rate of infection of total hip replacement ranges from 0.5-3.0% for primary total hip replacement and 3-6% after revision hip surgery. However, the increasing number of joint replacements being performed means the absolute number of such infections will remain significant and pose substantial costs to healthcare systems worldwide. Often, the key for the management of these infections, designated as periprosthetic-joint infections, is the removal of the infected prosthesis and debridement of the infected bone tissues, although recent studies suggest the retention of the infected implants may be an acceptable option in selected patients (Legout and Senneville, 2013; Song et al., 2013).

Difficulty on the treatment arises on the diagnosing of less explicit infection. Many of the current laboratory tests are indirect measurements lacking specificity for diagnosis of infection as they can also be seen in aseptic, mechanical loosening. Researchers are engaged on finding new diagnosis methods to improve specificity and sensitivity, such as sonication of removed implants, molecular methods, and mass spectrometry (Hendricks et al., 2004; Legout and Senneville, 2013; Springer, 2015).

## 2.1. Aetiology and pathogenesis of periprosthetic-joint infection

Understanding both aetiology and pathogenesis of bone infection is relevant to improve precautions and measures towards prevention and/or treatment.

Every operation harbours the risk of surgical infection because it is impossible to avoid completely the bacterial contamination in the operating theatre. The environment conditions along with the

patient bacterial flora provide the source for these infections. Whether contamination leads to infection depends on the number of bacteria introduced during surgery, the virulence of the bacteria and the condition of the patient's defence. If the latter is unable to balance the bacterial attack, surgical infection will result (Frommelt, 2005). Despite considerable progress in prevention the use of PMMA, as other biomaterials, suppresses the patient's defence locally entailing the risk of attracting infectious microorganisms (Van de Belt et al., 2001).

According to the mechanism of bacterial inoculation Hendricks et al. (2004), grouped in three the causes of prosthesis-related infections: direct contamination of the biomaterial or the surrounding tissues; contamination by the spread of a superficial infection; and blood-borne contamination. In spite of the importance of the latter two, the direct relation to the occurrence of deep-infection is not yet clarified. However, direct contamination is likely to cause the greater part of prosthesis-related infections. There is a generalised agreement that 90% of infections during the first year after implantation are due to bacterial contamination during surgery (Charnley, 1972; Zimmerli, 2015). Therefore, precautions before surgery are well established as the most effective way of protecting orthopaedic devices from contamination and eventual infection. Prophylactic actions regarding the patient and the operating room are carefully monitored and implemented, including: the patient good general health condition; previous patient screening for most prevalent microorganisms causing bone infections and eventual decontamination; and proper hygiene of patient's skin (Frommelt, 2005; Van de Belt et al., 2001).

Implant-associated infections result from bacteria adhesion to an implant surface and subsequent biofilm formation at the implantation site (Ribeiro et al., 2012; Zimmerli, 2015). The interaction between the device and the patient's bacterial flora starts as soon as the implant is fixed with BC. At that moment the foreign-body remains devoided of a microcirculation, which is crucial for host defence and the systemic delivery of antimicrobial agents. From that interaction, specialised bacteria begin a self-protection process from the patient's defence mechanism, resisting against external and internal environmental factors, by growing in structures known as biofilms, which are highly resistant to antibiotics (Trampuz and Widmer, 2006). Biofilms allow bacteria to spread along the surface of the implant and BC. Periprosthetic infection starts when some of the bacteria switch back to planktonic forms (freely suspended bacteria) (Fig. 5) inducing infection in the adjacent bone tissue, in a process known as periprosthetic osteomyelitis (Frommelt 2005; Legout and Senneville, 2013; Ribeiro et al., 2012).

The process of bacterial adhesion to implant is influenced by environmental factors, bacterial properties, biomaterial surface properties and by the presence of serum or tissue proteins. Also, the nature of the surface material, such as chemical composition of the material, surface charge, hydrophobicity, surface roughness and the presence of specific proteins at the surface, are all thought to be important in the initial cell attachment process. Consequently adjustments of the biomaterial´s surface may contribute to inhibiting bacterial adhesion and prevent implant-associated infection (Ribeiro et al., 2012).

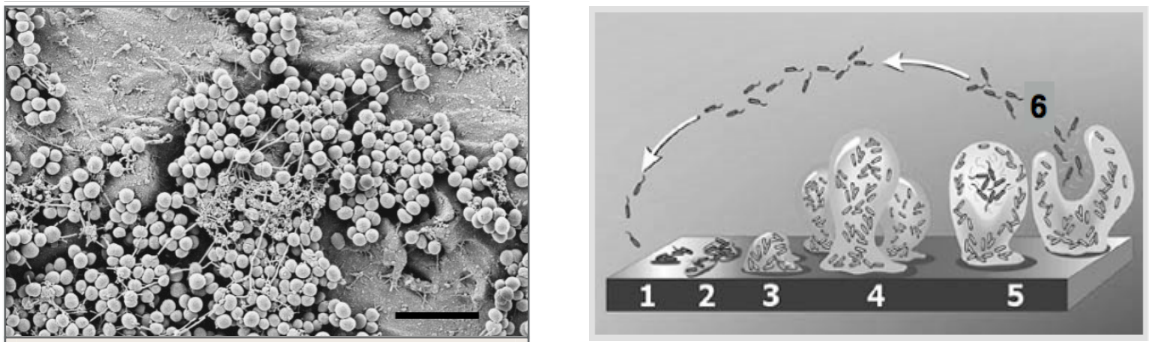



**Fig. 5** A scanning electron micrograph of a *Staphylococcus epidermidis* biofilm on a foreign material (adapted from Zimmerli et al., 2004) (left); Schematic representation of the stages of a biofilm development on a medical device surface (right). Label for right image: 1 - Bacteria attraction; 2 - Joint replacement surface adhesion; 3 - Embedded biofilm growth; 4 - adhesive exopolymers composed mainly of polysaccharides; 5 - Small canaliculi; 6 – Transmission (adapted from http://www.entkent.com/biofilms.html, Graphic by Peg Dirckx and David Davies, ©2003 Center for Biofilm Engineering Montana State University).

## 2.2. Microbiology of periprosthetic-joint infection

When on biofilm form, even low-virulence microorganisms, such as Gram-positive staphylococci and *Propionibacterium acnes* may cause infection. Staphylococci, as *S. aureus* and *S. epidermidis,* account for more than 50% of the periprosthetic infections related with hip and knee arthroplasties (Table 3). They represent, in absolute, the main causative agents in orthopedics (Ribeiro et al., 2012; Zimmerli, 2015) as they possess several cell-surface adhesion molecules that facilitate its binding to bone matrix, stronger for *S. aureus*. In addition, *S. aureus* produces virulence factors and rapidly develops antimicrobial resistance. As shown on Table 3, *S. epidermidis* is the most frequently isolated member of the group of coagulase-negative staphylococci from implant-associated infections mainly related to nosocomial infections, and have been found to be more antibiotic resistant than *S. aureus*. The strong virulence of *S. epidermidis* is attributed to its ability to adhere strongly to surfaces, form a biofilm that confers protection from phagocytosis and other major components of the host defence system (Ribeiro et al., 2012).

The period between colonisation and clinically detectable infection may last for 3 months (early infection), for 3—24 months (delayed infection) or even up to two years (late infection). In prosthetic joint infections, early infection occurs perioperatively and is generally caused by virulent microorganisms as *S. aureus* (Trampuz and Widmer, 2006). This problem is aggravated by the emergence of multirresistant bacteria against which applied therapy is not efficient (Anagnostakos et al. 2008; Lewis, 2009). Delayed infection is mainly caused by microorganisms of low virulence, *e.g. Propionibacterium acnes*. Late infection results mostly from haematogenous seeding originating from skin, respiratory, dental, and urinary tract infections. In internal fixation devices, haematogenous infections are less frequent than in prosthetic joint infections (Trampuz and Widmer, 2006).

**Table 3.** Microbiology of prosthetic joint infections (adapted from Zimmerli et al., 2004; Zimmerli, 2015).

| Microorganism | Frequency (%) |
|---|---|
| **Gram-positive cocci** | ~ 65% |
| *Staphylococcus epidermidis* (50% of late infections) | ~ 30—43% |
| *Staphylococcus aureus* (early infections) | ~ 12—23% |
| *Streptococcus* spp. | ~ 10% |
| *Enterococcus* spp. | ~ 3—7% |
| **Aerobic Gram-negative bacilli** | ~ 6% |
| *Enterobacteriaceae (such as Escherichia coli)* | |
| *Pseudomonas aeruginosa* | |
| **Anaerobic bacteria (Gram-positive)** | ~ 4% |
| *Propionibacterium* spp. | |
| *Finegoldia magna* | |
| **Polimicrobial** | ~ 20% |
| **Culture-negative** | ~ 7% |
| **Fungi** | ~ 1% |

Systemic administration of antimicrobial agents is an easy and frequently used way to control biofilms. However, the majority of antimicrobial agents that are effective against planktonic bacterial cells are ineffective against the same bacteria when growing in a biofilm. Combinations of multiple antimicrobial agents with different spectrum of activity and modes of action are being used as strategy to improve the performance of these antimicrobial agents and circumvent bacterial adaptation. The tremendous resistance of biofilms to conventional antibiotic therapy has prompted intense research on finding solutions of modifying surfaces capable of resisting to bacterial colonization.

One of these solutions is the use of ALABC for prophylaxis and treatment of implant-associated infections.

# 3. Antibiotic-Loaded Bone Cement

Albeit the success of acrylic BC for anchoring implants to the bone on arthroplasties, the problematic of infections remained unsolved. Buchholz and Engelbrecht (1970) envisaged the inclusion of antibiotics into the PMMA-bone cement using it as a local antibiotic delivery to treat implant-related infections. They hypothesised antimicrobial agents could release from the BC matrix as the monomer did. At that time, it was discovered a new broad-spectrum aminoglycoside, gentamicin, which presented good antimicrobial activity against the main pathogens causing prosthetic joint infections, such as, *Staphylococcus* spp. (coagulase-positive and coagulase-negative), *P. aeruginosa* or *E. coli* (Frommelt and Kuhn, 2005). Hence, Buchholz and Engelbrecht decided to add powdered gentamicin to the BC powder component and performed one-stage revision in patients suffering from periprosthetic infection using that ALABC. Although the release mechanism of gentamicin was largely unknown (Buchholz et al., 1981), it proved to be very effective in producing long-term high-level drug concentrations. With this procedure, infection rate after primary implantation of artificial joint replacement reduced from about 7% to lower than 1%. Since then, the use of ALABC has been considered to be the standard of care for patients with chronic infection, for local delivery of antibiotics.

In spite of its current and well-established use this biomaterial presents some drawbacks related to the:

- matrix structure: influence on the antibiotic deliver and on biofilm development;
- limited number of antibiotics pre-loaded: only a reduced number of antibiotics are available on commercial formulations;
- bioactivity: due to the smooth surface there is poor osteointegration, which is often referred as one of the main causes for implant failure.

Proposed solutions to address these drawbacks often imply the addition of other substances over the base-components of the BC, which directly affect its mechanical performance, hampering its use for prophylaxis. Therefore, improve ALABCs performance without jeopardising the mechanical behaviour represent the big challenge of research on this domain.

## 3.1. Rationale for ALABC use

Considering that bone is a mineralised tissue and cannot expand, when inflammation occurs blood flow is reduced. Consequently, drugs cannot be transported via blood circulation and do not reach the inflamed bone tissues, which, ultimately became infected. This is the main reason why local application is the preferable way to administer antibiotics in a situation of bone infection (Frommelt and Kuhn, 2005).

Moreover, whenever an arthroplasty is performed, both the implant and the BC, used to anchor it to the bone tissue, are indwelling medical devices intended for long-term presence inside the patient. All biomaterials in man entail the risk of attracting infectious microorganism to the surface where they may develop biofilm. To effectively eliminate bacteria in a biofilm, local antibiotic concentrations must achieve 10 to 100 times the usual bactericidal concentration. This renders treatment with systemic antibiotics ineffective against biofilm (Samuel, 2012). As a consequence periprosthetic infection arise and a surgical revision is needed for total eradication of these pathogens, which may include partial or total removal of the foreign-materials and debridement of the infected tissues along with the local administration of antibiotics at the site of infection.

Under these conditions, ALABC is useful for both treatment, by delivering extraordinary high levels of antibiotic concentration at the site of infection, and prophylaxis, by direct delivery of antimicrobial agents on the surface of implants at risk for bacterial colonisation.

There is also an economic reasoning as the ratio cost-efectiveness has a great influence on decision-making. Recent data show that cemented implants outperform cementless besides being cheaper. The development of non-cemented arthroplasty dates back to the 1980s, as an attempt to avoid BC use due to the reported early loosening and implant failure attributed to the effect of BC application procedure on the surrounding implant bone tissues. The implants used in non-cemented arthroplasty possessed modified-surfaces to directly fix to the bone tissues without cement. However, these implants continued to fail, although, differently, due to metal wear debris reactions (Haddad, 2011; Smith et al., 2012).

Reports on BC failure are now seen as being influenced by previous generations of implants with poor cement mixing and inadequate cementation techniques. With evolution of materials, acquired knowledge from failing and improved mixing techniques, cemented arthroplasty has regain followers and contemporary data reflects equivalent or better survival of cemented compared to cementless implants. Jameson et al. (2015) found that cementless implant almost doubles cemented implant costs, on needed materials. Furthermore, cementless implants did not reduce rates of aseptic revision and demands adequacy to the patient, *e.g.* depending on femoral size the implant must be different, whereas on using BC the same implant fits to different users.

## 3.2. Types of ALABC and Clinical use

ALABC is used for both prophylaxis and therapy of periprosthetic infections. The requirements for these two purposes are different, as prophylactic use is determined by the pathogens expected at the site of the prosthesis, and therapeutic use demands identification of the pathogens prior to the revision surgery (Frommelt and Kuhn, 2005).

In either case there are two ALABC alternatives:

1) premixed commercially available brands, in which the powdered antimicrobial agent(s) is mixed in the cement powder;

2) off-label formulations in which the selected antibiotic is hand-mixed with the cement powder of a plain commercial brand, by the surgeon, in the surgery room under strict and ruled hygienic conditions, during the arthroplasty implantation procedure (Lewis, 2009).

When proposed for prophylaxis, low doses of antibiotics are required in order to avoid adverse drug effects and jeopardising of the mechanical properties of the BC intended for mechanical fixation of the implant (Jiranek et al., 2006). In general, a low-dose ALABC contains 0.5—1 g of powdered antibiotic(s) per 40 g of BC powder. In Europe, it is used regularly in primary arthroplasties aiming to prevent bacteria from colonising the surface of the artificial joint replacement. However, since 2006, six commercial low-dose ALABC have been approved by the FDA for prophylactic use in the second stage of a two-stage total joint revision, after the complete eradication of infection, and specifically not for prevention on primary or first revision total joint arthroplasty (Lewis, 2015). Gentamicin turned out to be a suitable agent for prophylactic use in ALABC and it is consensual for all commercial brands.

ALABC is used therapeutically in situations of arthroplasty revision when infection is already installed. After debridement of infected tissues and depending on the extension of the infection, ALABC is used differently: when implant is substituted during revision surgery, ALABC is used prophylactically for the new prosthesis fixation; in cases of deeper debridement ALABC is used to prepare a spacer (Fig. 6) for implantation during several weeks before a second surgery to insert a new prosthesis (Liu et al., 2014). Also ALABC beads (Fig. 7) have been used to treat chronic osteomyelitis. These two devices are self-made off-label ALABCs that are loaded with high amounts of antibiotic for local delivery in high concentrations to fight bacteria; it is also used during revision to fill up the space of debrided tissues at the site of bone infection (Gomes et al., 2013).

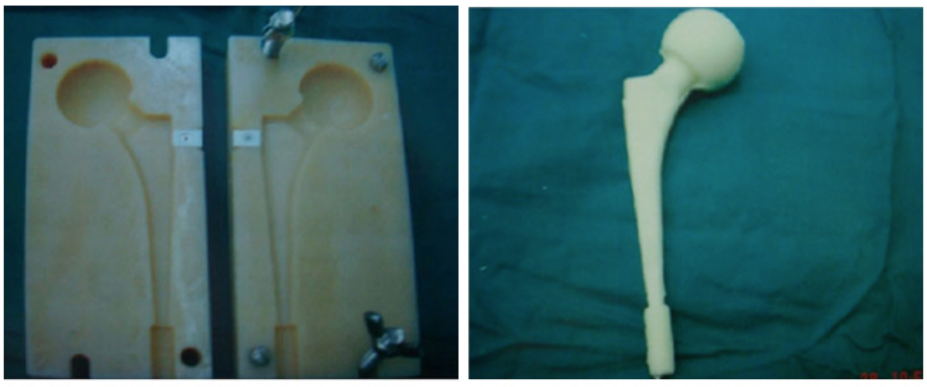


**Fig. 6** Photo of a spacer mould (left) and of the spacer containing the antibiotic ready to insert inside the patient as a functional prosthesis (right) (adapted from Liu et al., 2014).

On first revision situation, ALABCs may contain up to 4 g of powdered antibiotic(s) per 40 g of BC powder for an effective release of the drug with therapeutic concentrations. To circumvent cases of known resistance to gentamicin, confirmed allergic reactions to antibiotics from patient, or even in the case of chronic recurrent infection, there are other options of antibiotics with different mechanisms of action commercially available. For example, tobramycin alone or combinations of clindamycin or vancomycin with gentamicin are available as commercial brands to use in revision surgery (Frommelt and Kuhn, 2005). Moreover, higher doses ranging 6 to 8 g of powdered antibiotic(s) per 40 g of BC powder are used in the form of beads or spacers to guarantee a local sustained release of the antibiotics to eradicate the biofilm causing the infection (Jiranek et al., 2006). Retrospective studies suggest that the combination with antibiotic systemic administration has a favourable effect in the prevention and treatment of periprosthetic infection (Frommelt and Kuhn, 2005).

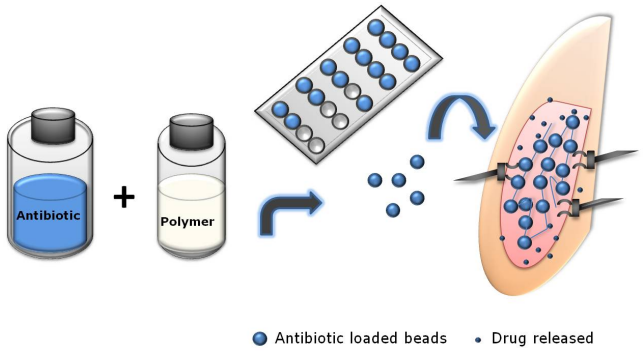



**Fig. 7** Illustration of antibiotic-loaded beads preparation and *in situ* implantation close to bone tissue (adapted from Gomes et al., 2013).

## 3.3. The antibiotics

Antibiotics to be loaded into BC must present not only a broad-spectrum of activity but also fulfil requirements as availability in powder form, termostability and water-solubility (Samuel, 2012). Table 4 shows the main antibiotics used in systemic treatment of orthopaedic infections.

As referred earlier, the first antibiotic to fulfil those requirements used in ALABC was gentamicin which is an aminoglycoside antibiotic with both bactericidal and bacteriostatic effect, depending on concentration, against the main pathogens causing prosthetic joint infections, such as, *Staphylococcus* spp. (coagulase-positive and coagulase-negative), *P. aeruginosa* or *E. coli.*

The main commercial brands of BC intended for prophylaxis consist of a gentamicin- and tobramycin-loaded PMMA-BC, with broad-spectrum activity regarding bone infections (Arora et al., 2013; Gallo et al., 2005). In revision surgery for treatment of infection commercial-brands may also offer alternatives to gentamicin by adding specific antibiotics (*e.g.* clindamycin and

vancomycin) according with the identified microorganisms. In the case of clindamycin, its combination with aminoglycosides is known to have an antimicrobial effect on more than 90% of the bacteria common to infected arthroplasties (Rice and Mendez-Vigo, 2009; Van de Belt *et al.,* 2001).

However, the increasing emergence of multiresistant bacterial strains especially methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant *enterococci* (VRE), multiressistant *Acinetobacter baumannii* and extended spectrum *beta-lactamase* producing *Enterobacteriaceae* (Cui *et al*., 2007; Trampuz and Widmer, 2006), makes most commercially-available BCs inadequate for local antibiotic treatment (Anagnostakos et al., 2008).

**Table 4.** Classification of common antibiotics used in systemic treatment of orthopaedic infections (adapted from Lewis, 2013).

| Antibiotic class; Example | Mechanism of action | Activity or target species | Effect on Bacteria |
|---|---|---|---|
| β-lactams; penicillin | Inhibition of cell wall biosynthesis | Broad-spectrum activity | Bactericidal |
| Aminoglycosides; gentamicin tobramycin | Binding of 30S ribosomal subunit | Broad-spectrum activity | Bacteriostatic or Bactericidal (depending on concentration) |
| Chloramphenicols; chloramphenicol | Binding of 50S ribosomal subunit | Broad-spectrum activity | Bacteriostatic |
| Macrolides; erythromycin | Binding of 50S ribosomal subunit | Broad-spectrum activity | Bacteriostatic or Bactericidal (depending on concentration) |
| Lincosamides clindamycin | Binding of 50S ribosomal subunit | Broad-spectrum activity | Bacteriostatic |
| Tetracyclines; minocycline | Binding of 30S ribosomal subunit | Broad-spectrum activity | Bacteriostatic |
| Rifamycins; rifampicin | Binding of RNA polymerase β-subunit | Gram-positive bacteria | Bactericidal |
| Glycopeptides; vancomycin | Inhibition of cell wall biosynthesis | Gram-positive bacteria | Bactericidal |
| Quinolones; ciprofloxacin levofloxacin | Inhibition of DNA synthesis | Broad-spectrum activity | Bactericidal |
| Lipopetides; daptomycin | Depolarization of cell membrane | Gram-positive bacteria | Bactericidal |

Many researchers are engaged in finding newer broad-spectrum antibiotics able to be loaded into BC but there is no agreement concerning the choice or dose to be mixed with the cement (Samuel, 2012). It should be emphasised that these decisions have direct influence on other crucial properties of BC, thus conditioning the application to which it may be used for. For example, if a high-dose of antibiotic is loaded, the resultant ALABC may result useless for load-bearing functions because of unsuitable mechanical properties. In this case ALABC is moulded in other forms, such as beads or spacers.

Hence, most studies reporting alternative antibiotics loaded into BC are proposals for spacers or beads intended for the treatment of bone-related infections. Anagnostakos et al. (2008) suggested the use of linezolid, which rapidly penetrates bone and tissue and offers little resistance, to load hip-spacers with good results against MRSA. Anguita-Alonso et al. (2006), tested different high-loading antimicrobials into PMMA beads, *i.e.* cefazolin, ciprofloxacin, gatifloxacin, levofloxacin, linezolid and rifampicin and reported successful release of all antimicrobials, without giving any information on the mechanical performance of such cements. Rifampicin, however, was already reported to hinder BC polymerisation (De Palma et al., 1982; Galvez-Lopez et al., 2014).

Daptomycin, a last-generation lipopetide antibiotic effective against multidrug-resistant Gram-positive pathogens commonly found in osteomyelitis and joint infections, is one of the most coveted drugs for loading into BC and use against *Staphylococci* and *Streptococci*. The first report dates from 1991 when daptomycin was combined with vancomycin and amykacin, with improved release due to the presence of a poragen (dextran) but only for applications when structural integrity was unimportant (Kuechle et al., 1991). As already pointed out, the results greatly depended on the amount of daptomycin loaded, which in turn, determines its application (Cortes et al., 2013; Gálvez-López et al., 2014; Hsu et al., 2014, Rouse et al., 2006). More recently, Peñalba Arias et al. (2015), have reported the use of a low-dose BC loaded with daptomycin combined with gentamycin to successfully and completely inhibit *S. epidermidis* biofilm. Nevertheless, none of these studies have tested the compliance with the international standards for BC mechanical properties (ISO 5833), specifically regarding the flexural strength determination. As the specific literature is scarce, further studies on the mechanical performance of daptomycin-loaded BC are still needed to exploit the potential therapeutic utility of this well tolerated antibiotic with a low risk of spontaneous appearance of resistance (Rice and Mendez-Vigo, 2009).

There is, however, a worldwide controversy concerning the use of novel antibiotics, like daptomycin (authorized by FDA in 2003) without well-established rules considering that bacterial resistance is spreading faster than the discovery of new antibiotics (Lewis, 2013). The danger of reaching the end of antibiotic pipeline is real and close in time, suggesting that those antibiotics should be preserved and only used when there is no therapeutic alternative (Chong and Sullivan, 2007; Cooper and Shlaes, 2011; Editorials, 2013).

### *Minocycline and Levofloxacin*

As a valuable alternative to the lack of new antibiotics, some researchers are proposing the upraising of old antibiotic compounds, once they have remained active against a large number of currently resistant bacteria (Garrido-Mesa et al., 2013).

Within this context two well known antibiotics have found recently a renewed interest in literature and have been referred as presenting a broad-spectrum of activity against the main organisms responsible for prosthetic joint infections, especially against MRSA. Minocycline, a second-generation tetracycline (Fig. 8, left), and levofloxacin, a third-generation fluoroquinolone (Fig. 8, right) (Bishburg and Bishburg, 2009; Landersdorfer et al., 2009; Lima et al., 2014).

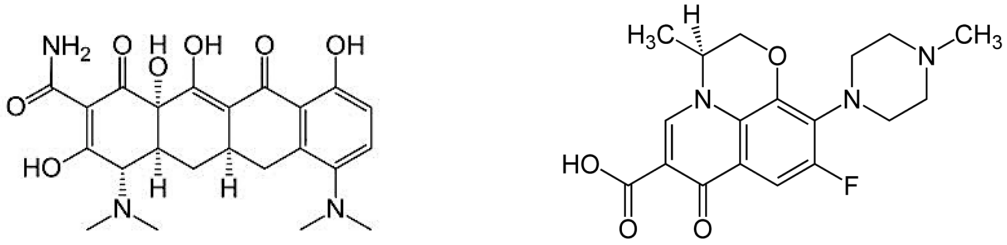



**Fig. 8** Chemical structures of minocycline (left) and levofloxacin (right).

Minocycline is in clinical use for over 40 years and has been considered well tolerated. It has excellent oral bioavailability, tissue penetration and tolerability, presenting a prolonged half-life of 15–19 h, probably due to extensive tissue penetration and protein binding (ca. 75%) (Bishburg and Bishburg, 2009). Recent *in vitro* studies have reported high rates of susceptibility to minocycline both among community and nosocomial MRSA isolates. Moreover, Gomes and Fernandes (2007) reported that low minocycline concentrations (1 μg/mL) are able to stimulate the proliferation of osteoblastic-induced bone marrow cells. Minocycline hydrochloride ($M_w$ 493.94 g/mol) is soluble in water preferably at pH < 5.6, with a greater partition coefficient at neutral pH indicating enhanced lipophilic properties for pH > 7 (Zbinosvky and Chrekian, 1977). Regarding stability, minocycline is photosensitive and retains 90% of its initial potency for 1 week at room temperature at acidic pH (pH < 5.2). At pH 6.6 minocycline potency is reduced to 73%. Buffers solution containing components of formate, acetate, phosphate and borate are strong catalysers of minocycline degradation (Pawelczyk and Matlak, 1982).

Levofloxacin is a broad-spectrum antibiotic with excellent tissue penetration and availability in both oral and intravenous formulations, generally well tolerated (Zimmerli, 2015). Stereochemically stable in body fluids, it binds to plasma proteins at ca. 24 to 38%. The elimination half-life after single (500 mg) or multiple doses of levofloxacin ranged from 6.4 to 7.4 h and 6.9 to 7.6 h, respectively. Levofloxacin is active against a range of Gram-positive organisms.

Due to reported adequate penetration into uninfected osteoarticular tissues, achieving effective microbiological concentrations in synovial tissue, cancellous and cortical bone tissues for susceptible pathogens generally encountered in bone and joint infections. Levofloxacin is being used in the treatment of most osteoarticular infections caused by susceptible microorganisms such as *S. aureus*, *S. epidermidis*, *Enterococcus* spp. and *Enterobacteriaceae* (Gunasekaran et al., 2013; Hurst et al., 2002; Rimmelé et al., 2004). Levofloxacin ($M_w$ 361.37 g/mol) presents high kinetic stability but, like other fluoroquinolones, may chelate alkaline earth and transition metal cations. Little data is found about experimental solubility of levofloxacin, however Zhang et al. (2012), reported a strong dependence on the pH value, with lower pH promoting higher levofloxacin solubility due to the stronger hydrogen bonds between the solute and water molecules. Levofloxacin solubility is reported 100 mg/mL for $0.6 < pH < 5.8$. Maximum solubility, 272 mg/mL, is attained at pH 6.7. For $pH > 6.7$, levofloxacin solubility decreases and reaches a minimum value of 50 mg/mL at a pH of approximately 6.9 (North et al., 1998).

Both minocycline and levofloxacin are available in powder form, with melting points over 200ºC, and soluble either in organic solvents as in water, depending on pH values. In aqueous solutions they are both more soluble and stable at acidic pH. This is an important feature considering that the pH value of the human body is normally maintained at 7.4, but may change from 3 to 9 due to several causes such as accidents, imbalance in the biological system due to diseases, infections and other factors. Also, after surgery, a localized acidity occurs, with the pH value near the implant varying typically from 5.3 to 5.6 (Manivasagam et al., 2010).

Hence, in the present research work levofloxacin and minocycline were selected as model-drugs, to load into a lactose-modified acrylic BC in an attempt to investigate the improvement of the antibiotics release from the inner BC matrix.

## 3.4. Importance of bulk and surface of ALABC

Bulk and surface properties of the BC are determinant for the performance of this biomaterial. Porosity and surface hydrophobicity are key issues regarding the release of an antibiotic from the BC inner matrix. In addition, porosity plays an important role on the mechanical behaviour of the BC.

During the BC preparation, air entrapment inside the polymer matrix may be created by mixing, monomer evaporation and resultant viscosity, which is responsible for the porosity after BC setting. The final porosity of the polymer matrix is of critical importance to the BC mechanical properties, when the material is to perform a load-bearing function, and to the release of the

antibiotic from the inner matrix. When needed, porosity may be decreased of about 2.5% through the use of vacuum mixing. Also, high viscosity cements possess a higher porosity than low viscosity ones because of the greater difficulty for entrapped air to escape from the polymer inner matrix. However, porosity in ALABCs is beneficial as it facilitates the release of the antibiotics by creating inner channels that allow the aqueous release medium to penetrate, to dissolve the antibiotic and diffuse out through the voids and cracks to the surrounding bone tissues (Lewis, 2009).

The easiness of the BC to adsorb water is determined by its hydrophobicity, which is a surface characteristic. The more hydrophilic a surface is the greater is its ability to adsorb water. Surface hydrophobic of BC is related to its wettability, a concept herein used to assess the extent to which a solid will come in contact with water. A surface is considered to be hydrophobic when water does not spread on the surface producing a high contact angle (> 90º), and oppositely, when water spreads decreasing the contact angle, the surface is considered to be hydrophilic.

Actually, the BC surface properties dictate the interaction between the implant, the bone tissues and potentially infecting microorganisms. BCs are moderately hydrophobic materials considering the values of their water contact angles, around ~80º (Bettencourt et al., 2004). The release of water-soluble substances like antimicrobial agents from BC depends directly on the ability to water permeability (Lewis, 2009), which, in turn, is determined by the hydrophobicity of BC components.

Typically, antibiotic loading have been reported as having little effect on BCs contact angles with water suggesting the loaded antibiotic is mainly located inside the matrix (Van de Belt et al., 2001). Also, variations of the contact angle values between different ALABCs, ranging between 70º and 80º, are dependent on the hydrophobicity of their components (Frommelt and Kuhn, 2005).

Surface roughness also plays a role on the interaction of aqueous mediums and the biomaterial once it refrain aqueous medium from spreading evenly along the surface. Surface roughness depends greatly on the finishing procedure and surgeon application. However, considering identical finishing procedures, roughness depends on the brand of the ALABC. Most probably, size and proportion of pre-polymer beads and radiopacifiers used in each brand determines the final surface roughness (Van de Belt, 2001).

Moreover, considering that hydrophobic biomaterials are less disposed to biofilm formation than hydrophilic biomaterials new solutions for the design of infection-resistant biomaterials must be found, aiming at an optimal balance on surface properties to either favour release of the antimicrobial agents or prevent biofilm adhesion (Van de Belt, 2001).

In short, porosity, roughness and hydrophobicity of BC determine the adsorption and absorption of water, the antibiotic release profile and propensity to bacteria adhesion. Antibiotic release, however, is also strongly dependent on the antibiotic physico-chemical characteristics,

hydrophobicity, molecular size and charge, which ultimately determine interaction with the polymer matrix.

## 3.5. Antibiotic Release from ALABC

Identifying and understanding the mechanisms involved in the antibiotic release process from a polymer matrix, such as the acrylic BC, it is not always obvious. Often, more than one mechanism is involved, at a given time, or different mechanisms may dominate, at different stages, throughout the drug delivery process (Siegel and Rathbone, 2012).

The ALABC matrix is considered a monolithic system where the antibiotic is quite homogeneously dispersed in the continuous matrix that controls the release rate. In these systems antibiotic release occurs through the pores filled with the release medium, where antibiotic or other components of the BC first dissolve and then can diffuse out, albeit slowly, through the impermeable matrix eventually reaching the surface (Siepmann et al., 2012). Therefore, porosity plays a major role in this system and factors such as pores form, length, diameter as well as the existence of tortuous pathways connecting pores to surface, strongly influence the diffusion process. Also steric and hydrodynamic interactions between pores and antibiotic molecules interfere with their release.

Due to the tortuosity between internal pores and surface, typically, the release of antibiotic from the BC matrix occurs in three main phases (Lewis, 2009):

(i) an initial rapid burst release of the antibiotic located at surface, normally within the first 24 h;

(ii) a second phase where the antibiotic located inside the matrix continues releasing but with a lowered rate;

(iii) a final phase in which the release rate is very slow and steady, eventually attaining a constant value.

Considering the hydrophobicity of the ALABC matrix, the first phase is ruled by surface area porosity and wettability, which determines the amount of the fluid that reaches the inner voids, cracks and paths in the BC. As the fluid progresses into tortuous pathways of the matrix, it may occur delay and eventually blocking of the antibiotic release due to tortuosity and the existence of “dead ends” in the pore space that difficult the diffusion-out of the drug (Fig. 9), resulting in a decrease of the antibiotic release rate. The existence of the steady release stage suggests a role for specific solution chemistry equilibrium phenomena (Lewis, 2009).

The quantitative interpretation of the values obtained during dissolution/release tests of drugs and other substances from BC is facilitated when mathematical formulae express the dissolution results as a function of some parameters.

**Fig. 9** Schematic representation of a pore structure evidencing a curved, tortuous single path from bottom surface to upper surface (left); a tangle of curved pores with intersections and dead ends (right) (adapted from Siegel, 2012).

Drug diffusion and dissolution from reservoir and matrix type systems has been described by kinetic models in which the dissolved amount of drug (M) is a function of time (t), M=f(t). The physicochemical properties of the drug, including cristallinity and polymorphism, particle size, solubility and amount in the BC can influence the release kinetics (Costa and Sousa Lobo, 2001). Also, the system characteristics are important, such as porosity, swelling and degradability (bulk or surface).

The drug transport inside BC system and its release involves multiple steps triggered by different physical or chemical phenomena, making it difficult, to get a mathematical model describing it in the correct way. As a general rule water-soluble drug incorporated in a matrix is mainly released by diffusion, while for a low water-insoluble drug the erosion of the matrix will be the principal release mechanism (Costa and Sousa Lobo, 2001). However, it should not be overestimated because crucial factors such as the drug's diffusion coefficient and molecular weight, partition coefficient and solubility in the excipient must be taken into consideration. Likewise, the system's properties are also relevant, *e.g.* polymer cross-linking, polymer crystallinity, presence of diluents or adjuvants and surface area (Chien and Lin, 2007).

The Higuchi model (1961) has a large application in governing the rate of release of solid drugs randomly dispersed in polymeric matrix systems. Higuchi showed that the total amount of the drug released is a function of the square root of time as a result of the increasing diffusion pathway with the progression of the dissolution front through the porous matrix (Table 5). Korsmeyer et al. (1983) and Peppas (1985) proposed a generalised expression, known as the Korsmeyer-Peppas model, that considered the characteristics of the macromolecular network system and the drug as well as the Fickian and non-Fickian drug release mechanism from the polymer matrix (Table 5). Kuhn and Wilson (1985) proposed a model with an approximation for the complete release process, where the cumulative amount of drug released was a combined result of the Higuchi, the KP models and an initial constant (Table 5).

Later Ritger and Peppas (1987) introduced a semi-empirical equation to express the general drug release behavior from polymers, by coupling of Fickian (diffusion-controlled) and non-Fickian mechanisms (Case II transport). Lindner and Lippold (1995) proposed the addition of the burst effect to the Korsmeyer equation (Table 5) (reviewed by Torrado et al., 2001).

**Table 5.** Summary of the mathematical models frequently used to evaluate the release kinetic of drugs and others substances from BC matrices (adapted from Bettencourt et al., 2000, 2001, 2004; Costa and Sousa Lobo, 2001; Torrado et al., 2001).

| **Equation Models** | | |
|---|---|---|
| Higuchi (1961) | $M_t = k_H\sqrt{t}$ | Semi-solid and/or solid matrixes containing dispersed water soluble and low soluble drugs.<br>Drug dissolution occurs after the concentration drops below the matrix drug solubility.<br>Describes drug release as a diffusion process based in the Fick's law, square root time dependent. |
| Korsmeyer-Peppas (Korsmeyer et al., 1983) (Peppas, 1985) | $\frac{M_t}{M_\infty} = k_{KP}t^n$ | $k_{KP}$ constant incorporates the characteristics of the macromolecular network system and of the drug<br>*n* exponent is determined using the portion of the release curve where $M_t/M_\infty < 0.6$, and characterises the different release mechanisms:<br>Release exponent n / Drug transport mechanism / Rate as a function of time<br>0.5 / Fickian diffusion / $t^{0.5}$<br>0.5 <n <1.0 / Anomalous transport / $t^{n-1}$<br>1.0 / Case-II transport / Zero order release<br>Higher than 1.0 / Super Case-II transport / $t^{n-1}$ |
| Kuhn and Wilson (1985) | $M_t = a + ct^{1/2} - be^{-kt}$ | *$M_t$ represents the cumulative drug release up to time t; a is associated with the initial burst effect; b is associated with the kinetics of a dissolution process; c is associated with a Fickian diffusion of the drug particle.* |
| Ritger and Peppas (1987) | $\frac{M_t}{M_\infty} = k_1t^{1/2}+k_2t$ | *$Mt/M_\infty$ represents the fractional release of drug; $k_1$ constant described the diffusion-controlled release process and $k_2$ constant the rate release (Case II transport)* |
| Lindner and Lippold (1995) | $\frac{M_t}{M_\infty} = kt^n+b$ | *$Mt/M_\infty$ represents the fractional release of drug; n is the release exponent of the Korsmeyer-Peppas model; b represents the burst effect.* |
| Frutos et al. (2010) | $M_t = a + b\left(1-e^{-kt}\right) + c\sqrt{t}$ | *$M_t$ denotes the cumulative drug released up to time t; k is a constant of the mathematical model; a, represents the burst effect on drug release; b, is related with the drug dissolution process; and c is related with the Fickian drug diffusion process.* |

Torrado et al. (2001), when studying the release of gentamicin from a commercial ALABC, rearranged the mathematical model parameters from Khun and Wilson (1985) to fit their experimental data. This kinetic model rearrangement resulted in the best fit for the antibiotic release from the BC matrix by admitting the occurrence of three mechanisms in the release behaviour of the drug (Table 5), a burst effect on drug release, a drug dissolution process and a Fickian drug diffusion process.

Frutos et al. (2010) has named this model as the "*coupled mechanism*" and also applied it to the release mechanism of gentamicin from the lactose-modified ALABC with good fitting results. Slane et al. (2014) on studying the release of the same antibiotic but from a different ALABC commercial brand also used the coupled-mechanism with very good fitting results.

### *Antibiotic stability after release*

As a first step in a BC formulation development, *in vitro* release assays, mimetising physiological conditions (*e.g.* saline solution at 37ºC, pH of infected site) are performed aiming to select the formulation with the most promising properties.

During *in vitro* assays, the amount of released antibiotic is monitored and quantified by common analytical techniques. Simultaneously, it is important to evaluate the stability of released antibiotic using microbiological assays where selected bacteria strains are subject to withdrawn antibiotic release aliquots and susceptibility is measured.

Testing methods include the disk diffusion test and the MIC test, with this being the gold standard for determining the susceptibility of bacteria to antibiotics. MIC is the acronym to minimum inhibitory concentration and correspond to the lowest (*i.e*. minimal) 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; Cavalieri et al., 2005). Also $MIC_{50}$ and $MIC_{90}$ are often determined and represent the lowest antibiotic concentration that inhibits 50% or 90% of bacterial isolates, respectively. MIC tests can be performed using broth or agar media, but broth microdilution is the most widely used method in clinical laboratories. When bacteria strains are biofilm producers, like *S. aureus* and *S. epidermidis*, the biofilm inhibitory concentration (BIC) is also determined using the correspondent guidelines (Høiby et al., 2015). BIC corresponds to the antibiotic concentration that no longer prevents bacteria growth and biofilm formation.

Determination is guideline-dependent and is based on a known quantity of bacteria with specified dilutions of the antimicrobial agent. Using interpretive criteria from the chosen guideline, CLSI, EUCAST or BSAC, results are interpreted as susceptible, intermediate, or resistant (Davidson et al., 2000). Control bacteria are always included in tests, but important sources of variation include the culture medium, bacterial inoculum, antibiotic preparation and incubation conditions. The available guidelines differ in their recommendations, *e.g.* the CLSI recommends larger inocula and higher MICs than does the BSAC. This however is more important regarding epidemiological studies where clear thresholds and validated methodologies must be standardized within countries (Andrews, 2001).

To determine if the antibiotic from the release study is microbiologically active an assay is performed according to a chosen guideline and the result is compared with the established MIC for a given bacteria strain.

### *Porosity promoters*

The porosity of commercially available ALABCs depends on its composition and on mixing

methods during preparation with porosity resultant from hand mixing being higher than that of BC produced via vacuum mixing. Therefore, for the same type and amount of antibiotic different commercial ALABCs deliver different amounts of antibiotic (Lewis, 2009).

However, envisaging higher antibiotic release from ALABCs porosity may be modulated and increased by adding space fillers, also called as poragens or release modulators (Table 6). Most of these are water-soluble components available in powdered form that allows mixing during BC preparation. The physical characteristics of these fillers, such as solubility, particle size, or volume fraction would be expected to affect matrix porosity, and therefore, water permeability.

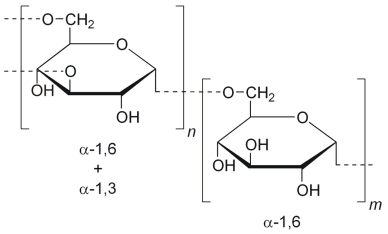
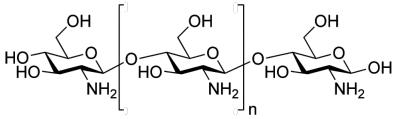
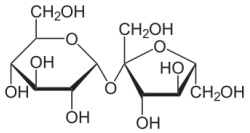
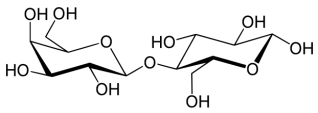
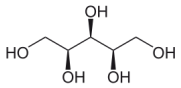
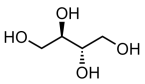
In the early 1990s Kuechle et al. (1991) added dextran, a high molecular-weight polysaccharide, to vancomycin-loaded PMMA. The released concentrations were approximately four times higher than those determined from plain vancomycin-loaded BC. More recently, Jackson et al. (2011) also included dextran into acrylic BC with major improvement on release of vancomycin, linezolid and fusidic acid. McLaren et al. (2006, 2007a, 2007b) reported an increase of daptomycin release from ALABC beads when using xylitol, glycine, sucrose, and erythritol, with better results with xylitol smaller size particles (100 μm). These smaller particles led to smaller pores, greater pore interconnectivity, smaller areas between the pores with no fluid penetration and greater increase in the effective surface area causing a greater release rate. Salehi et al. (2013), Slane et al., (2014) and Weiss et al. (2009), also evaluated xylitol-modified ALABC for gentamicin and daptomycin release with increased results but for cement spacer or beads and not for implant fixation.

Chitosan, a natural high molecular weight polymer (Table 6), biodegradable and with intrinsic antibacterial activity against a broad spectrum of bacteria, filamentous fungi, and yeasts (Tan et al., 2014), is found to be a useful additive for the treatment of bone infection when loaded on ALABCs, either in powder or in nanoparticulate form. It is expected to enhance antibiotic release while preventing bacterial colonisation and biofilm formation. However, these outcomes are dependent on chitosan concentration, on microorganisms strains used to test biofilm formation and on time of assay, as described by Shi et al. (2006) and Tunney et al. (2008), who reached opposite results.

A research group has mentioned the addition of lactose (Table 6) to commercial ALABCs (Diez-Peña et al., 2002; Frutos et al., 2010; Virto et al., 2003) to increase gentamicin release without hampering the mechanical properties due to lactose high elastic modulus (Perkins et al., 2007).

However, the mechanical properties were not acquired in those studies in accordance with international standards. As lactose, a water-soluble filler, is an hydrophilic compound it emerges as a promising PMMA surface modifier for preventing biofilm growth. Lactose has a long track history of safe use in pharmaceutical formulations explained by the high stability, soluble in water at acidic pH, with melting point over 200ºC and relatively low cost.

**Table 6.** Examples of release modulators used to increase antibiotic release from ALABCs.

| Chemical Group | Name | Structural Formula | Reference |
|---|---|---|---|
| **Polysaccharides** | Dextran |  | Jackson et al. (2011)<br>Kuechle et al. (1991) |
| | Chitosan |  | Shi et al., (2006)<br>Tan et al. (2012a; 2012b; 2014)<br>Tunney et al. (2008) |
| **Disaccharide** | Sucrose |  | McLaren et al. (2007a; 2007b) |
| | Lactose |  | Diez-Peña et al. (2002)<br>Frutos et al. (2010)<br>Virto et al. (2003) |
| **Polyalcohols** | Xylitol |  | McLaren et al. (2006; 2007a; 2007b)<br>Slane et al. (2014)<br>Salehi et al. (2013) |
| | Erythritol |  | McLaren et al. (2007b) |
| **Amino acid** | Glycine |  | McLaren et al. (2006) |

## 3.6. Appraisal of biocompatibility of ALABC

Biocompatibility is a complex concept associated with the extensive domain of medical devices and with the multiple interactions related to their safety and performance. Since the moment the biomaterial is placed inside the patient, a host response is triggered, which it will be acceptable if the biomaterial is safe. Also, performance of the biomaterial is related to a specific application, in a specific type of tissue, interacting with specific type of interfaces (Eloy, 2012). Thus, biocompatibility testing is the fundamental requirement when developing new materials and their surfaces for medical devices and tissue engineered medical products (Bruinink and Luginbuehl, 2012).

The International Standard ISO 10993 describes test methods to assess the *in vitro* cytotoxicity of

medical devices on extracts of the test sample, and/or on the test sample itself. A cell line of mouse fibroblasts (L929; NCTC) is recommended although the complementary use of other cell lines is not discouraged. A cell line of human osteoblasts (MG63; ATCC®CRL-1427) is often also used to follow the standard requirements (ISO 10993-5).

This standard proposes three categories of tests for assessing the cytotoxicity of potentially released materials: (1) extract tests, (2) direct-contact tests and (3) indirect-contact tests (agar diffusion test, filter diffusion test). One or more tests may be used depending on the nature of the sample material. Cytotoxicity is assessed using different parameters based on cell and culture morphology (qualitatively), quantitative measurement of cell impairment, such as effects on cell growth (proliferation), and specific aspects of cell metabolism. Qualitative and quantitative measurements are reported to correlate very well (Bruinink and Luginbuehl, 2012).

The most striking limitation of all suggested tests in ISO 10993-5 is the short test period, *i.e.* 2 h (filter diffusion), 24 h (extract acute cytotoxicity), or 24–72 h (agar diffusion). By defining a reduction of 30% as the threshold for an extract to be toxic, only a cell lysing compound, a compound inducing apoptosis in a very short term, or very strong inhibitor of cell proliferation will be able to give rise to such a reduction after a treatment period of 24 h.

According to Williams (2014) the term “Biocompatible” should only be considered in the context of the characteristics of both the material and the biological host within which it is placed, as it is a property of a system and not of a material and states *“It follows that there can be no such thing as a biocompatible material”*. Interactions between biomaterials and tissues are time dependent and some materials may be effectively conditioned after contact with the tissues, and this has to be taken into account in the characterisation of the material and biological host system. These interactions may be controlled by surface properties as energy, topography, functionality and substrate stiffness. The control of biocompatibility involves, therefore, much more than non-citotoxicity (Williams, 2014).

Therefore, these tests do not fully determine the biocompatibility of a material, and represent no more than a primarily, but important, step towards the *in vivo* testing by determining the interaction cell-material as suitable for a given clinical application or not (ISO 10993-5).

## 3.7. Bioactivity of ALABC

Two biomechanical phenomena characterise the bone response to the cemented implants: 1) the smooth surface of all PMMA-bone cements; and 2) the penetration of the cement mass into the bony honeycombs thus stiffening the scaffolding of cancellous bone, converting it into a stiffer compound material that represents a living symbiosis between bone and its PMMA »inlay« (Draenert and Draenert, 2005). Some authors designate this behaviour as recognised

"osteointegration" in the sense of lack of a negative tissue response suggesting that the foreign-body surface is positively recognisable from the osteogenic cells as a biomimetic scaffold which may favour early peri-implant osteogenesis (Mavrogenis et al., 2009). However, the fibrous tissue layer between BC and bone is known as the weak-link zone and can lead to loosening of the prosthesis (He et al., 2012).

Therefore, in an attempt to address the poor bone tissue integration of commercial ALABC, osteoconductive materials, like ceramics, are being considered to load ALABCs. The smooth surface of BCs does not allow the same bone bonding as that from ceramics, where bone contacts are regularly formed indicating interface stability, as a consequence of their osteoconductive and osteophilic nature (Kim[a] et al., 2004).

The incorporation of bioceramics into ALABCs, such as glass and calcium phosphate (CaPs), are increasingly being reported as successful additives on promoting superficial interaction between the BC and the bone tissue. Furthermore, the properties of calcium phosphates are not modified when incorporated in the PMMA matrix (Lopez-Heredia et al., 2012).

One of the most important applications of bioactive calcium phosphates, such as hydroxyapatite (HA), has been as coatings of orthopaedic metal implants, at locations where a strong interface with bone is required, *i.e.*, femoral stems and acetabular metal-backs for the hip joints and tibial and femoral components for the knee joints, as well as injectable bone filling material (Ferraz et al., 2007). For both applications particle size is an important consideration when optimal degradation and release profiles are needed. NanoHA was tested *in vitro* for the periodontitis treatment with beneficial effects on sustained antibiotic release, osteoconduction and resorbability. Moreover, preparation methods of nanoHA do not impair properties such as biocompatibility, bioactivity, osteoconductivity and osteoinduction (Ferraz et al., 2007).

Nevertheless, the potential of nanostructured materials are huge and improvements are to be expected when combined with other materials, such as BC, where antibiotic delivery and tissue regeneration are central aspects of bone infection treatment (Uskokovic, 2015).

Furthermore, research on this domain has reported the inclusion of cations, such as magnesium and strontium, into the calcium phosphates crystal lattice as an added value for improving osteointegration, as these ions share the same pathway as the calcium in humans inducing the increase of bone forming sites (Dall'Oca et al., 2014; Guo et al., 2005; Laurencin et al., 2011; Ren et al., 2010).

Nevertheless, bone ingrowth into ALABC is still a concern (Fini et al., 2002; He et al., 2012; Sa et al., 2015). The combination of ceramics with poragens or with another ceramic, with a different resorption rate, is being explored as an attempt to address this downside. The combination of PMMA with tricalciumphosphate (TCP) and HA, two bioactive calcium phosphates with different resorption rates, coated on PMMA-bone cement surface seemed to offer potential benefits over classic PMMA-bone cement (Draenert and Draenert, 2005). The HA, structurally similar to the

inorganic phase of human bone, warrants the superficial interaction with bone tissue cells, and the TCP increases porosity pores, while being reabsorbed, promoting bone ingrowth.

The immediate drawback of this strategy is the risk of hampering the mechanical properties due to porosity increase. Nonetheless, He et al. (2012) described the combined use of low-content of chitosan (10%), as poragen, with bioactive glass, as bioactive filler, into PMMA–bone cement and achieved satisfactory results regarding the bonding strengths between bone and implant and without compromising compressive strength of the bioactive BC. Other authors studied the addition of HA and chitosan (10%) into the PMMA matrix and reported a more preferable environment for cell attachment and proliferation than for plain PMMA, concluding that a viable bone/cement interface could result in a better orthopedic implant fixation system by combining BC rigidity with biological ingrowth for long-term stability (Kim[b] et al., 2004).

Moreover, calcium phosphates, which are also widely used as drug delivery materials in medicine and are inherently radiopaque, could bring an extra advantage if considered to load BC as drug-loaded bioactive agents. It would be like a merging of the desired mechanical and biological properties of a BC by combining the vast clinical experience and structural function of PMMA and the biological potential of calcium phosphates materials (Arcos and Vallet-Regi, 2013; Ginebra et al. 2006; Lopez-Heredia et al., 2012).

This innovative approach deserves, in our opinion, further research and thorough evaluation.

## 3.8. Mechanical Properties of ALABC

The main function of BC in a cemented arthroplasty is to transmit loads through the interface into the bone and muscle surrounding for long periods of time. The mechanism of loading is especially complex as the total load affecting BC is a mixture of compressive loading combined with bending, tension, shear, and torsion, turning difficult to define what strength property actually leads this phenomenon (Hosseinzadeh et al., 2013). Therefore, it has been extremely difficult to simulate this complex situation in the laboratory.

Mechanical evaluation of acrylic BCs has varied considerably. Until 1987, when ISO-standard organisation added the bending modulus and bending strength determination, the only required testing was the compressive strength (Hansen and Jensen, 1992). The standard ASTM F451 (2008) still includes the compression test only.

Consequently, a significant number of papers started to be published providing data on the mechanical properties of the available commercial-brands of BCs as well as of research formulations not commercially available (Lee, 2005). Most studies are referred to quasi-static mechanical evaluation, *i.e.* compressive strength, bending modulus, bending strength, shear stress, tensile stress and surface hardness, which are important for short-term BC

characterisation specially giving information about the BC composition.

Still, and considering that the most common cause of arthroplasty revision is aseptic loosening, it still lacks information about dynamic properties, useful for a long-term BC characterisation, such as creep, stress relaxation or fatigue, either in *in vitro* physiological simulated conditions as in *in vivo* conditions, which may affect the transmission load into the bone or the bone/BC interface tension, compromising the expected life of a total arthroplasty (Lewis, 2015).

### *Quasi-static mechanical properties of bone cement as a preliminary evaluation*

Very rarely, if ever, is failure due to compression or bending of BC, meaning that the requirements of the international standard are most useful for quality control on the evaluation for novel ALABC formulations for prophylactic use in cemented total joint replacement surgery.

In Europe, the main standard for controlling the properties of acrylic BC is the ISO 5833:2002 which specifies preparation, packaging, set and cured cement requirements (ISO, 2002).

Regarding the latter, three requirements are mandatory: compressive strength (≥70 MPa), bending modulus (≥ 1800 MPa) and bending strength (≥ 50 MPa). The compressive strength is tested on cylindrical samples of BC 24 ± 2 h after forming and storage in dry air at 23°C (Fig. 10).

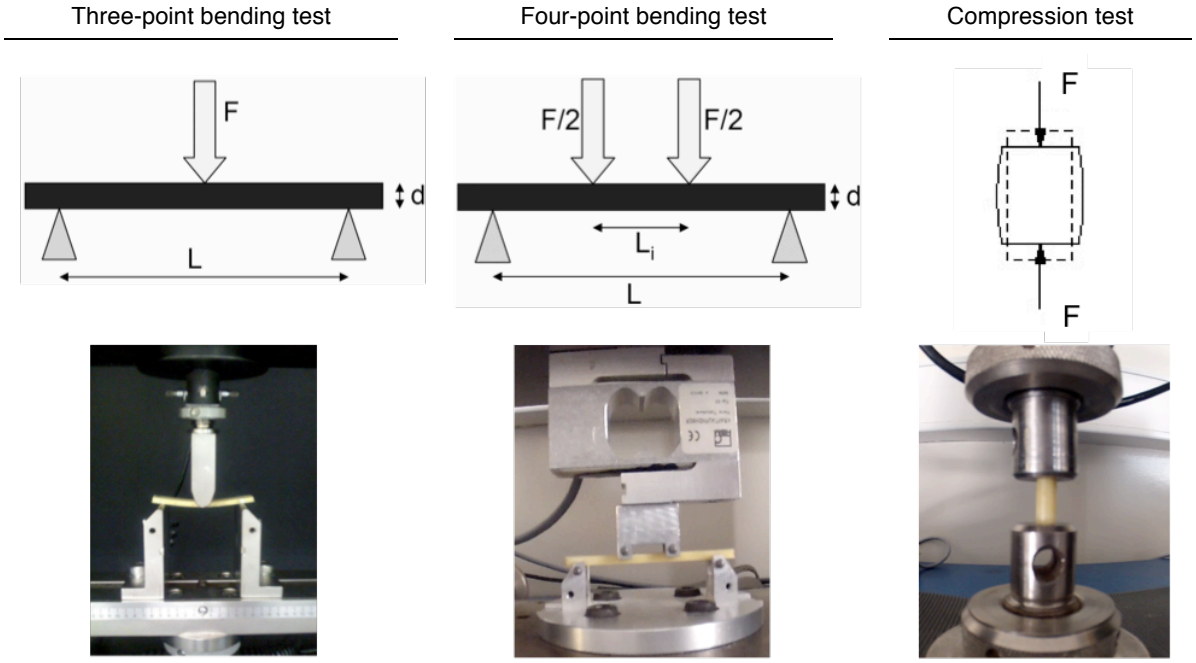



**Fig. 10** Schematic representation of the applied forces on a three-point flexural test (left), on a four-point flexural test (center) and on a compression test on a ductile material as BC (right). The bottom row shows instant photos during tests.

The strength is calculated from fracture load, 2% offset load or upper yield point load, whichever

occurs first. The bending modulus and bending strength is measured using a four-point bending test on beam specimens of BC 24 ± 2 h after forming and storage in dry air at 23°C. Formulae are given for the calculation of bending modulus and bending strength. All bone cements that are commercially available – plain and antibiotic-loaded – must comply with the requirements of ISO 5833 (Lee, 2005).

Considering the published data on compressive strength, bending strength and modulus, commercial-available BCs are strong in compression, weak in tension and have a medium bending modulus of elasticity. In consequence, BC should be loaded in compression wherever possible. It should be supported by cortical bone to allow the compression to be generated and to restrict tensile stresses (reviewed by Lee, 2005).

When suggesting any variation on the composition of a BC formulation, there must be awareness for the influence of that change on the mechanical properties. As referred before, by adding a second antibiotic, or increasing the amount of antibiotic, or by adding a poragen or a bioactive ceramic, changes of the bulk structure occur and the compliance with the ISO 5833 requirements may be compromised.

Moreover, on deciding what percentage of a new component to add to a commercial BC powder it must be taken into consideration the base-proportions of each constituent of that BC to avoid hindering their function on cured BC. For example, the amount of radiopacifier should not decrease to levels that might compromise radiopacity ranging between 8.0% and 15.0% in commercial brands (Hosseinzadeh et al., 2013).

Not seldom, on evaluating the mechanical properties of a commercial or an experimental BC matrix, ISO 5833 or ASTM 451 are not strictly followed, in many studies, especially regarding the flexural strength evaluation, which is the most restrictive mechanical property when including novel components into a formulation. Authors often report the use of a three-point flexural test instead the 4-point recommended by ISO 5833 (ASTM does not include flexural evaluation) or instead they only report compressive strength results, which hardly is affected.

This is an important subject because it has influence on final conclusions considering that BC tested by three-point flexural test result in a higher flexural strength than BC tested by four-point flexural test (Vallo, 2002). The three-point flexural test produces its peak stress at the specimen mid-point with reduced stress elsewhere along the specimen. The four-point flexural test produces peak stresses along an extended region of the specimen ($L_i$ on Fig. 10) thus exposing a larger length of the specimen with more potential for defects and flaws to be highlighted. This is the case of the BC, which inner matrix has a porous structure, as already presented, where propagation of cracks may occur starting on pores.

Still, as a preliminary study, the non-compliance results from three-point bending test excludes the execution for a four-point bending test.

Several parameters affect the interpretation of BCs mechanical analysis. There is a plethora of

studies either for different commercial-brands as for novel BC alternatives containing several additives (reviewed by Lewis, 2015). These include varied amounts of one or two antibiotics or other drugs, poragens, bioactive ceramics and fibbers for reinforcement. Unfortunately most data cannot be compared because each author may decide not to use a standard test proceeding; may use a standard but for any reason do not follow described settings; may use a standard, follow settings but only for one of the mechanical properties described - usually compressive strength is chosen as it involves lower risk of failure; may not use controls to which other results should be compare with to drove conclusions. The variability of options is such that even completely opposite results may be found when using exactly the same cement, address the same properties and characterisation, the same additives but just one difference on amounts or forms of the additives (Shi et al., 2006; Tunney et al., 2008).

Improvements at standardisation level may help decreasing the randomness of results and balance interpretations. For example by clearly defining methods of mixture according to BC intended application, limiting the amount of additives to be considered for loading, increasing the number of time-points after setting on which data must be acquired.

Overall, efforts must be joined to gather and systematise information to identify clearly the pros and cons of the choices of a certain additive or a particular antibiotic. Research should continue to focus on ALABCs tailored alternatives for a successful prophylaxis and treatment of bone infection, guaranteeing long-term stability of the implant and, more important, the patient's quality of life.

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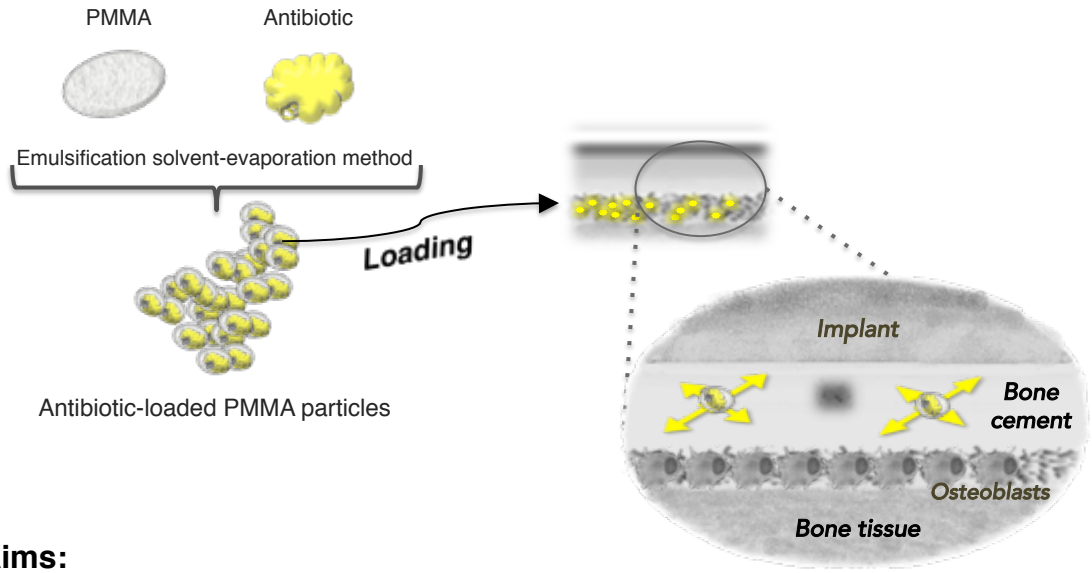
# Chapter 2

## INCORPORATION OF ANTIBIOTIC-LOADED PMMA PARTICLES IN ACRYLIC BONE CEMENT

## Graphical Abstract




**Aims:**

- Antibiotic stability after particle formulation and loading
- Antibiotic controlled release rate from particles and from BC
- Mechanical properties maintenance
- Biocompatibility maintenance

# Abstract

The use of antibiotic-loaded acrylic bone cement (ALABC) is still considered the standard of care for patients with chronic bone and joint infection, providing local delivery of high levels of antibiotics for an extended period without exceeding systemic toxicity. However, these ALABCs present a major drawback related with the incomplete and inadequate kinetic release of the antibiotic. New strategies must be found to improve the mechanisms of release while maintaining antibiotic antibacterial activity as well as the mechanical stability of the resultant bone cement (BC). The inclusion of antibiotic-loaded PMMA particulate systems in the BC matrix appears as an interesting approach to be tested fulfilling the aforementioned premises, given both, particles and BC, are PMMA-based materials.

The double-emulsion solvent evaporation method was used to prepare minocycline- and levofloxacin-loaded PMMAp. Antibiotic selection (levofloxacin and minocycline) was based on their broad-spectrum of activity against the main organisms responsible for bone and joint infections. Prepared PMMA particles, plain and antibiotic-loaded, presented suitable surface morphology, yield of preparation, stability and size. Antibiotic encapsulation and release strongly depended on drug's hydrophobicity, and formulation conditions have not interfered with antibiotic microbiological activity. Because only minocycline released from particles, just Mino(PMMA)p were included into the BC matrix. However, as a 15% ($w_{particles}/w_{BC}$) amount of Mino(PMMA)p hindered BC setting, further studies proceeded with calcium phosphate particles.

**Keywords**: Particulate systems, drug delivery, double emulsion solvent evaporation method, polymethylmethacrylate particles, antibiotic release

# 1. Introduction

The development of local drug delivery systems able to release drugs in a controlled way providing optimal drug concentrations at the site of illness has strongly evolved since the late 1960's. This is of particular importance in the case of orthopaedic biomaterial-related infections where different materials loaded with antibiotics for local delivery are under investigation (Van de Belt et al., 2001). Among these, polymethylmethacrylate (PMMA) has been widely explored as a carrier material for the development of local drug delivery systems due to its biocompatibility and its appealing mechanical and chemical characteristics (Kreuter, 2007).

Since 1970, when Buchholz and Engelbrecht added gentamicin to PMMA for the prophylaxis and treatment of prosthetic joint infection, this biomaterial is being continuously used as a local delivery system in orthopaedic field. Mainly there are two forms of use: that of antibiotic-loaded acrylic bone cement (ALABC) applied in arthroplasties for prophylaxis, and of antibiotic impregnated bead chains or spacers for musculoskeletal infections treatment (Bettencourt and Almeida, 2012).

The use of ALABC is still considered the standard of care for patients with chronic bone and joint infection, providing local delivery of high levels of antibiotics for an extended period without exceeding systemic toxicity, while being a more cost-effective procedure when compared to cementless implants (Jameson et al., 2015; Zilberman and Elsner, 2008). However, these ALABCs present a major drawback related with the incomplete and inadequate kinetic release of the antibiotic (Jiranek et al., 2006; Lewis, 2009). New strategies must be found to improve the mechanisms of release while maintaining the antibacterial activity as well as the mechanical stability of the bone cement (BC) (Jiranek et al., 2006).

Ensuing the use of PMMA beads as antibiotic delivery systems in bone infections, the inclusion of antibiotic-loaded PMMA particulate systems in the BC matrix to improve drug delivery appears as an interesting approach to be tested. The use of PMMA as a biocompatible polymer for particle preparation was first described in the late 1970's although its use as a drug carrier system has been neglected because it is not biodegradable, which however is an interesting property when considering loading into BC (Bettencourt et al., 2010).

To date there are some examples of drugs encapsulated in PMMA particles namely, coenzyme Q10 (Kwon et al., 2002), α-tocopherol (Bettencourt et al., 2010) and more recently of daptomycin and vancomycin (Ferreira et al., 2015), describing the production of the PMMA particles to be used as drug carriers, however none are commercialised.

Moreover, the idea of encapsulating drugs in PMMA particles and then loading them into BC was already reported for antioxidants with successful results, circumventing the impossibility of loading directly the antioxidant into the BC matrix, which hampered BC setting (Bettencourt et al., 2010).

Within this context the intent was to encapsulate both minocycline and levofloxacin on PMMA

particles (PMMAp) prior to loading them into the BC matrix. This approach would protect the antibiotic molecules against eventual degradation during BC setting while controlling the burst effect of antibiotic release extending it for a longer period. Moreover, the non-biodegradability of PMMA represented *the asset* to the ideal carrier systems for controlled antibiotic release after inclusion in BC since both the particles and the cement are made of the same biomaterial. As so, the resultant biomechanical properties and biocompatibility of the PMMAp-loaded BC, expectantly, would not be compromised. The reason for the antibiotics selection was their broad-spectrum of activity against the main organisms responsible for bone and joint infections (Bishburg and Bishburg, 2009; Zimmerli, 2015).

On deciding to prepare particulate carriers, size distribution, type and preparation methodology are important subjects to consider. PMMA particles (micro- or nanoparticles) can be prepared either by direct polymerization of the methylmethacrylate monomer using polymerization reactions or from pre-formed PMMA polymer. Particle preparation is fairly described in literature with primacy to the emulsion polymerization method, which includes different procedures (*i.e.* conventional, surfactant-free and micro- and miniemulsion), and allows fast-yielding varied diameter particles with low polydispersity and easy-scalability. However, toxicological issues regarding the use of organic solvents, initiators, surfactants or the liberation of residual monomers, during or after polymerization, haunt these techniques, when the produced particles are intended for biomedical applications, such as vaccine delivery (Bettencourt and Almeida, 2012).

Pre-formed polymer-based techniques are an alternative method to produce particles on-demand from pre-formed PMMA, either of different molecular weights or blended with different polymers. Those include emulsion solvent evaporation/extraction (Ferreira et al., 2015; Zigoneanu et al., 2008), nanoprecipitation (Aubry et al., 2009), supercritical fluid methods (Elvira et al., 2004; Matos et al., 2010), spray-drying and crystallization (Zhou et al., 2001). All methods allow producing nano- or microparticles, or a mixture of both, depending on the formulation conditions. Exception made to nanoprecipitation method that yields only nanoparticles (Bettencourt and Almeida, 2012).

Nevertheless, the most widely used method for PMMAp preparation is the emulsion solvent evaporation due to its known advantages, as being a simple and inexpensive technique, use of accessible equipment, possible encapsulation of different types of drugs (hydrophobic or hydrophilic) at room temperature and versatility allowing customisation of the key-parameters in order to obtain the required particles. Selecting the starting pre-formed polymer (type and molecular weight), ratio to the organic solvent where it solubilises; the type, volume and concentration of the surfactant aqueous solutions; the nature and solubility of the drug to be encapsulated; the drug/polymer ratio; the time and speed of homogenisations and evaporation; the time, temperature and speed of centrifugation; are all customisable parameters that enable preparation of particles with different yields, size distribution, surface charge and drug loading

(Zydowicz et al., 2002). This method may be executed as a simple (o/w) or a double ($w_1/o/w_2$) emulsification depending on the drug hydrophobicity characteristics. Usually more hydrophobic drugs are encapsulated using simple-emulsion solvent evaporation (being directly dissolved in the organic solvent) and more hydrophilic drugs with the double-emulsion solvent evaporation.

In the present study, the double-emulsion solvent evaporation (DESE) $w_1/o/w_2$ method was used to prepare minocycline- and levofloxacin-loaded PMMAp.

Formulation and performance parameters were assessed, including encapsulation efficiency and drug loading, surface morphology, size and surface charge. Furthermore, antibiotic *in vitro* release and antimicrobial activity were evaluated.

Afterwards, an *in vitro* proof of concept experiment was designed by preparing thin BC pilot-specimens to load antibiotic-loaded PMMAp. The aim was to evaluate the effect on BC setting as well as facilitating the release medium to percolate the BC matrix during the *in vitro* release study. The first aim is related to the utmost need of maintaining the structural properties of the BC after loading the particles. The second was reasoned by the BC matrix characteristics, non-erodible, hydrophobic and low porosity, which hampers antibiotic release, which can only dissolve and diffuse through the existent voids and cracks reached by the release medium (Siepmann, 2012).

# 2. Materials and Methods

## 2.1. Materials

Polymethylmethacrylate powder (PMMA, $M_w$ 350 000 g/mol), levofloxacin (Lev, on particles designation), polyvinylalcohol (PVA, $M_w$ 13 000 – 23 000 g/mol, 87-89% hydrolysed), polysorbate 20 (Tween® 20) and phosphate buffer saline (PBS) were purchased from Sigma-Aldrich (Spain). Minocycline hydrochloride (Mino, on particles designation) was kindly donated by Atral-Cipan (Castanheira do Ribatejo, Portugal). Dicloromethane (DCM) (analytical grade) was obtained from Merck (Portugal). Sucrose was obtained from Labonal (Portugal). Sodium chloride was obtained from AppliChem (AppliChemGmbH, Darmstadt, Germany). Distilled water was of Milli-Q quality (Millipore, Bedford, MD, USA). All other reagents were of analytical grade and were used without further purification. Commercial acrylic BC CMW1® Radiopaque (high viscosity BC intended for digital application) was purchased from Depuy Synthes (Portugal).

## 2.2. Preparation of PMMA particles by DESE method

Particles were prepared at room temperature using the DESE method ($w_1/o/w_2$) described elsewhere (Bettencourt et al., 2010; Florindo et al., 2010) (Fig.1).

In brief, an inner aqueous solution ($w_1$) was emulsified into an organic phase (o), composed by a 50:1 ratio of PMMA:DCM (mg/mL), by homogenisation using an IKA® T10 Basic Ultra-Turrax (IKA, Germany) for 3 min at ca. 25000 rpm. To prepare the plain particles, $w_1$ consisted on 1mL of a 10% (w/V) PVA solution. When preparing drug-loaded particles, $w_1$ corresponded to 1mL of a drug solution prepared with the 10% (w/V) PVA solution.

The resultant ($w_1/o$) emulsion was then added drop wise into a 6-fold larger volume of a 1.25% (w/V) PVA solution ($w_2$) and homogenised for 7 min at 9999 rpm using a Silverson Laboratory Mixer Emulsifier L5M (Silverson Machines Inc., UK). The resulting ($w_1/o/w_2$) emulsion was magnetically stirred at 200 rpm for 4 h, to guarantee complete evaporation of the organic solvent. Particles were then harvested by centrifugation (15.000 rpm, 20 min, 18ºC; Beckman Coulter Inc., Allegra 64R High speed centrifuge, USA), washed twice and subsequently dispersed in 5 mL of a cryoprotectant solution, 10% ($w/w_{PMMA}$) sucrose, before freeze-dried (Christ Alpha 1-4, B. Braun Biotech International, Germany) to obtain a fine, free-flowing dry powder of particles. The mean yield of preparation was calculated relating the obtained mass of particles to the initial mass of all components, and obtained values were always comprised between 70% and 80% (w/w). All batches were prepared in triplicate and plain particles were used as controls whenever necessary. All produced particles were at a 7.5% ($w/w_{PMMA}$) of each antibiotic content.

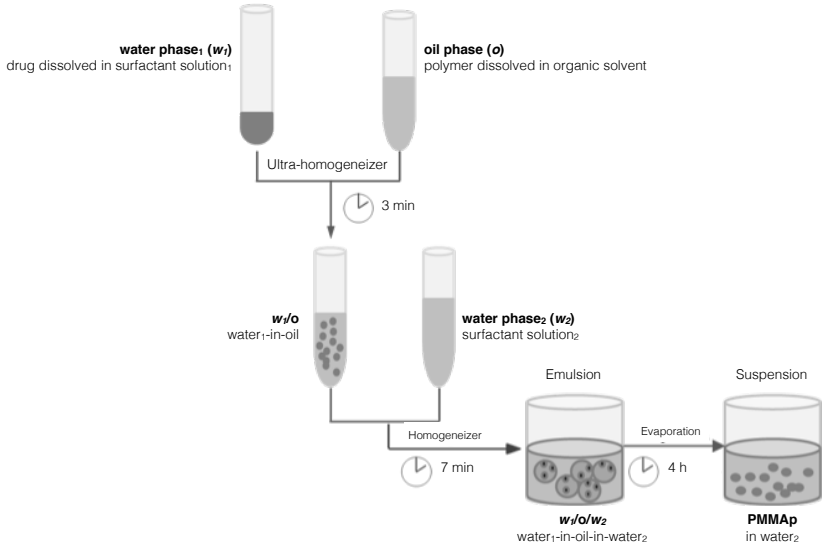



**Fig. 1** Schematic representation of the DESE method (solution volumes are not drawn at proportion).

## 2.3. Particles surface morphology

The surface morphology of particles was analysed and photographed using a scanning electron microscope (SEM). The particles, as dry powder, were mounted on the SEM sample stab using a double-sided sticking tape and were coated with a gold film (20 nm) by sputtering under vacuum. Photomicrographs of suitable magnifications were obtained. For plain and Mino-loaded particles it was used a Hitachi S2400 equipment and for Lev-loaded particles a Hitachi S450 (Hitachi, Japan).

## 2.4. Particle size analysis

Particle size was determined by dynamic light scattering, using the Malvern Mastersizer 2000 (Malvern Instruments, UK). The adequate diffraction index for PMMA material was selected and lyophilised particles were dispersed in filtered purified water directly in the sample dispersion unit (Hydro SM, Malvern Instruments, UK). The system was maintained at 25°C with constant agitation until an obscuration of 4 to 6% was reached. For each sample four independent measurements were performed and at least three replicates were obtained.

## 2.5. Surface Charge

Particle surface charge was obtained measuring the zeta potential (ZP) of the lyophilised particles by electrophoretic light scattering (Malvern Zetasizer Nano, Malvern Instruments, UK).

Measurements of ZP were performed for each particle batch after appropriate dilution and dispersion with filtered purified water. For each sample five independent measurements were performed and at least three replicates were obtained.

## 2.6. Particle encapsulation efficiency and drug loading

Encapsulation efficiency (EE) and drug loading (DL) percentages of the antibiotics in the PMMAp were determined indirectly by measuring the antibiotic amount present in the supernatant phase after the particle centrifugation step. Supernatants were adequately diluted before analysis.

Minocycline content was obtained by UVvis-spectrophotometry (Hitachi U-2001, Hitachi Instruments Inc., USA) using a calibration curve obtained at 350 nm. This procedure was performed in triplicate.

Levofloxacin content was determined by HPLC-UV (Shimadzu LC-6A and SPD-6A, Kyoto Japan), using an adjusted method described in literature (*see* Annex; Hart et al., 2010). Briefly, the chromatographic analysis was performed using a 125-4, 5 µm, LiChrosphere® 100 RP-18 (Merck, Darmstadt, Germany) column, a degassed mobile phase of water:acetonitrile and triethylamine (85:15(V/V), 0.6%(V/V)) adjusted to pH 3 using ortho-phosphoric acid (Sigma Aldrich and Panreac Quimica, Spain), a 1.2 mL/min flow rate and UV detection at 284 nm. All chromatographic separations were carried out at 25ºC.

For both antibiotics EE was expressed as the percentage of encapsulated antibiotic in PMMA particles related to the initial amount of antibiotic used for particles preparation ($w_i$), according to Eq. (1):

$$\%EE = \frac{w_i - w_s}{w_i} \times 100 \qquad \text{Eq. (1)}$$

where $w_s$ is the amount of antibiotic present in the supernatant after particles harvesting.

Also, DL was expressed as the percentage of the encapsulated antibiotic in PMMA related to the initial amount of polymer and antibiotic ($w_{p+a}$), according to Eq. (2):

$$\%DL = \frac{w_i}{w_{p+a}} \times \%EE \qquad \text{Eq. (2)}$$

## 2.7. Antibiotic stability throughout formulation procedure

Qualitative analysis of antibiotic stability during formulation procedures was assessed by microbiological assay. In brief, it was performed by the Mueller-Hinton agar diffusion method against reference antibiotic-susceptible isolates of *Escherichia coli* (ATCC 25922) and of

*Staplylococcus aureus* (ATCC 25923). Bacterial strains were seeded with microbial inoculums from 2 to $3\times10^5$ cfu/mL. Commercial discs of the studied antibiotics and the solutions of non-processed antibiotics (1mg/mL) were used as controls. Commercial discs were Oxoid MH 30μg CT0030B, for minocycline, Bio-rad 5μg LVX 66858, for levofloxacin and Oxoid Blank Discs CT0998B (Oxoid Ltd. Basingstoke, Hampshire, England) for controls. To obtain the same concentration as the commercial disc, the necessary volume of each antibiotic standard solution was placed in a blank disc: 30 μL for minocycline and 5 μL for levofloxacin.

The evaluated test samples were aliquots of the first supernatant obtained after particles centrifugation and diluted to obtain adequate antibiotic concentration (1.0 mg/mL). Culture plates were incubated at 37°C for 24 h after what the zones of growth inhibition were observed around the dried discs and diameters were measured in mm, indicating antibiotics microbiological activity.

## 2.8. Fourier Transform Infrared spectroscopy (FTIR)

FTIR spectra were obtained at 400 – 4000 $cm^{-1}$ scanning range using the KBr pellet technique. Powder samples of minocycline, levofloxacin, PMMA and lyophilized particles of (PMMA), Mino(PMMA) and Lev(PMMA) were incorporated with potassium bromide in an agate mortar. A pellet was obtained by compressing the powder mixture into discs in a hydraulic press, under a pressure of 740 GPa for 3 min. The pellet was placed in the light path and spectra obtained were the results of averaging thirty scans. Different spectrophotometers were used, a Jasco FT/IR – 460 Plus spectrophotometer (JASCO Europe s.r.l., Italy) for Mino(PMMA) particles and a IRA ffinity-1 spectrophotometer (Shimadzu, Kyoto, Japan) for Lev(PMMA) particles.

## 2.9. *In vitro* release studies

Minocycline release from the Mino(PMMA) particles was determined by suspending an adequate amount of particles in PBS buffer (pH 7.4) in a proportion of 1:1 mg particles per mL of release medium. Containers were placed in a horizontal-shaking water bath under gentle agitation (130 rpm) at 37°C (JULABO SW 21, Julabo Labortechnik GmbH, Germany).

At pre-determined time-points throughout a one-week period (168 h), tubes were collected, centrifuged at 6000 rpm for 20 min at ambient temperature (Centrifuge Heraeus, Megafuge 1.0R, Heraeus, Germany) and 2.0 mL of the supernatants were measured for antibiotic content using methods previously described for minocycline (Section 2.7, Chapter 2). The withdrawn samples were then replaced with equal volumes of fresh PBS and stirring continued until next collection.

Levofloxacin release from the Lev(PMMA) particles was determined by suspending an adequate amount of particles in NaCl (0.9% w/w) with Tween 20® (0.05% w/V) (pH 5.6) in a proportion of 1.5:1, mg particles per mL of release medium. Containers were placed in a horizontal-shaking

water bath under gentle agitation (130 rpm) at 37°C (JULABO SW 21, Julabo Labortechnik GmbH, Germany).

Three independent containers were prepared to each pre-determined time-point, throughout a one-week period (168 h). At each time-period tubes were collected, centrifuged at 12000 rpm for 10 min at ambient temperature (Centrifuge Heraeus, Megafuge 1.0R, Heraeus, Germany) and 1.0 mL of the supernatants were measured, in triplicate, for antibiotic content using methods previously described for levofloxacin (Section 2.7, Chapter 2).

## 2.10. Preparation of acrylic bone cement specimens

Thin specimens of acrylic BC were prepared, at room temperature (25±1ºC) and atmospheric pressure, by loading 15% ($w/w_{BC}$) of Mino(PMMA) particles. The commercial supplier recommended proportion [CMW1® powder]:[Monomer liquid] (1 g of powder and 0.5 mL of liquid) was maintained. Mino(PMMA) particles were previously well-dispersed in the CMW1 powder in a glass mortar before adding the monomer. Both components were then thoroughly mixed. BC dough was then moulded in thin plates with an average thickness of 1.30 ± 0.02 mm, an average length of 23.40 ± 0.03 mm and an average width of 19.50 ± 0.01 mm. BC setting proceeded for one hour.

# 3. Results

## 3.1. Particle characterisation

SEM micrographs revealed the formation of microparticles with a smooth surface and spherical shape (Fig. 2) with no evident presence of pores or cracks on particle surface. Interestingly, Lev(PMMA) particles presented a clustered appearance due to the sucrose layer, that remained in the formulation after the freeze-drying step.

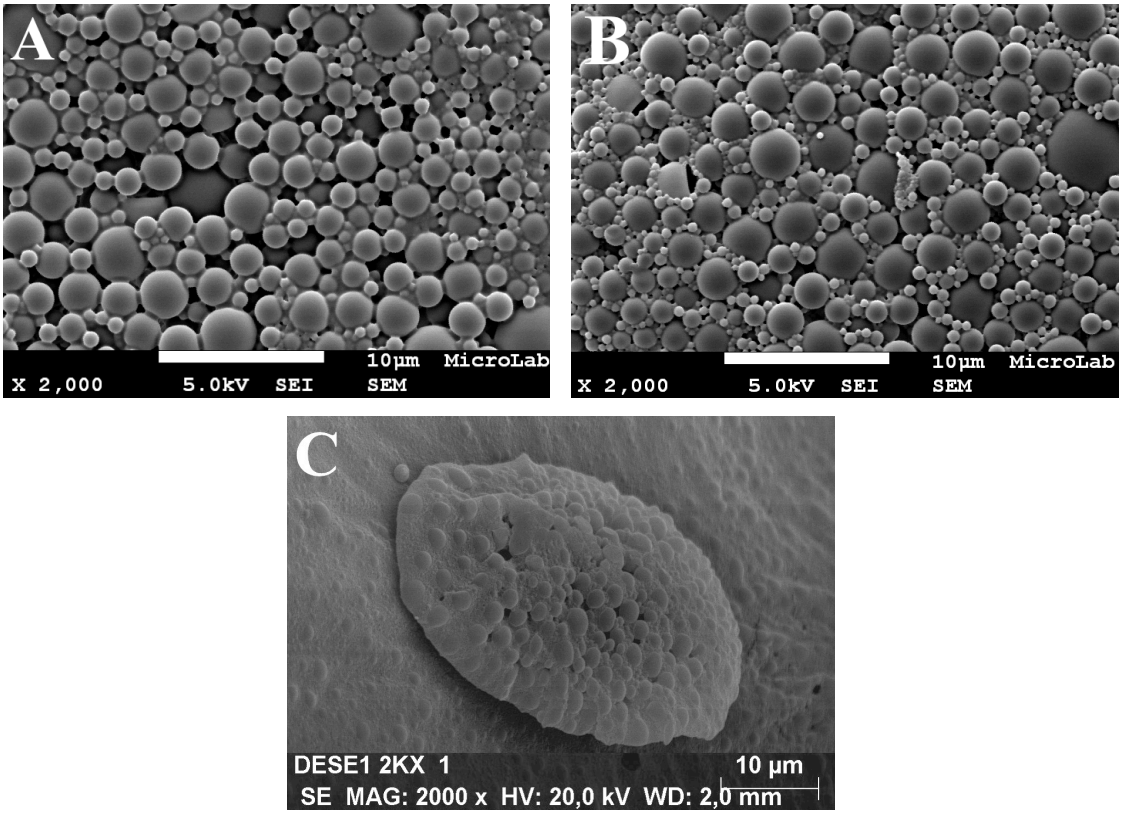



**Fig. 2** SEM micrographs for plain PMMA particles (A), Mino(PMMA) (B) and Levo(PMMA) (C) particles.

Table 1 summarises the results regarding ZP, EE and DL and for both plain and antibiotic-loaded PMMA particles. All PMMAp presented negative values of ZP, below -30 mV, more accentuated for antibiotic-loaded particles. Values of EE and DL for Lev(PMMA) particles were higher than those obtained for Mino(PMMA) particles.

**Table 1.** Summary of the resultant properties of produced PMMA particles, presented as mean±SD.

| Particle batch | ZP (mV) | EE (%, w/w) | DL (%, w/w) |
|---|---|---|---|
| PMMA | - 31.0 ± 0.1 | - | - |
| Mino(PMMA) | - 35.8 ± 0.4 | 38.6 ± 1.3 | 2.8 ± 0.1 |
| Lev(PMMA) | - 35.7 ± 1.8 | 65.0 ± 1.6 | 4.5 ± 0.2 |

Concerning the PMMAp size there were no noteworthy differences between plain and loaded particles (Table 2). From d(0.1), d(0.5) and d(0.9) parameters, which represent the diameter range of 10%, 50% or 90% of the particle population distribution, it can be concluded that the preparation method has mainly produced PMMA microparticles with a average volume weight mean of 3 μm.

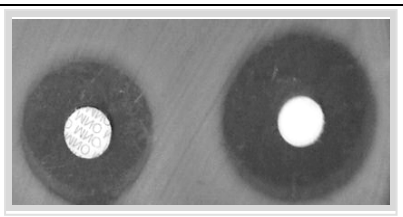
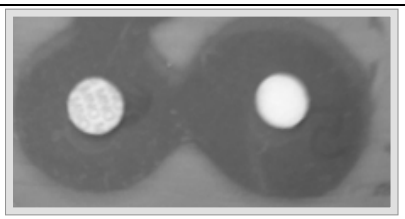
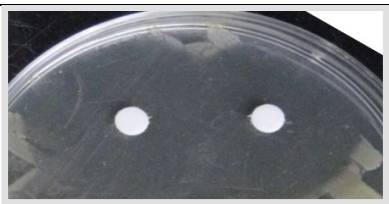
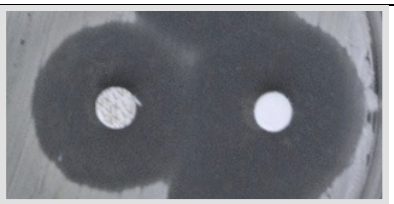
**Table 2.** Size distribution of PMMA particles, presented as mean±SD.

| Particle batch | Span | VWM (μm) | d (0.1) | d (0.5) | d (0.9) |
|---|---|---|---|---|---|
| PMMA | 2.07 ± 0.03 | 2.57 ± 0.51 | 0.23 ± 0.01 | 2.28 ± 0.31 | 4.94 ± 0.58 |
| Mino(PMMA) | 1.21 ± 0.00 | 2.96 ± 0.00 | 1.53 ± 0.00 | 2.70 ± 0.00 | 4.81 ± 0.00 |
| Lev(PMMA) | 1.52 ± 0.32 | 2.53 ± 0.18 | 0.79 ± 0.63 | 2.40 ± 0.06 | 4.43 ± 0.05 |

## 3.2. Antibiotic stability throughout formulation procedure

Microbiological activity of the antibiotics in the formulation supernatants, against *E. coli* and *S. aureus* are presented in Table 3. For both Mino(PMMA) and Lev(PMMA) formulations, images show similar growth inhibition zones between the commercial disc and that containing the aliquot of the first supernatant, obtained after the first centrifugation step.

**Table 3.** Bacteria inhibition zones obtained from first supernatant solutions of the antibiotic-loaded particle formulations.

| **Bacteria Strain** | ***E. coli*** | | ***S. aureus*** | |
|---|---|---|---|---|
| **Sample** | Commercial | Mino/PMMAp | Commercial | Mino/PMMAp |
| **Minocycline** |  | |  | |
| **Sample** | Commercial | Levo/PMMAp | Commercial | Levo/PMMAp |
| **Levofloxacin** |  | |  | |

## 3.3. Fourier Transform Infrared spectroscopy (FTIR)

Fig. 3 shows the spectra obtained for plain PMMAp (A and D), Mino(PMMA)p (B) and Lev(PMMA)p (E). To ease comprehension spectra of plain and loaded particles were overlaid (C and F). Spectra analyses allow concluding that no significant structure differences were observed.

It could be hypothesized that minocycline and levofloxacin were physically entrapped instead of chemically bonded to the PMMA particles.

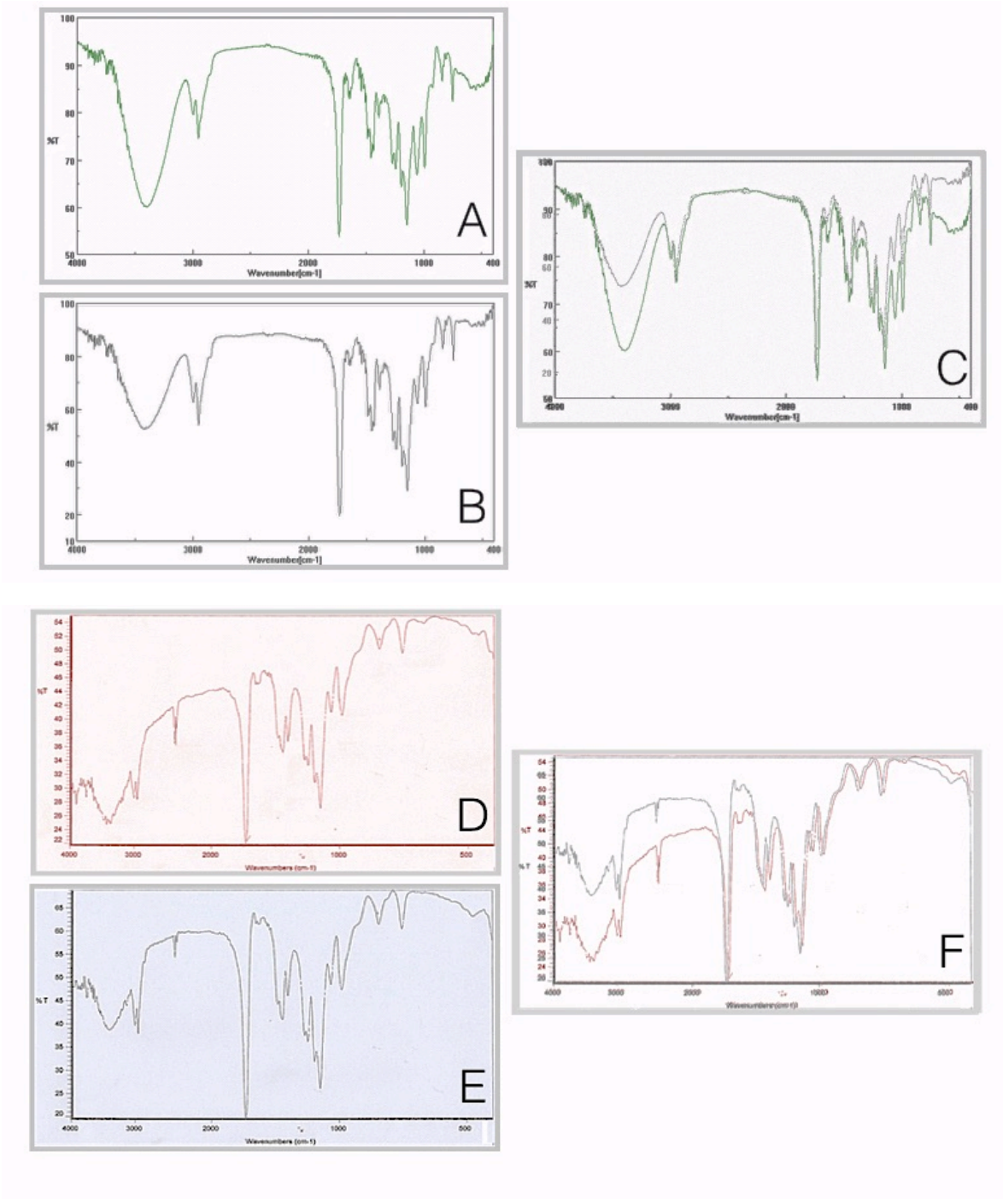



**Fig. 3** FTIR spectra for particles: plain PMMA (A and D), Mino(PMMA) (B) and Lev(PMMA) (E). Overlay of the spectra to better identify the presence of peaks before and after, minocycline (C) and levofloxacin (F) loading.

Considering the plain PMMA particles spectrum (Fig. 3A and 3D), the broader band observed from 3100 to 3600 $cm^{-1}$ on both spectra is assigned to O-H stretching of the abundant hydroxyl

groups. In addition, the spectra display three-absorption bands between 2800 and 3000 $cm^{-1}$, which are due to C-H stretching (Ayre el al., 2014; Sivakumar and Rao 2002).

The C=O band stretching vibration of the ester group is located near 1700 $cm^{-1}$. For both antibiotic-loaded particles, overlaid spectra were fairly superimposable (Fig. 3C and 3F) and it was not observed new peaks appearance or the disappearance of others.

## 3.4. *In vitro* Release Studies

Concerning Mino(PMMA)p, minocycline release showed an initial burst of 35.1% followed by an increased constant release until 72 h reaching 72.5% of cumulative release (Fig. 4). After a one-week period there was evidence of minocycline degradation due to the pH of the release medium (Fig. 5). Regarding Lev(PMMA)p, and even using a release medium favourable to levofloxacin solubility, results showed no antibiotic release from particles (Fig. 4). An amount of 0.1% was detected at time zero but afterwards no further levofloxacin release was detected above its limit of detection (< 0.25 μg/mL).

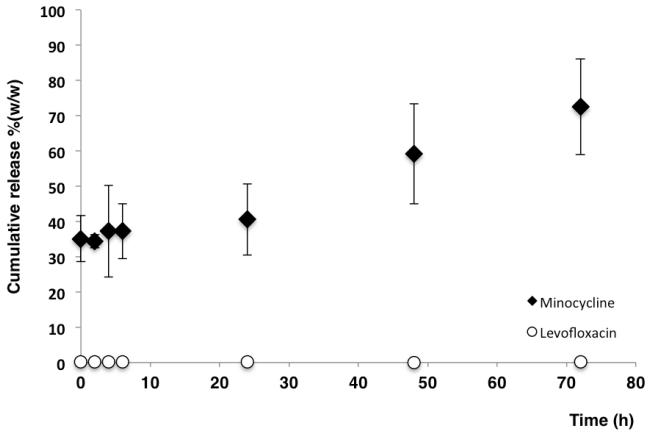



**Fig. 4** *In vitro* release profiles from Mino(PMMA)p and Lev(PMMA)p (mean±SD; n=3).

**Fig. 5** Photo of minocycline solutions: a just-prepared (in yellow) and after one-week, in PBS, evidencing degradation (in brown).

# 4. Discussion

The inadequate and often incomplete release rate of antibiotics from the available commercial ALABC is well known and described (Lewis, 2009). The ALABCs are PMMA-based BCs, which is a biocompatible polymer with remarkable performance in the orthopaedic field due to the excellent mechanical performance, and that has being reported with increasing interest on its applications as a drug particulate carrier (Bettencourt and Almeida, 2012; Coelho et al., 2010; Shi et al., 2010).

This study aimed at improving the controlled release of antibiotics through the inclusion of antibiotic-loaded PMMAp into the BC matrix. The rationale was to guarantee antibiotic protection during BC setting and maintenance of the mechanical performance of the particle-loaded BC, assuring its important structural function on prosthesis fixation to bone. The selected antibiotics were minocycline and levofloxacin, both with a broad-spectrum of activity against susceptible pathogens generally present in bone and joint infections (Zimmerli, 2015). Physicochemically both present amphiphilic characteristics - greater for levofloxacin; soluble in acidic aqueous media; melting point over 200ºC.

Focusing on particle preparation, these were obtained by the DESE method, using a pre-formed PMMA polymer. Complete evaporation of the organic solvent used (DCM) was guaranteed by the continuous stirring for a long period of time. Although DCM is classified as a Class 2 solvent, according to ICH classification, which may lead to toxicological effects (Bettencourt and Almeida, 2012), a previous study performed in our laboratories using the same DESE method, demonstrated that DCM final amount was below the safety limit (600 ppm) determined by the ICH classification (EMA, 2006; Florindo et al., 2010). Moreover, PVA was the surfactant used to stabilise both emulsions, with the first solution 8-fold higher concentrated than the second due to the most demanding need of emulsion stability of the smaller nanodroplets formed during ultra-homogenization (Budhian et al., 2007). It has been reported as an adequate surfactant to prepare polymer particles, not only from biodegradable polymers, but also for PMMA particles (Ferreira et al., 2015; Florindo et al., 2010; Kwon et al., 2002).

Mino(PMMA)p and Lev(PMMA)p were prepared and fully characterised regarding key-parameters as morphology, size distribution, surface charge, encapsulation efficiency and drug loading, antibiotic microbiological stability and *in vitro* release.

Electron microscopy (SEM) analyses revealed, for all formulations, particles with a spherical shape and a smooth surface with no discernible pores or cracks (Fig. 2). Higher EE and DL values were obtained for levofloxacin than for minocycline (Table 1), which was expected since the latter presents higher water solubility. As intermediate values were obtained using the same method to encapsulate a hydrophilic dye, these results may be considered as promising (Zydowicz et al., 2002). Surface charge was determined measuring the ZP, and plain PMMAp and antibiotic-loaded-PMMAp all presented negative values (Table 1), which is consistent with the

literature (Ahlin et al., 2002). ZP values below -30 mV are conventionally considered as suitable to assure that these PMMAp would remain stable in suspension (Everett, 1994). Moreover, the ZP of antibiotic-loaded PMMAp was slightly more negative when compared to the plain PMMAp, which means an increased stability of these formulations. Size analysis led to conclude that DESE method was consistent and mainly produced microparticles, with roughly 2.5 — 3 μm in volume mean diameter (Table 2). It could also be concluded that minocycline or levofloxacin loading into PMMAp did not affect the particle size.

In the rationale for including these particles into BC, particle size was not a critical parameter, considering that particles were not intended to deliver directly the antibiotic in the infection site but rather to be included in a larger PMMA matrix, from where it should diffuse-out after release from the particles. Thus, the limiting factor of antibiotic release would be the hydrophobic BC matrix instead of particle size.

However, knowledge of the antibiotic stability throughout particle formulation was an important issue, regarding their antimicrobial activity. Data in Table 3 shows that both minocycline and levofloxacin retained the antibacterial activity properties after microencapsulation, against both *E. coli* and *S. aureus*, two of most common pathogens isolated in bone-related infections. Moreover, a FTIR study was performed to disclose any eventual chemical bonding between the antibiotic molecules and the particle polymer-matrix (Fig. 3). From spectra comparison between plain PMMAp and loaded-PMMAp no significant structure differences were observed (Fig. 3C and 3F), suggesting that neither minocycline nor levofloxacin were chemically bonded to PMMA particles but rather entrapped inside the polymer network.

*In vitro* drug release from the Mino(PMMA)p showed a marked burst effect with 35.1% of the antibiotic being released at first contact with release medium, followed by a constant release until 72 h (Fig. 4), which is consistent with the literature. In fact, publications point out that drug release from hydrophobic PMMA-based materials during the first 72 h is due to the drug molecules closer to the surface of the material (Lewis, 2009). Nonetheless, in the present studies 72.5% minocycline was eluted from particles within a 3-day period. As mentioned, the study was interrupted after one week due to minocycline degradation in the PBS (pH 7.4) (Pawełczyk and Matlak, 1982; Zbinovsky and Chrekian, 1977). It was a promising result considering that PMMA beads have been reported to deliver only an average of 25% of the loaded antibiotic, gentamicin, an aminoglyscoside (Kanellakopoulou and Giamarellos-Bourboulis, 2000) and that microparticles have higher surface area than beads, which, at least theoretically, increases drug percentage delivery.

On the other hand, *in vitro* levofloxacin release from the Lev(PMMA)p proceeded in a more acidic release medium due to levofloxacin high solubility in aqueous solutions with pH< 6.7. Although a release medium at pH 5.6 was used, levofloxacin release from Lev(PMMA)p resulted unexpectedly low, with only 0.1% (w/w) of levofloxacin released. As no increase was observed

since time zero it was likely that the drug detected in the release medium corresponded to adsorbed levofloxacin molecules on the outer surface of the particle (Fig. 4). Other authors have reported a satisfactory levofloxacin release from other polymeric particles (PLGA) produced using the same method, in spite the low EE achieved (Cheow and Hadinoto, 2011). As levofloxacin is an amphiphilic molecule, it is prone to interact both with the hydrophobic PMMA surface and the release medium. In this case, the interaction with the PMMA surface was apparently more significant than the solubility in the aqueous release medium.

Completed the particle characterisation, the second aim of this work was to load the prepared particles into the BC matrix. Given the obtained results, the study proceeded only with the Mino(PMMA)p. An *in vitro* experiment was designed to evaluate the ability of the release medium to percolate the BC matrix and promote minocycline release from the microparticles therein incorporated. Hence, very thin BC specimens were prepared containing 15% (w/w) of Mino(PMMA)p, which unfortunately hampered BC setting making impossible to obtain the cement (Fig. 6). The inclusion of foreign-particles into the polymeric matrix always interferes with the polymerisation by hampering the establishment of polymer structure. By adding particles of the same biomaterial, most probably, the monomer also reacted with the PMMA (from the particles), which have a higher average molecular weight ($M_w$ 350 000 g/mol) than the PMMA from BC ($M_w$ 120 000 g/mol). This imbalanced the proportion to the liquid monomer added and polymerisation did not occur, as it should.

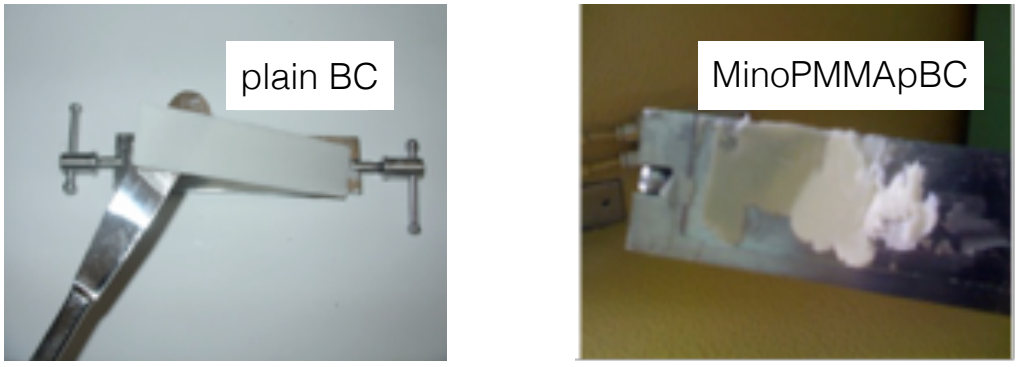



**Fig. 6** Photos of a well-set plain BC specimen and of a Mino(PMMA)$_p$BC specimen, which has not set.

As the percentage of added particles represented a minocycline loading of 0.4% ($w_{Mino}/w_{BC}$), considering the obtained minocycline DL (Table 1), lower amounts of particles were not considered for loading into BC because the corresponding amount of minocycline would not have any antimicrobial significance. It is noteworthy to refer that commercial antibiotic-loaded BCs typically contain 2.5% ($w_{antib}/w_{BC}$) of free antibiotic (Jiranek et al., 2006).

# 5. Conclusion

In short, PMMA particles, plain and antibiotic-loaded, were successfully prepared by the DESE method with suitable surface morphology, yield of preparation, stability and size. Antibiotic encapsulation and release strongly depended on drug's hydrophobicity, and formulation conditions have not interfered with antibiotic microbiological activity.

However, PMMAp inclusion in the BC matrix did not evolved as expected, suggesting that loading other type of biomaterial particles as drug delivery systems in BC should be explored (Chapter 4).

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# Chapter 3

## ANTIBIOTIC LOADING INTO ACRYLIC BONE CEMENT

# Section 1

# A NOVEL MODIFIED ACRYLIC BONE CEMENT MATRIX. A STEP FORWARD ON ANTIBIOTIC DELIVERY AGAINST MULTIRESISTANT BACTERIA RESPONSIBLE FOR PROSTHETIC JOINT INFECTIONS

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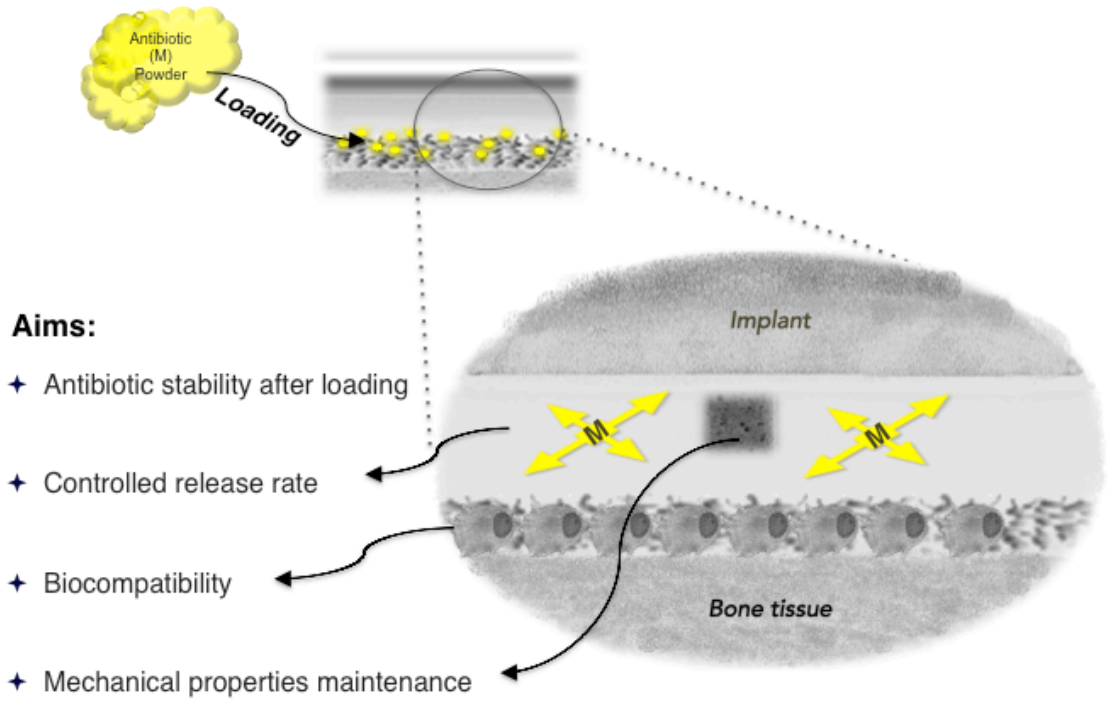
# Graphical Abstract




## Highlights

- A novel modified BC matrix loaded with minocycline
- Complete in vitro release of the loaded minocycline
- Increased antimicrobial activity of minocycline against representative strains of orthopaedic infections, including MRSA
- Full mechanical properties compliance and biocompatibility maintenance of the novel BC matrix

# Abstract

Currently the safe and responsible use of antibiotics is a world-wide concern as it promotes prevention of the increasing emergence of multiresistant bacterial strains. Considering that there is a noticeable decline of the available antibiotics pipeline able to combat emerging resistance in serious infection a major concern grows around the prosthetic joint infections once the available commercial antibiotic loaded polymethylmethacrylate bone cements (BC) are inadequate for local antibiotic treatment, especially against MRSA, the most commonly isolated and antibiotic-resistant pathogen in bone infections. In this paper a novel modified BC matrix loaded with minocycline is proposed. A renewed interest in this tetracycline arises due to its broad-spectrum of activity against the main organisms responsible for prosthetic joint infections, especially against MRSA. The modified BC matrices were evaluated concerning minocycline release profile, biomechanical properties, solid-state characterization, antimicrobial stability and biocompatibility under *in vitro* conditions. BC matrix loaded with 2.5% ($w/w_{BC}$) of minocycline and 10.0% ($w/w_{BC}$) of lactose presented the best properties since it completely released the loaded minocycline, maintained the mechanical properties and the antimicrobial activity against representative strains of orthopaedic infections. *In vitro* biocompatibility was assessed for the elected matrix and neither minocycline nor lactose loading enhanced BC cytotoxicity.

**Keywords:** bone cement, arthroplasty, minocycline, mechanical properties, antibiotic release, biocompatibility.

# 1. Introduction

Due to bacterial resistance, the continuing rise of infections resistant to common treatments is a growing threat and has won place in the worldwide agenda (Editorials, 2013; Cooper and Shlaes, 2011]. There is a growing tendency to reserve novel antibiotics with activity against resistant bacteria for treating serious infections. Some researchers are proposing as valuable alternatives the upraise of old antibiotic compounds, once they have remained active against a large number of currently resistant bacteria (Editorials, 2013; Garrido-Mesa et al., 2013).

Within the presented context, this research work had the intention to use minocycline, which is a tetracycline that has found recently a renewed interest in literature and has been referred as presenting a broad-spectrum of activity against the main organisms responsible for prosthetic joint infections, especially against MRSA, the most commonly isolated and antibiotic-resistant pathogen in bone infections (Bishburg and Bishburg, 2009).

The use of antibiotic-loaded polymethylmethacrylate bone cement, herein after BC, is considered to be the standard of care for total hip replacement patients with chronic infection, providing local delivery of antibiotics without invasive procedures. Single or combinations of different antibiotics are being considered to load into BC for more effective orthopaedic infection eradication; for example, gentamicin and tobramycin, vancomycin and clindamycine (Gallo et al., 2005; Jiranek et al., 2006; Shi et al., 2010; Van de Belt et al., 2001). However, the inadequate release kinetics of antibiotics from the hydrophobic acrylic BC matrix often leads 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,* which hinders the use of local antibiotic therapy for prosthetic joint infections. For that reason, recent research engagement is on finding novel BC formulations able to achieve a total release of the antibiotic, in order to prevent the emergence of the multiresistant strains.

When evaluating the possible applicability of antibiotic agents to load into BC it must be considered not only a suitable antimicrobial spectrum and an adequate release profile but also other important aspects as the resultant biomechanical properties and biocompatibility of the modified BC (Bruinink and Luginbuehl, 2012; Hendricks et al., 2004; Jiranek et al., 2006). The biomechanical properties will be of utmost importance, as BC serves as the primary fixation material between bone and the prosthetic element in cemented arthroplasty by forming a mechanical anchorage between the two components (Ries et al., 2006). Therefore, attention must be given to mechanical integrity once the inclusion of antibiotics often impairs the mechanical properties of acrylic BC (Shi et al., 2006). Also the biocompatibility is imperative once it evidences the success of the interaction between the implanted material and cells nearby.

This research work was designed to develop and fully characterize, a novel minocycline-loaded

BC matrix modified with lactose. Considering that BC is not an erodible or biodegradable drug delivery system and that porosity of a monolithic matrix has a relevant role in drug release from the matrix core, we have elected lactose to increase BC matrix porosity (Siepmann et al., 2012). Lactose has a long track history of safe use in pharmaceutical formulations explained by the high stability, with no significant tendency to react with the drug or other components of a formulation, free solubility in water and relatively low cost. Recently, lactose has been described has a successful additive of matrixes with good results related to antibiotic release (Frutos et al., 2010; Virto et al., 2003).

The main aim of this work was to assess the feasibility of minocycline loading into lactose-modified BC considering the following key aspects: drug release, microbiological activity, mechanical and structural properties and biocompatibility.

# 2. Materials and Methods

## 2.1. Materials

Commercial acrylic BC, CMW1® Radiopaque, and minocycline hydrochloride were kindly donated by DePuy Iberia (Spain&Portugal) and Atral-Cipan (Castanheira do Ribatejo, Portugal), respectively. Sodium chloride was obtained from AppliChem (AppliChemGmbH, Darmstadt, Germany). Polysorbate 20 (Tween20®), ammonium hydroxide solution, and tetrahydrofurane were all obtained from Sigma-Aldrich (Spain). Lactose monohydrate, di-ammonium oxalate monohydrate, ethylenediaminetetraacetic acid disodium salt 85ulphate85 (EDTA), Titriplex® III and 1,2-propanediol, were obtained from Merck (Merck Millipore, Lisbon, Portugal). Dimethylformamide for HPLC was obtained from BDH Prolabo (VWR, Portugal). Deionized water was obtained from a Millipore analytical deionization system (F9KN225218).

## 2.2. Preparation of acrylic bone cement specimens

Different specimen matrices (parallelepiped and cylindrical) with varied compositions (Table 1) were prepared at room temperature (23±1ºC) and atmospheric pressure, according to commercial BC specifications and ISO 5833 recommendations (ISO 5833, 2002). CMW1® is high viscosity BC intended for digital application. Solid components (CMW1® powder, minocycline and lactose) were thoroughly but carefully mixed in a glass mortar and then the proportional quantity of liquid monomer was added. When the desired consistency was obtained, BC dough was manually casted, into an aluminium mould, for bending tests specimens, and into PTFE mould, for compression tests specimens, accordingly with the ISO 5833 moulds dimensions. Cure proceeded at room temperature for 1 h also accordingly with ISO 5833. All specimens were finished to careful polishing, measured with a digital micrometer (Mitutoyo Digimatic, Painesville, Ohio, USA) with an accuracy of 0.01 mm, and stored in a vacuum desiccator (23±1°C, 24±2 h) before use. Cylindrical specimens were used for compressive strength determination. Parallelepiped specimens were used for all the other tests and treated accordingly.

**Table 1.** Composition of the loaded BC specimens, expressed as wt.% of CMW1® powder.

| BC Matrix | Minocycline (%) | Lactose (%) |
|---|---|---|
| BC | 0 | 0 |
| BC10L | 0 | 10.0 |
| BC20L | 0 | 20.0 |
| M[BC] | 2.5 | 0 |
| M[BC10L] | 2.5 | 10.0 |
| M[BC20L] | 2.5 | 20.0 |

M= minocycline; L=lactose

## 2.3. *In vitro* release studies

In vitro minocycline release from parallelepiped BC specimens (75 × 10 × 3.3 mm) was featured with each BC sample (± 3.0 g) incubated in 10 mL of a solution of NaCl 0.9%(w/V) with 0.05%(V/V) Tween20® (hereinafter release medium) in a shaking water-bath at 37°C. At predetermined intervals, throughout a one-week period (168 h), aliquots of the supernatant were collected and analysed in triplicate. The withdrawn aliquots were then replaced with equal volumes of fresh release solution and sink conditions were guaranteed during the whole study. Minocycline content was determined by HPLC, using an adaptation of a method described in USP29 (US Pharmacopeia). Briefly, the HPLC procedure employed an i.d. 5 mm column (Lichrospher® 100 RP-18, Merck KgaA, Darmstadt, Germany), a mobile phase of ammonium oxalate 0.2 M/EDTA 0.01 M/dimethylformamide/tetrahydrofurane (600:180:120:80, V/V), pH 7.2, 1 mL/min flow rate and UV detection at 350 nm.

## 2.4. Biomechanical Tests

The mechanical properties, compressive strength, bending modulus and bending strength were measured according to ISO 5833 (ISO 5833, 2002). Tests were performed at room temperature in a servo-hydraulic universal machine (TIRAtest® 2705). Testing was carried out on at least five specimens of each BC matrix and results were expressed as mean±SD.

### *Compressive strength*

Compressive strength tests were performed on cylindrical specimens (6 × 12 mm), placed between a flat non-compressible surface and the platen of the testing machine. Applied crosshead rate was 20 mm/min (Fig. 1, left) until reaching the upper yield point or the cylinder fracture.

### *Bending strength and bending modulus*

Bending strength and bending modulus tests were performed on parallelepiped specimens (75 × 10 × 3.3 mm) by means of a four-point loading apparatus (Fig. 1, right) operating with a crosshead speed of 5mm/min until breakage.

## 2.5. Solid-state characterization

### *Porosity*

The percent porosity, $\phi$, of BC specimens was determined with recourse to Eq.1. $D_{skt}$ and $D_B$ are, respectively, the skeletal and bulk densities of BC specimens and were both determined using gas pycnometry (Micromeritics AccuPyc 1330 Gas Pycnometer; Micromeritics Analytical Services,

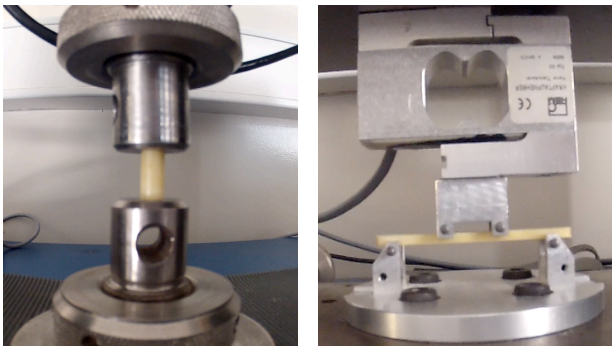


**Fig. 1** Biomechanical apparatus: on the left, for the BC cylinder specimen compressive strength testing; on the right for the BC parallelepiped specimen flexural testing.

Norcross, USA). Helium was the gas used and all assays were performed at room temperature. $D_B$ values were obtained from a powder mixture sample, weighed before cure, with the composition of each of the chosen matrix. $D_{skt}$ values were obtained after curing, using a weighed parallelepiped fractured sample of each of the chosen matrices. For each matrix a minimum of three determinations were made.

$$\varphi = \left(1 - \frac{D_{skt}}{D_B}\right) \times 100 \qquad \text{Eq.1}$$

### *Microstructure analysis*

The fracture surface morphology of parallelepiped BC specimens was analysed and photographed through a thermal field emission scanning electron microscopy, FEG-SEM, model JSM7001F (JEOL, Japan) operated at 5 kV. Briefly, 3 mm samples of a selected group of representative fractured parallelepiped specimens were cut (Fig. 2), 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).

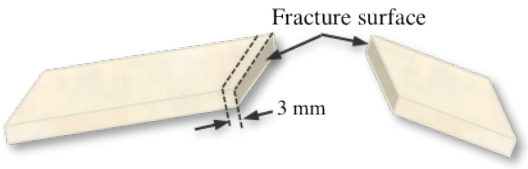



**Fig. 2** Schematic representation of the BC samples cut for FEG-SEM observation.

### *Contact angle and surface energy determination*

For contact angle measurements, BC matrices were cut in parallelepipeds (1.2 × 25.0 × 17.8 mm) using the same cut-off machine referred earlier. Assays were performed with a Kruss K100 tensiometer (Kruss GMBH, Hamburg, Germany) using the Wilhelmy Plate method, by immersing the plates 5 mm into the test liquids (water and 1,2-propanediol) at a speed of 3 mm/min, at 25.0±0.1°C. Advancing contact angles were used for surface energy ($\gamma$) estimation of all BC matrices, as well as its dispersive ($\gamma_d$) and polar components ($\gamma_p$) based on the harmonic mean method proposed by Wu (1971) and expressed by the Eq.2, where $\gamma_{12}$ is the interfacial tension between phases 1 and 2, which each have a surface tension consisting of a polar and dispersive component (Bettencourt et al, 2002; Wu, 1971). 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).

$$\gamma_{12} = \gamma_1 + \gamma_2 - \left(\frac{4\gamma_1^d\gamma_2^d}{\gamma_1^d+\gamma_2^d}\right) - \left(\frac{4\gamma_1^p\gamma_2^p}{\gamma_1^p+\gamma_2^p}\right) \qquad \text{Eq. 2}$$

### *Differential scanning calorimetry*

Differential scanning calorimetry (DSC) measurements were conducted in a DSC Q2000 (TA Instruments, New Castle, DE, USA) using sample powders accurately weighed and sealed in an aluminium pan. An empty sealed pan was used as reference. Heating proceeded from 0ºC to 250°C, at a scan rate of 10ºC/min, under a nitrogen atmosphere. The heat flow as a function of temperature was measured. BC matrices were analysed and compared with raw materials (CMW1® powder, lactose and minocycline) and analysed in the % (w/w) proportion present in the BC blend.

### *X-Ray Diffraction Studies*

X-ray diffraction studies on BC with and without minocycline to study the possible changes in crystallinity of the antibiotic were carried out using a high-resolution Rigaku Geigerflex D/Mac, C Series diffractometer with Cu Kα radiation (k=1.5406 Å) produced at 30 kV and 25 mA, which scanned the diffraction angles (2θ) between 0° and 80° with a step size of 0.02º 2θ $s^{-1}$.

### *Fourier Transform Infrared spectroscopy (FTIR)*

FTIR spectra were obtained with IRAffinity-1 spectrophotometer (Shimadzu, Kyoto, Japan) at 400–4000 $cm^{-1}$ scanning range. Powder samples of minocycline and lactose, and powdered samples of all the BC matrices, were incorporated with potassium bromide in an agate mortar. A pellet was obtained by compressing the powder mixture into discs in a hydraulic press, under a pressure of 740 GPa for 3 min. The pellet was placed in the light path and spectra obtained were the results of averaging thirty scans.

## 2.6. Microbiological assay

Aliquots of the supernatants of a one-week minocycline release study (2.3) were used to determine the minocycline minimum inhibitory concentrations (MICs) by means of a two-fold serial broth microdilution assay (CLSI, 2011). Microdilution method was carried out using, as microbial strains, Gram-positive bacteria *S. aureus* ATCC 25923 (MSSA), *S. aureus* CIP106760 (MRSA), *Enterococcus faecalis* ATCC 51299 *(E. faecalis* low-level VR*)*, *S. epidermidis* ATCC12228. All aliquots were diluted at concentrations ranging from 2 to 0.0313 mg/mL with Müeller-Hinton broth medium (Biokar Diagnostics, France). The antimicrobial activity of the release medium was also evaluated to serve as control. The MIC values were taken as the lowest concentration of minocycline, presented in µg/mL, that inhibited the growth of the microorganisms, after 24 h of incubation at 37°C. The bacterial growth was measured with an absorbance Microplate Reader set to 620 nm (Thermo Scientific Multiskan® FC, Thermo Fischer Scientific Inc.). Assays were carried out in triplicate for each tested microorganism.

## 2.7. Biocompatibility studies under *in vitro* conditions

### *Cell viability studies*

Extracts from the BC specimen matrix M[BC10L] were obtained by immersing the specimens in an adequate volume of release medium, agreeing to a ratio of 1g / 5mL. Aliquots of the extracts were taken at the time of 10 minutes and 24 h of contact with the release medium. BC and BC10L matrices were used for comparison. The cytotoxicity was assessed using the cell viability MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reduction test on osteoblasts-like cells MG-63-human (ATCC CRL-1427™) (Mosmann, 1983). The day before the experiment, cells were seeded in 96 well tissue culture plates, in RPMI 1640 culture medium supplemented with 10% fetal serum bovine, 100 units of penicillin G (sodium salt), 100 $\mu$g of streptomycin sulphate and 2 mM L-glutamine, at a concentration that allow cells to grow exponentially during the time of the assay. The BC extracts of minocycline standard and sodium dodecyl sulphate (SDS, used as positive control) to be tested were diluted in culture medium. Each extract was tested in six wells in a single experiment, which was repeated at least 3 times. After 24 h, cell media was removed and replaced with fresh medium. The MTT dye solution was then added to each well (stock solution 5 mg/mL in 10 mM phosphate buffer solution at pH 7.4). After 3 h of incubation the media was completely removed and the intracellular formazan crystals were solubilised and extracted with DMSO. After 15 min at room temperature the absorbance was measured at 570 nm in a microplate reader (FLUOstar Omega, BMG LABTECH, Germany). The percentage of cell viability was determined for each extract solution.

### *Direct contact assay*

In order to characterise, morphologically, the cell behaviour in the presence of the previously tested BC matrices, MG63 cells were cultured at a $2.5\times10^4$ cells/cm$^2$ cell density in tissue culture Petri dish 60 cm (Nunc, Denmark) in direct contact with the BC matrix. Sterile filter papers with and without SDS (10 mg/mL) were used as negative and positive controls, respectively. After 24 h, MTT solution was added to a final concentration of 0.5 mg/mL, for the evaluation of cell viability.

## 2.8. Statistical Analysis

All data sets presented as mean±SD, are the result of at least five determinations and were examined by one-way analysis of variance (ANOVA) with a post-hoc Tukey test for comparison of each studied matrix specimen with the control specimen using GraphPad PRISM 5® (GraphPad Software, Inc., La Jolla, CA). The level of statistical difference was defined at a $p < 0.05$ level.

# 3. Results

## 3.1. *In vitro* release studies

Minocycline release profiles are shown in Fig. 3. Lactose loading influenced the minocycline release. A complete minocycline release was achieved after a one-week period, from the lactose-loaded BC matrices; minocycline release from M[BC] matrix, increased two-fold with lactose loading, that is, from 56.3±2.1% to 108.4±3.1% (M[BC10L]) and to 109.0±10.0% (M[BC20L]). Also, M[BC20L] matrix, showed a different release profile, when compared to M[BC] or M[BC10L], with more minocycline being released over the experiment time.

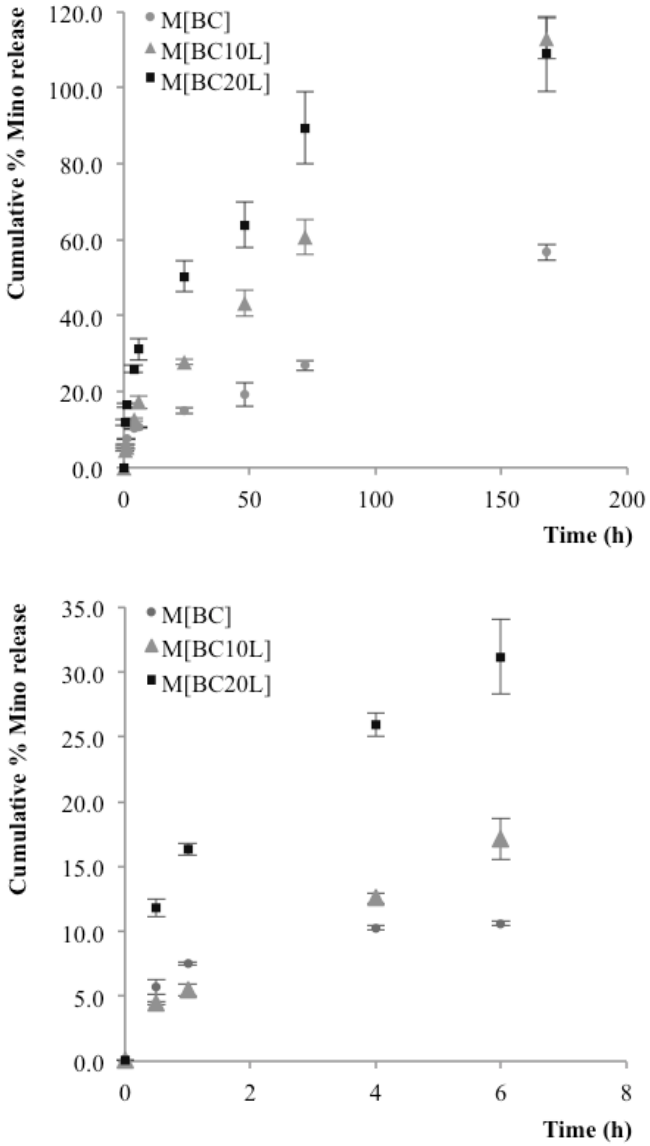



**Fig. 3** *In vitro* release profiles until 168 h, a one-week period time (above). Zoom of the *in vitro* release profile until 6h (below). Markers and error bars represent mean±SD (n=3).

The release kinetic mechanism was fitted with the experimental data to standard equations (Table 2). The best fitting model was the designated “coupled mechanism” described elsewhere for the same type of BC (Frutos et al., 2010). It is noticeable that, for all matrices, there was a faster release in the first 4h (burst effect) with a minocycline release over 10%. The fitted *b* and *c* parameters of the “coupled mechanism” model varied inversely, with the diffusion process (parameter *c*) prevailing over the dissolution process (parameter *b*) for the M[BC20L] (Fig. 4).

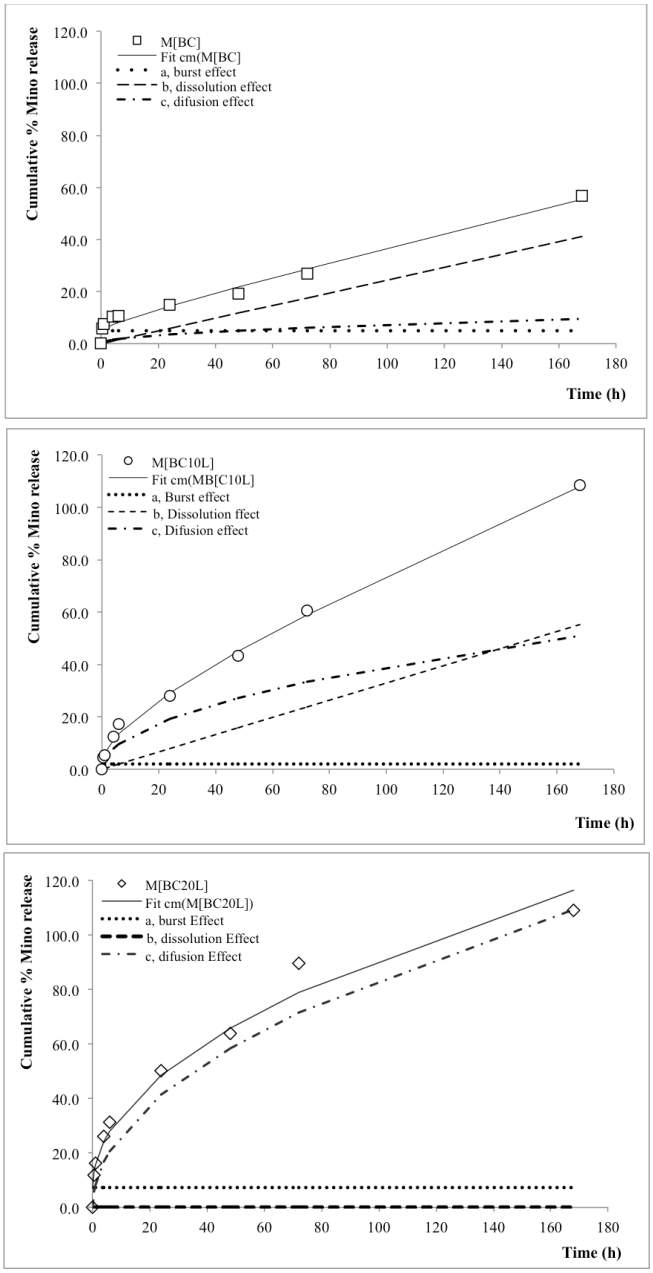



**Fig. 4** Minocycline release fitted curves, with the “coupled mechanism” (Frutos et al., 2010) with each of the parameters of the equation model, *a*, *b* and *c*, being presented.

**Table 2.** Fitted parameter values and $r^2$ for the different equation models used to determine the release mechanism of minocycline from BC matrices.

| Equation Models | Model Parameters | BC Matrix | | |
|---|---|---|---|---|
| | | M[BC] | M[BC10L] | M[BC20L] |
| Zero Order | $k_0$ ($h^{-1}$) | 0.119 | 0.228 | 0.269 |
| $M_t = k_0 t$ | $r^2$ | 0.862 | 0.964 | 0.555 |
| First Order | $k_1$($h^{-1}$) | 0.002 | 0.005 | 0.019 |
| $M_t = 1 - e^{-k_1 t}$ | $r^2$ | 0.974 | 0.992 | 0.969 |
| Higuchi | $k_H$($h^{-1/2}$) | 1.019 | 2.24 | 2.881 |
| $M_t = k_H \sqrt{t}$ | $r^2$ | 0.922 | 0.974 | 0.978 |
| Korsmeyer-Peppas | $k_{KP}$($h^{-1/2}$) | 2.1 | 3.77 | 15.96 |
| | n | 0.6 | 0.7 | 0.4 |
| $M_t = k_{KP} t^n$ | $r^2$ | 0.934 | 0.994 | 0.999 |
| Coupled Mechanism | $k_{cm}$($h^{-1}$) | 7.04E-06 | 5.62E-06 | 5.65E-07 |
| | a | 4.89 | 1.994 | 7.31 |
| $M_t = a + b(1 - e^{-k_{cm} t}) + c\sqrt{t}$ | b | 3.48E+04 | 5.84E+04 | 0 |
| | $c$ ($h^{-1/2}$) | 0.73 | 3.923 | 8.42 |
| | $r^2$ | 0.977 | 0.997 | 0.978 |

$M_t$ denotes fraction of minocycline released up to time $t$; $k_0$, $k_1$, $k_H$, $k_{KP}$ and $k_{cm}$ are constants of the mathematical models; $n$ is the release exponent of the Korsmeyer-Peppas model; $a$ represents the burst effect, $b$ is related with the dissolution process and $c$ with the diffusion process, of the "coupled mechanism" model.

## 3.2. Biomechanical Tests

Fig. 5 presents the biomechanical testing results set for the BC matrices under study. All values were in accordance with the ISO 5833 required values (compressive strength ≥70 MPa, flexural strength ≥50 MPa, and flexural modulus ≥1800 MPa) (ISO 5833, 2002), except for flexural strength of M[BC20L] matrix. All the modified BC matrices showed a compressive strength similar ($p > 0.05$) to that of the plain BC matrix (93.3±1.3 MPa).

Considering flexural strength related to plain BC (56.6±6.3 MPa), all matrices resulted in comparable values (BC10L, 50.0±5.5 MPa; BC20L, 47.1±6.7 MPa; M[BC], 55.0±4.7; M[BC10L], 50.3±4.2 MPa; $p > 0.05$) with exception to M[BC20L] matrix, which besides being statistically different (45.0±4.4 MPa, $p < 0.05$) was also below the required value of 50 MPa.

Also, the obtained values for flexural modulus of the modified BC matrices were not statistically different ($p > 0.05$) from that of the plain BC matrix (3005±51 MPa).

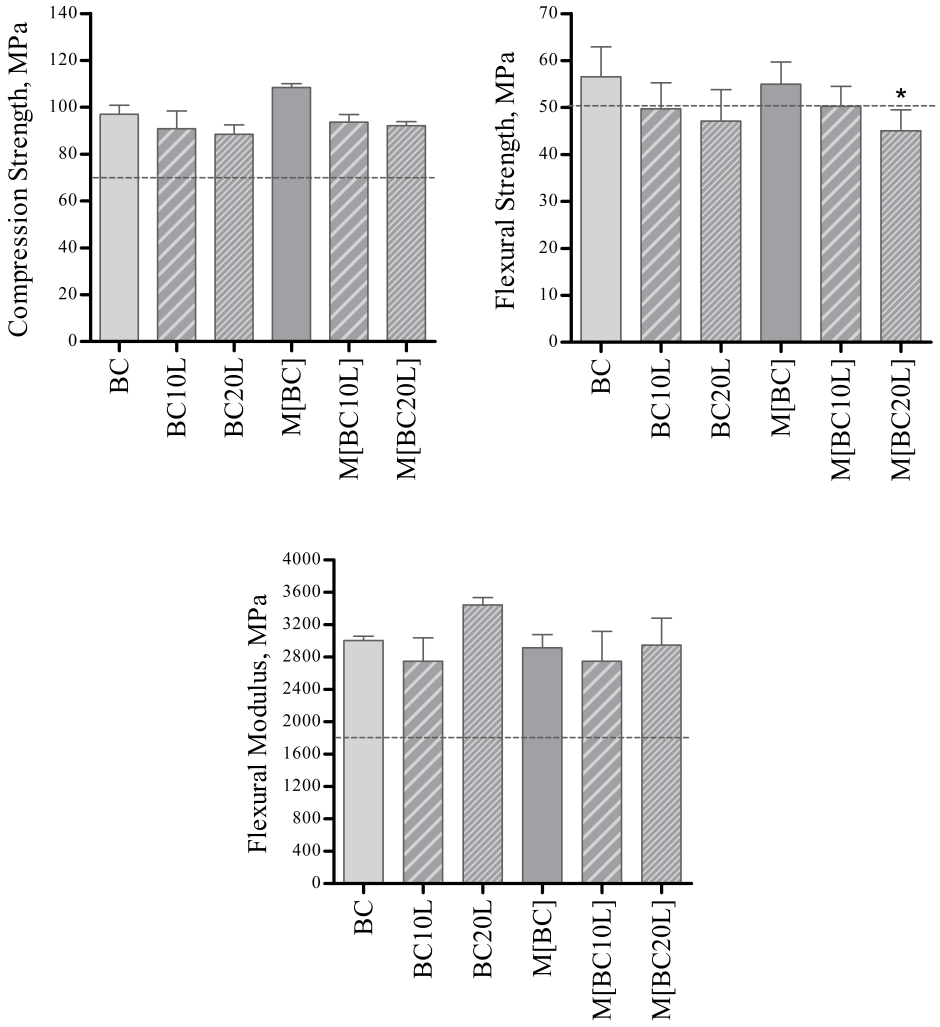



**Fig. 5** Biomechanical properties of the BC matrices under study (mean±SD; n=5). Compressive strength (MPa); Flexural strength (MPa); and Flexural modulus (MPa). *significant difference to BC matrix, $p < 0.05$. Dashed lines represent the ISO 5833 required values for each biomechanical property.

## 3.3. Solid-State Characterization

Results of BC matrices porosity are presented in Table 3. Minocycline or lactose individually loaded into BC increased porosity. When both minocycline and lactose were loaded into BC the porosity increased, achieving a limit of 9.6% for both M[BC10L] and M[BC20L]. The SEM analysis of the BC core matrix, through fracture surface observation, revealed differences in pore size among the matrices studied.

Plain BC specimen presented a smooth surface with a few small voids and cracks, whereas minocycline-loaded BC matrix revealed a growth of the number of pores. As the loaded lactose increased, those voids and cracks also increased, either in number as in size, resulting in a more rugged surface (Fig. 6).

To get further insight into the effect of either minocycline as lactose in BC structure, surface

energy, DSC, XRD and FT-IR analysis were also performed. Minocycline or lactose loading did not affect the BC surface energy. Fig. 7 shows that there were no significant differences ($p > 0.05$) comparatively to BC matrix in what concerns total surface energy ($\gamma$) as well as its dispersive ($\gamma^d$) and polar components ($\gamma^p$).

**Table 3.** Porosity results for BC matrices (mean±SD; n=3).

| BC Matrix | Porosity (%) |
|---|---|
| BC | 6.1 ± 0.2 |
| M[BC] | 7.3 ± 0.2 |
| BC10L | 8.2 ± 0.4 |
| M[BC10L] | 9.6 ± 0.1 |
| BC20L | 9.1 ± 0.1 |
| M[BC20L] | 9.6 ± 0.8 |

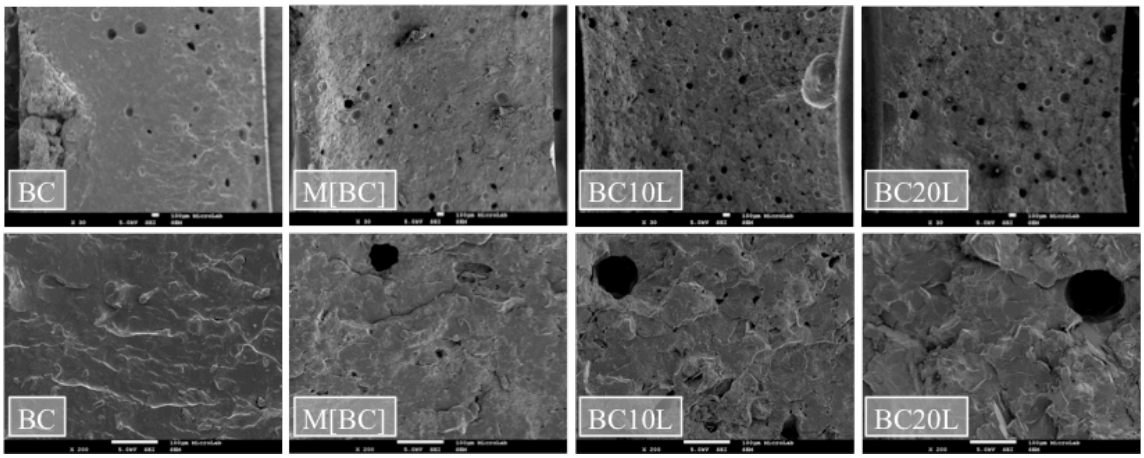



**Fig. 6** FEG-SEM micrographs of the BC matrices rupture surface. First row presents a ×30 magnification of the BC matrices the second row presents a ×200 magnification of the same BC matrices.

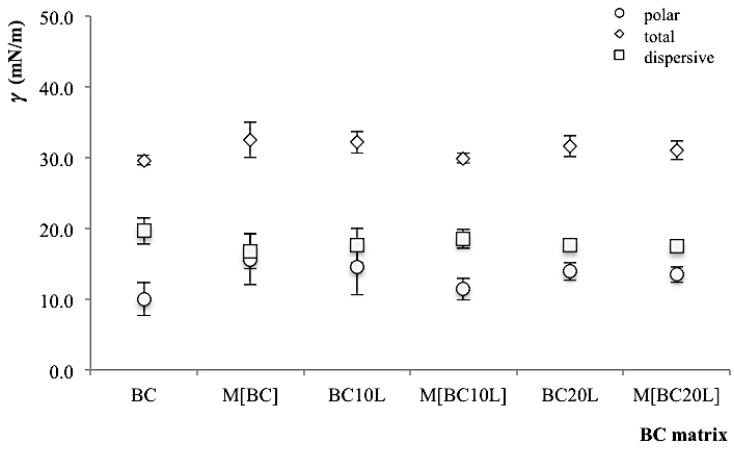



**Fig. 7** BC experimental values for total ($\gamma$±SD; n=3), polar ($\gamma$±SD; n=3) and dispersive ($\gamma^d$±SD; n=3) surface energy (no significant differences found when compared to BC matrix, $p > 0.05$).

Concerning possible modifications of the BC matrices physical state throughout the temperature range achieved during BC cure (≈ 50ºC), DSC thermograms of the BC matrices and of the raw materials were obtained (Fig. 8). Throughout the range of 0-100ºC no thermal events were observable concerning minocycline and lactose loading, besides the glass transition temperature of the BC occurring between 60 – 70ºC (Kühn, 2005). Throughout the range of 0-250ºC, the relevant difference is with the minocycline melting peak, at ca. 190ºC, and the minocycline degradation peak, at ca. 220ºC. The melting peak was not observed for the BC matrix, meaning that no crystalline minocycline was detected. These same observations were noticed for all minocycline–loaded BC matrices (data not shown).

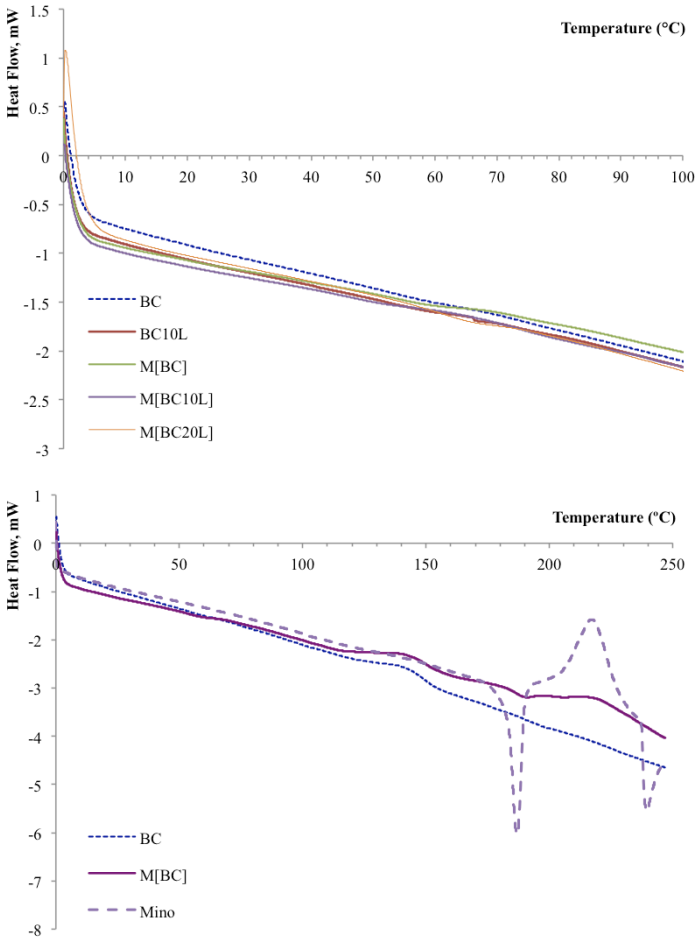



**Fig. 8** Thermograms (DSC) of the BC matrices and raw materials. Above: a thermal cycle 0-100ºC; below: a thermal cycle 0-250ºC.

From the XRD graphs, Fig. 9, it is noticeable that M[BC] matrix graph do not present any peak between 5º and 15º (2θ) which is the range where the most intense peaks of the minocycline hydrochloride crystalline structure appears.

From FTIR spectra observation (Fig. 10) no major differences were observed on spectra profiles

when, either minocycline or lactose, were loaded into BC. Lactose inclusion did not change the BC structure once the profiles of both spectra are fairly superimposable with respect to the main peak absorptions. The same conclusions may be taken with respect to minocycline inclusion on matrices M[BC] and M[BC10L].

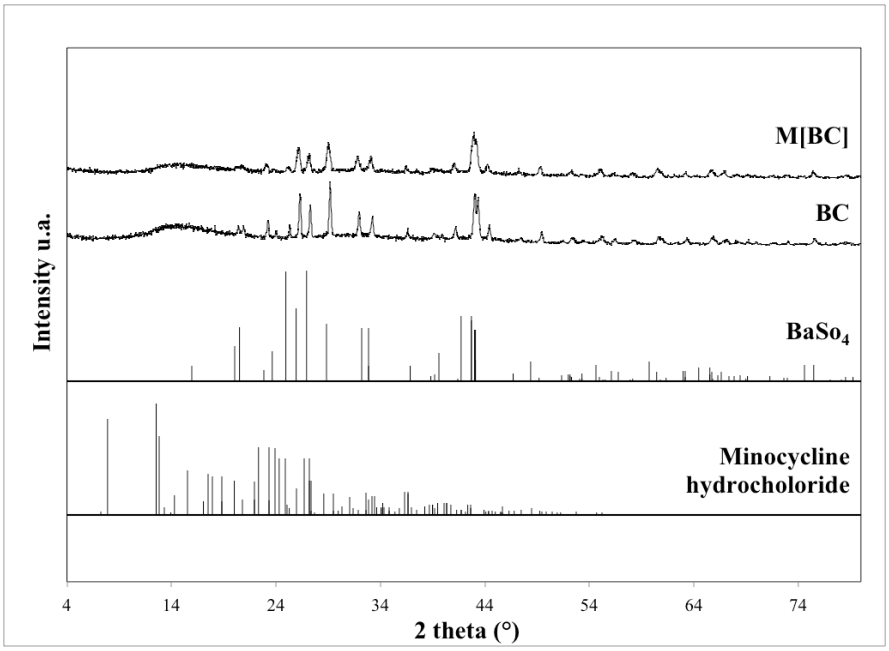



**Fig. 9** XRD patterns for comparison of the minocycline hydrochloride powder, of the radiopacifier used on CMW1 ($BaSO_4$), of the plain BC and of the M[BC] matrices.

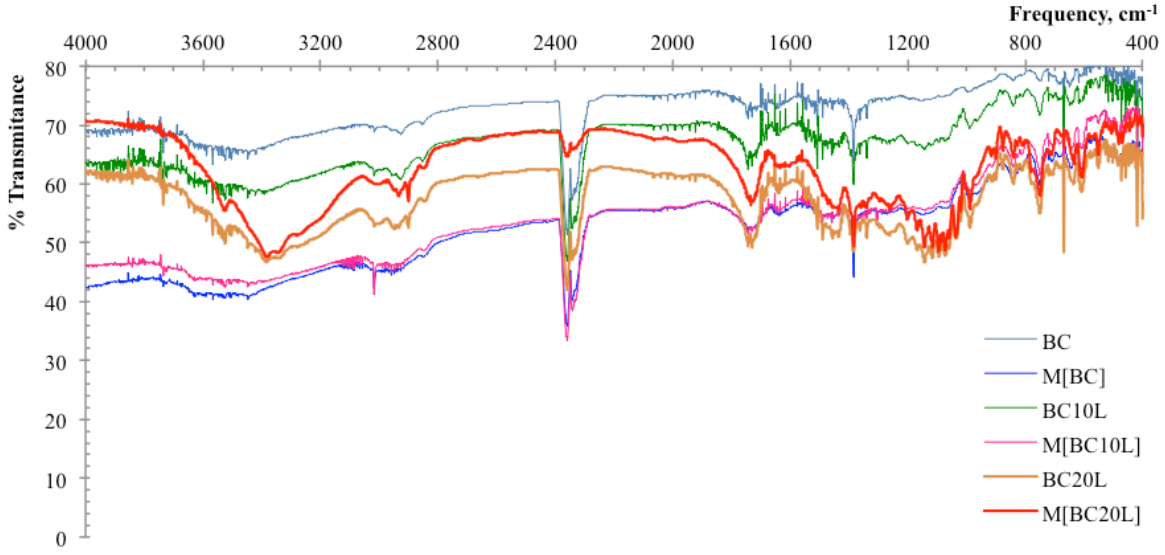



**Fig. 10** FTIR spectra of the different BC matrices, with or without minocycline.

## 3.4. Microbiological assay

Microbiological results, presented on Table 4, shows that MICs values found for a standard minocycline solution, prepared with release medium, are in agreement with literature (Bishburg and Bishburg, 2009). Nor release medium or plain BC matrix showed any antimicrobial effect against the bacterial strain tested. Also noticeable is that, for all the bacterial strains studied and for all the other BC matrices, the MICs values for minocycline released from the BC specimens were lower than those obtained with the standard minocycline solution.

For *S. epidermidis* and *E. faecalis* strains, MICs of minocycline released from BC matrices were eight-fold less (0.125 mg/mL) than MICs of the standard minocycline solution (1.0 mg/mL), with exception to M[BC20L] matrix that was four-fold less (0.25 mg/mL). In the case of MSSA, MICs of minocycline released were four-fold less (0.125 mg/mL) than MICs of standard minocycline solution (0.5 mg/mL), again with exception to M[BC20L] matrix that was two-fold less (0.25 mg/mL). With respect to MRSA strain, MICs of minocycline released from M[BC] was four-fold less (0.5 mg/mL) than MICs of standard minocycline solution (2.0 mg/mL), with exception to M[BC10L] and M[BC20L] matrices that were two-fold less (0.25 mg/mL).

## 3.5. Biocompatibility studies under *in vitro* conditions

The biocompatibility evaluation proceeded with the selected matrix M[BC10L] in what concerns adequate release and biomechanical properties. Results showed that minocycline and lactose loading did not enhance cytotoxicity for the 10 min and the 24h extract solutions (Fig. 11), since no statistical difference to plain BC was found ($p > 0.05$).

Regarding direct contact assay, phase-contrast micrographs were taken to the interface of the matrix cell layer with outer contact areas of the BC matrices (Fig. 12). Only the cells in contact with filter paper containing SDS (positive control) did not grow, all the others presented a monolayer of viable cells.

**Table 4.** MIC of free minocycline and of minocycline release aliquots from BC matrices.

| Bacteria strain | MIC of standard minocycline solution (μg/mL) | MIC of minocycline released from BC (μg/mL) | | | | |
|---|---|---|---|---|---|---|
| | Experimental | Release Medium | BC | M[BC] | M[BC10L] | M[BC20L] |
| MRSA | 2 | >2 | >2 | 0.5 | 1 | 1 |
| MSSA | 0.5 | >2 | >2 | 0.125 | 0.125 | 0.25 |
| *S.epidermidis* | 1 | >2 | >2 | 0.125 | 0.125 | 0.25 |
| *E.faecalis* (Low-level VR) | 1 | >2 | >2 | 0.125 | 0.125 | 0.25 |

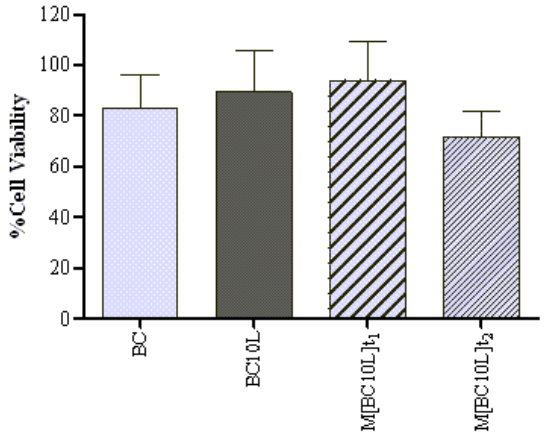



**Fig. 11** Cell viability of BC matrices showed no significant differences towards BC matrix, $p > 0.05$. $t_1$ and $t_2$ represent the release time of 10 min and 24 h, respectively, of minocycline extract solutions. Results are expressed as mean±SD (n=15).

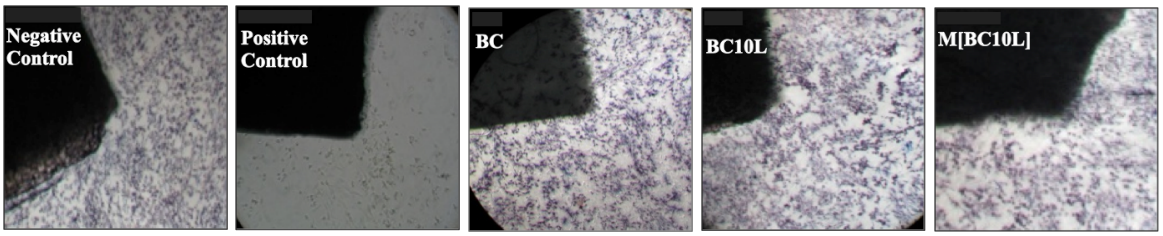



**Fig. 12** Phase-contrast micrographs of the interface of matrix cell layer with outer contact areas of the BC matrices.

# 4. Discussion

The acrylic BC is a long-standing successful solution to deliver antibiotics to patients after a cemented arthroplasty surgery. However, the constant rise of multiresistant pathogens causing complicated infections in orthopaedic surgery turns urgent the need for newer modified acrylic BCs, with novel mechanisms of release and antibacterial activity against responsible pathogens (Jiranek et al., 2006). Thus, this research work aimed at developing and fully characterizing a novel acrylic BC able to deliver, in a controlled manner, an amount of antibiotic above its MIC value, in order to prevent bacterial resistance and, at the same time, without hampering either the functional properties or the way biological tissues react when in contact with the BC. We propose to load a therapeutic concentration of minocycline (2.5% ($w/w_{BC}$)) into two different BC matrices: 1) plain acrylic BC and 2) lactose modified acrylic BC. Minocycline renewed interest application in orthopaedic infections due to the broad-spectrum of activity against MRSA, and its lipophilic affinity to BC matrix were the main reasons for its choice as a suitable antibiotic. Lactose was chosen to increase the porosity of the non-biodegradable monolithic BC matrix due to its well-known application in pharmaceutical formulations.

Minocycline *in vitro* release studies revealed very promising results with a complete drug release from the lactose-loaded BC matrices after a one-week period. This result is clinical significant as a residual presence of the drug after a long-term implantation period will contribute to induce and/or select resistant strains (Neut et al., 2003).

To the best of our knowledge, the release kinetics of minocycline from acrylic BC has not been previously reported. Most studies of antibiotic loaded into different commercial BC, refer to an incomplete release of the drug from the BC matrix, in some cases in very low concentrations and through long periods of time, increasing the risk of resistance development (Anagnostakos and Kelm, 2009; Chang et al, 2011; Lewis, 2008; Van de Belt et al., 2000).

Minocycline release profiles were quite similar among all matrices under study, evidencing a two-phase profile kinetic, with an initial burst followed by a sustained release. The best kinetic fit (Table 2) was the “coupled mechanism” model. Frutos et al. (2010) have already proposed the same pattern of release on their study on gentamicin release from similar BC modified matrices.

For all matrices, the minocycline burst was over 10% in the first 4h, which is a very important advantage, as a high initial concentration of antibiotic can help prevent the risk of infections in the first hours after surgery. This result might be related with the low molecular weight of minocycline (493.94), which tends to enhance the burst release, as reported by Huang *et al.* (Huang and Brazel, 2001). Lactose-loaded matrix doubled the amount of antibiotic released when compared with the lactose-unloaded matrix, which is in agreement with previously reported data (Frutos et al., 2010).

Over the sustained release period diffusion prevailed over the dissolution process only for

M[BC20L] matrix (Table 2). For this matrix, after the initial burst, the release was mainly controlled by diffusion. This might be related with the significant number of pores increase where minocycline promptly dissolves in the release medium and rapidly diffuses through the pathways within the matrix.

The BC microstructure study was of utmost importance to understand the influence of both minocycline and lactose loading on *in vitro* release behaviour. SEM screened possible structural changes of the BC matrices, before release, through observation of the fracture surface. Results evidenced that porosity increased either with minocycline as with lactose (Table 3). These changes could justify the release results once it was visible that small voids and cracks of plain BC specimen slightly increased with minocycline loading. SEM images clearly showed the depth of those cracks, which might have induced the development of a network of interconnected pathways inside the matrix allowing release medium to penetrate more deeply and contributing to complete minocycline release (Fig. 6). Other authors have referred the advantage of lactose loading due to its water-soluble and hydrophilic nature, which allows it to dissolve when in contact with release medium, thus increasing voids and cracks during the release period (Frutos et al., 2010; Tukaram et al., 2010).

However, the increased porosity due to lactose loading (Table 3) became the key issue to mechanical properties as it directly interferes with the inner structure of the BC. From SEM images it was visible a more rugged surface with minocycline and lactose loading. With exception to M[BC20L] matrix, our findings comply with the required biomechanical characteristics established by ISO 5833 for evaluation of acrylic cements for surgery. Loading up to 2.5% of minocycline and up to 10% of lactose to the BC plain matrix revealed no negative effects on BC biomechanical properties allowing an adequate mechanical performance (Fig. 5).

Thus, the M[BC10L] matrix was found as a very promising modified BC matrix, either in terms of *in vitro* drug release as in mechanical performance, but it had yet to be evaluated for minocycline microbiological activity after release from the BC matrices, otherwise those advantages would be meaningless. Microbiological studies were performed with bacteria strains that are major causative agents of the orthopaedic infection, the Gram-positive bacteria, *S. aureus* and *S. epidermidis*. Minocycline has been described to have significant *in vitro* activity against MRSA strain, very similar to that of vancomycin (Bishburg and Bishburg, 2009) with the advantage of no literature reference, to best of our knowledge, of bacteria resistance relating bone infections. In fact, minocycline released from the BC matrix has higher antibacterial activity against all the strains evaluated, including MRSA (Table 4) comparing to the standard minocycline solution.

To better understand this microbiological result it was necessary to investigate the minocycline dispersion in BC matrices. The DSC studies revealed the presence of amorphous minocycline in the BC matrices (Fig. 8). Minocycline powder has a crystalline structure that doesn´t change during heating scanning until reaching 190ºC. At 190ºC minocycline melting happens immediately

followed by degradation at 220ºC (exothermic peak). Since that endothermic peak at 190ºC has disappeared on minocycline-loaded BC matrices thermograms it might be suggested that minocycline acquired an amorphous phase, at least to some extent. It was hypothesized that BC polymerization reaction could have decreased minocycline crystallinity and increased the amorphous phase, which had a higher antibacterial effect than the crystalline minocycline. The increase in minocycline antibacterial activity due to crystallinity changes has also been found by other researchers when studying minocycline release from poly(lactic-co-glycolic acid) (PLGA) nanoparticles (Kashi et al, 2012). With XRD analysis, Fig. 9, it was verified that minocycline hydrochloride powder is a crystalline substance which presents intense peaks between 5° and 15° (2θ). These peaks, however, are not present on the M[BC] matrix XRD graph, rather evidencing a characteristic appearance of an amorphous phase in that range. This suggests that after inclusion on BC, minocycline suffers a decrease in cristallinity and an increase of the amorphous phase. Moreover, DSC analysis confirmed that up to 100ºC there are no other thermal events noteworthy (Fig. 8), only the expected glass transition temperature of the BC, around 70ºC (Kühn, 2005). Furthermore, FTIR results showed that no new bonds were obtained during BC matrix cure. Lactose inclusion did not change the BC structure since both spectra were fairly superimposable. The same conclusions were taken with respect to minocycline matrices M[BC] and M[BC10L].

Biocompatibility is another key aspect to consider regarding the development of any antibiotic-loaded modified BC. Being the first component to be in contact with the cells, the surface characteristics of the biomaterial strongly influence its biocompatibility, for example, the surface energy is related to the hydrophilic degree of the surface, which is responsible for the cells liaison (Menzies and Jones, 2010). Our BC matrices surface energy revealed that biomaterial surface properties did not change (Bettencourt et al., 2004) with minocycline and lactose loading, meaning that biological interaction between BC and biological tissues will not be compromised due to changes in surface characteristics of BC matrix.

Cytotoxicity assays with osteoblasts incubated either with extracts or in direct contact were also performed to get further insight on biomaterial biocompatibility. These assays were conducted with M[BC10L] matrix, which evidenced the best results concerning the release profiles and biomechanical properties. The MG-63 cell line was selected because it has been described as the best osteoblast-like cells for evaluation of acrylic BC *in vitro* biocompatibility (Ciapetti et al., 2002; Granchi et al., 1995). The cytotoxicity assay was performed either with the extracts of the materials or with direct contact in order to evaluate the short-term effect of polymerization by-products. The increase in porosity could have induced a leaching increase of cytotoxic compounds from the polymer matrix, such as residual monomers and polymerization additives (Bettencourt et al., 2000, 2002, 2007; Granchi et al., 1995). However, our study reveals no significant increase of cytotoxicity when the modified matrices were compared with plain BC (Figs.

11 and 12).

We have demonstrated that, globally, the modified BC matrix, M[BC10L] exhibits valuable and consistent properties to be a viable alternative to currently loaded BC, although we are conscious of the limitations of the experimental protocol, regarding the differences between *in vitro* ant the *in vivo* conditions.

Our findings are also valuable regarding the actual preoccupation with the noticeable decline of the available antibiotics pipeline able to fight emerging resistance in serious infections, which, throughout the years has evidenced a decline. For that reason, the use of both new antibiotics tends to be reserved as the last option to ensure an appropriate usage and minimize the risk of resistance. Hence, a responsible selection and use of antibiotics, either old or new, becomes crucial when developing innovative strategies to improve BC, in order to combat the development of resistant bacterial strains in bone infections. This was one of our majors concerns when electing minocycline to load into BC.

## 5. Conclusion

A novel modified acrylic BC matrix prepared with 2.5% ($w/w_{BC}$) of minocycline and 10% of lactose was developed and characterized. This matrix totally delivers the loaded antibiotic in a controlled manner, during a one-week period. It was demonstrated that the biomechanical and structural properties were not affected by the loading of the compounds. Furthermore the antimicrobial activity of the antibiotic and the in vitro biocompatibility of the novel acrylic BC were ensured. Considering the overall results, this matrix appears to be a valuable option to enrich the currently available acrylic BC used in orthopaedic surgery to prevent prosthetic joint infections due to multiresistant pathogens.

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# Section 2

## KEY-PROPERTIES OUTLOOK OF A LEVOFLOXACIN-LOADED ACRYLIC BONE CEMENT WITH IMPROVED ANTIBIOTIC DELIVERY

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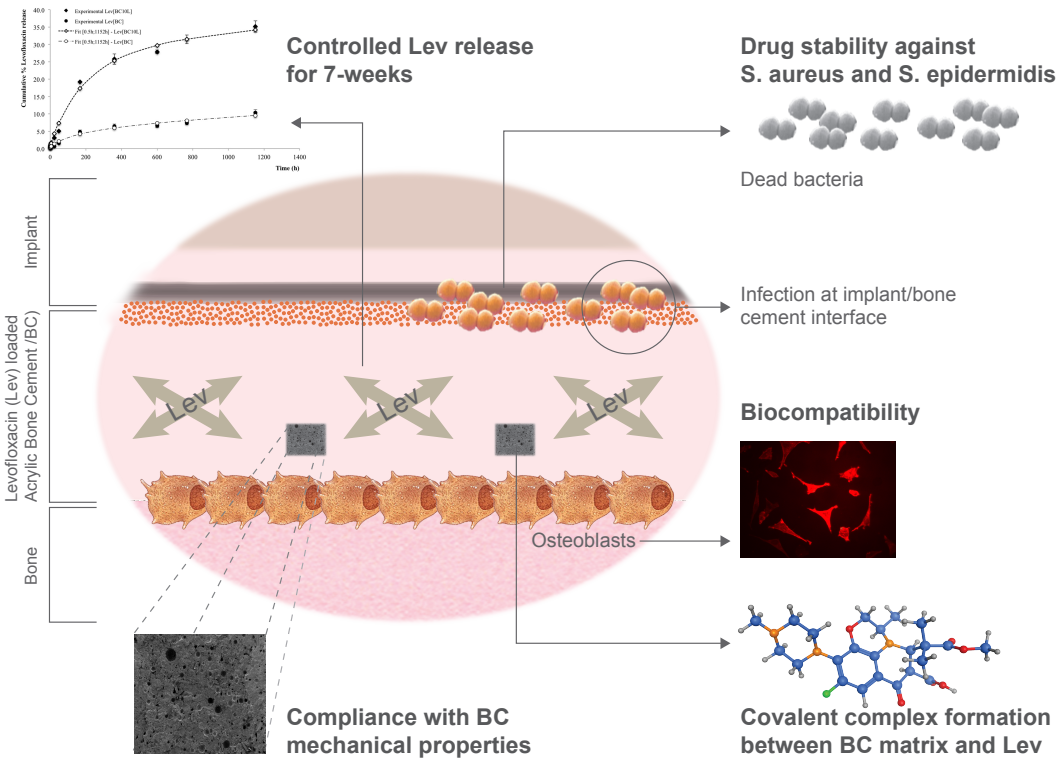
# Graphical Abstract




## Highlights

- Novel lactose-modified acrylic BC with improved antibiotic (levofloxacin) release.
- 7-week release of levofloxacin with antibacterial activity against *Staphylococcus aureus, Staphylococcus epidermidis* and *Escherichia coli*.
- Improved inhibition of *S. aureus* biofilm development by the lactose-modified BC matrix, when compared to the commercial acrylic BC matrix.
- Maintenance of both mechanical integrity and biocompatibility of the modified acrylic BC.
- Favourable covalent and non-covalent interactions between levofloxacin and the BC are evidenced by density functional calculations.

# Abstract

Antibiotic-loaded acrylic bone cements (ALABCs) are widely used to decrease the occurrence of bone infections in cemented arthroplasties and actually being considered as a more cost-effective procedure when compared to cementless implants. However, ALABCs have a major drawback, which is the incomplete release of the antibiotics and, as a result, pathogens that commonly are responsible for those infections are becoming resistant. Consequently, it is of most relevance to find new antibacterial agents to load into BC with an effective mechanism against those microorganisms. This research work intended to load levofloxacin, a fluoroquinolone with anti-staphylococcal activity and adequate penetration into osteoarticular tissues, on lactose-modified commercial bone cement (BC). This modified BC matrix exhibited increased levofloxacin release and delayed *Staphylococcus aureus* biofilm formation. Further insights on material-drug interaction during BC setting were investigated by density functional theory calculations. The obtained results suggested that favourable covalent and non-covalent interactions could be established between levofloxacin and the BC. Moreover, BC mechanical and biocompatibility properties were maintained. These features justify the potential of levofloxacin-loaded modified-BC as a valuable approach for local antibiotic delivery in bone infections management.

**Keywords:** poly(methylmethacrylate), fluoroquinolone, Staphylococcus-infection, controlled release, density functional theory.

# 1. Introduction

Bone infections remain a burden as a clinical complication of orthopaedic surgeries. Chemotherapeutic treatment includes the intravenously and oral administration of antibiotics for long periods of time along with local delivery, through the use of antibiotic-loaded acrylic bone cements (ALABCs) (Hendriks et al., 2004; Van de Belt et al., 2001). The advantage of the latter is to maintain a high local drug concentration for an extended period of release without exceeding systemic toxicity. To attain this purpose ALABC has been used in cemented arthroplasties, as a well-established procedure, which, however, presents a major drawback regarding the incomplete and inadequate kinetic release of the drug (Jiranek et al., 2006; Lewis, 2009; Shi et al., 2010). This fact is related to the structural properties of the non-erodible matrix of the bone cement (BC), as the hydrophobicity and low porosity hamper antibiotic diffusion from the BC core and only the adsorbed antibiotic molecules located in the path of the advancing fluid can dissolve, through voids and cracks, and elute from the matrix (Siepmann et al., 2012).

Recent studies favour the use of cemented over cementless implants, as these offer no net advantage while being more costly (Jameson et al., 2015). Therefore, it is important to pursue for improvements in ALABC specially on finding new antibiotics and additives to load and produce matrices able to perform a better antibiotic release, preventing the development of bacterial resistance.

This fact has achieved a worldwide concern regarding the danger of reaching the end of antibiotic pipeline, which triggered a growing tendency to preserve novel antibiotics and use alternative compounds with an effective action mechanism against the causative microorganisms (Cooper and Shlaes, 2011; Nature Editorials, 2013). Thereupon, several studies evidence a continued interest in improving the antibacterial activity of fluorinated quinolones on bone infections. Levofloxacin is being referred as the fluoroquinolone with the greater *in vitro,* and *in vivo,* anti-staphylococcal activity and lower toxicity (Giacometti et al., 2003; Holtom et al., 2000; Landersdorfer et al., 2009; Lima et al., 2014; Van Bambeke et al., 2005) besides an adequate penetration into osteoarticular tissues above the minimum inhibitory concentration (MIC) for susceptible pathogens generally encountered in bone and joint infections (Rimmelé et al., 2004). However, and to the best of our knowledge, only Anguita-Alonso *et al.* studied the use of levofloxacin in local drug delivery, as loaded acrylic BC in the form of beads, to be used in bone infections (Anguita-Alonso et al., 2006).

Moreover, when loading antibiotics to ALABC, it is very important to establish the drug-polymer interaction after cement setting, *i.e.*, if it is dispersed, chemically bonded or even both. Particularly, considering that acrylic BCs are a result of an exothermic free radical chain polymerization reaction between preformed polymethylmethacrylate (PMMA) powder and a liquid (containing a PMMA monomer). Reactivity conditions during BC setting may pose the hypothesis

that some free radicals might react with vicinal levofloxacin molecules during BC curing (Gallez and Beghein, 2002). This is an overshowed feature in the published literature with high relevance in the release of the antibiotic and, consequently, on ALABC microbiological activity. With this in mind several computational calculations were performed, using the density functional theory (DFT), to discuss the possibility of a non-covalent interaction or the establishment of a covalent bond between levofloxacin and BC.

Besides the structural evaluation of the drug dispersion on the inner polymeric matrix, and regarding the importance of the ALABC in cemented arthroplasties, which acts as a primary fixation material between bone and the prosthetic element, other important aspects must be considered and evaluated when developing a novel BC matrix, such as the resultant biomechanical properties and biocompatibility (Gallo et al., 2005; Van de Belt et al., 2001).

The present study focuses on developing a novel antibiotic-loaded modified BC matrix with possible application for prosthetic bone infections treatment. Levofloxacin was the chosen antibiotic, and lactose, a biocompatible poragen, was the agent selected to enhance drug release (Frutos et al., 2010; Matos et al., 2014). The selected amounts of levofloxacin and lactose were based on the currently used commercial ALABCs formulations (1 g of antibiotic to 40 g of BC powder) (Jiranek et al., 2006) and on previous studies that assessed different percentages of lactose to load into BC, without hampering the mechanical integrity (Frutos et al., 2010; Matos et al., 2014). The levofloxacin-BC interactions, crucial for ALABC performance were characterized using a DFT study, which is a step forward on the comprehension of the drug release mechanism. Furthermore, studies included the characterization of the key properties for ALABCs development, *e.g.* mechanical performance, drug release kinetics, microbiological activity and biocompatibility.

# 2. Materials and Methods

## 2.1. Materials

Commercial acrylic BC CMW1® Radiopaque (high viscosity BC intended for digital application) was purchased from Depuy Synthes (Portugal). Levofloxacin (Lev, on specimens designation), polysorbate 20 (Tween20®), sodium chloride, lactose monohydrate (L, on specimens designation) and the analytical grade solvents, acetonitrile, triethylamine and ortho-phosphoric acid 85% were commercially purchased. Deionized water was obtained from a Millipore analytical deionization system (F9KN225218).

## 2.2. Preparation of acrylic bone cement specimens

Parallelepiped and cylindrical BC specimens were prepared at room temperature (23±1ºC) and atmospheric pressure, with four different content compositions, referenced hereinafter as BC, BC10L, Lev[BC] and Lev[BC10L] matrices (Table 1). All the matrices maintained the proportion [CMW1® powder]:[Monomer liquid] recommended by the commercial supplier. Levofloxacin and lactose were added over the CMW1® powder. After careful mixing of those components, the monomer was added up to obtain dough with the desired consistency. The obtained BC dough was then manually casted into aluminium moulds according to the ISO 5833 recommendation (International Standard ISO Specification 5833, 2002). Cure proceeded for 1 h at room temperature. All specimens were finished to careful polishing, measured with a digital micrometer (Mitutoyo Digimatic, Painesville, Ohio, USA) with an accuracy of 0.01 mm, and stored in a vacuum desiccator (at 23±1°C for 24±2 h) before use. Cylindrical specimens were used for compressive strength determination and parallelepiped specimens were used for all the other tests and treated accordingly.

**Table 1.** Composition of the loaded BC specimens, expressed as wt.% of CMW1® powder.

| BC Matrix | Levofloxacin (%, w/w) | Lactose (%, w/w) |
|---|---|---|
| BC | 0 | 0 |
| BC10L | 0 | 10.0 |
| Lev[BC] | 2.5 | 0 |
| Lev[BC10L] | 2.5 | 10.0 |

## 2.3. Mechanical Assessment

Tests were performed at room temperature in a servo-hydraulic universal machine (TIRAtest® 2705). The compressive strength, bending modulus and bending strength were assessed

according to ISO 5833 (International Standard ISO Specification 5833, 2002) and are already described elsewhere (Matos et al., 2014). At least five specimens of each BC matrix were tested and results were expressed as mean±SD.

## 2.4. Microstructure analysis

### *Scanning Electron Microscopy*

The fracture surface morphology of parallelepiped BC specimens was adequately prepared (Matos et al., 2014), analysed and photographed through a thermal field emission scanning electron microscopy, FEG-SEM, model JSM7001F (JEOL, Japan) operated at 5 kV.

### *Contact angle and surface energy determination*

Assays were performed according with our teamwork-established procedure using BC matrices samples cut in adequate dimensions (Bettencourt et al., 2002; Matos et al., 2014). Assays were assessed with a Kruss K100 tensiometer (Kruss GMBH, Hamburg, Germany) using the Wilhelmy Plate method by immersing plates into the test liquids, water and 1,2-propanediol, at a speed of 3 mm/min, at 25±0.1°C. Advancing contact angles were used for surface energy ($\gamma$) estimation of the BC matrices, as well as its dispersive ($\gamma^d$) and polar components ($\gamma^p$) based on the harmonic mean method proposed by Wu (Wu, 1971). At least three plates were independently tested. Equations for surface tension estimation (Matos et al., 2014) were solved using the equation handling KRUSS-software program: contact angle measuring system K100 (version 2.05).

## 2.5. *In vitro* drug release studies

*In vitro* levofloxacin release was featured from parallelepiped BC specimens (25 × 10 × 3.3 mm) with ~1.0 g weight, incubated in 10 mL of a solution of NaCl 0.9%(w/V) with 0.05%(V/V) Tween20® (hereinafter release medium) in a shaking water-bath at 37°C. At predetermined timepoints, throughout a 7-week period, 1 mL aliquots of the supernatant were collected and analysed in triplicate. The withdrawn aliquots were then replaced with equal volume of fresh release medium solution and sink conditions were guaranteed during the whole study. Levofloxacin content was determined by HPLC-UV (Shimadzu LC-6A and SPD-6A, Kyoto Japan), using an adjusted method described in literature (*see* Annex; Hart et al., 2010). Briefly, the chromatographic analysis was performed using a 125-4, 5 µm, LiChrosphere® 100 RP-18 (Merck, Darmstadt, Germany) column, a degassed mobile phase of water:acetonitrile and triethylamine (85:15(V/V), 0.6%(V/V)) adjusted to pH 3 using ortho-phosphoric acid, a 1.2 mL/min flow rate and UV detection at 284 nm. All chromatographic separations were carried out at 25ºC.

The dissolution efficiency (DE) parameter was used to evaluate drug release profiles. DE is

defined as the area under the dissolution curve up to a certain time *t*, expressed as a percentage of the area of the rectangle arising from 100% dissolution in the same time and was calculated as follows on Equation (1),

$$DE = \frac{\int_0^t y \times dt}{y_{100} \times t} \times 100\% \qquad \text{Eq. (1)}$$

where *y* is the percentage of dissolved drug at time *t* (Khan and Rhodes, 1975).

Furthermore, the drug release mechanism was determined by fitting the experimental data to the "coupled mechanism" represented by the Equation (2) (Frutos et al., 2010; Matos et al., 2014),

$$M_t = a + b\left(1\text{-}e^{-kt}\right) + c\sqrt{t} \qquad \text{Eq. (2)}$$

where $M_t$ denotes the fraction of drug released up to time *t*, *k* is a constant of the mathematical model, *a*, represents the burst effect on drug release, *b*, is related with the drug dissolution process and *c* with the drug diffusion process.

## 2.6. Computational Study

Calculations were based on the density functional theory (DFT). This theory is dependent on the specific representation of the exchange-correlation functional and several possibilities are presently available. To represent exchange calculations with Becke's three-parameter the hybrid method (B3) was used (Becke, 1992). Correlation has been included using the Lee, Yang and Parr functionals (LYP) (Lee et al., 1988). All DFT calculations were performed using the Gaussian 03 Program Package (Frisch et al., 2004).

Full geometry optimizations were carried out for each molecule and for radicals at the B3LYP/6-31G(d,p) level of theory. The minima identified at this level of theory were confirmed (no imaginary frequencies). Vibrational spectra and (zero-point and thermal corrected) relative energies were computed also at the B3LYP/6-31G(d,p) level of theory (Ditchfield et al., 1971). For each molecule/radical/complex, several initial structures were considered and optimized and will be presented. All the molecules were built and initially minimized using MOE 2013.10 program (Molecular Operating Environment (MOE), 2013).

## 2.7. ATR-FTIR evaluation of the levofloxacin–BC interactions

A Thermo Scientific FTIR Spectrometer (San Jose, USA), Class 1 Laser Product Nicolet 6700, was used to proceed with a Fourier transform infrared spectroscopy (FTIR) attenuated total reflectance (ATR) analysis. To obtain the spectra, an 8 × 8 × 0.2 mm sample of each BC matrix was placed on the ATR diamond crystal, which provided an angle of incidence of 42°. At least four independent samples were analysed and, on each sample, data was collected from four

different locations. After each analysis, the crystal was rinsed with acetone and then dried with a soft tissue. As reference, the background spectrum of air was collected before the acquisition of each sample spectrum. Spectra were recorded with a resolution of 8 $cm^{-1}$, and 32 scans were averaged for each spectrum (scan range 4000–650 $cm^{-1}$). The software used for FTIR data collection was Omnic version 8.1 (Thermo Fisher Scientific Inc.).

## 2.8. Assessment of levofloxacin antimicrobial activity

### *Antimicrobial susceptibility testing*

The antimicrobial susceptibilities of selected strains to levofloxacin standard solution and to 7-week release medium supernatants, were obtained by the Broth Microdilution Method described by the Clinical and Laboratory Standards Institute (CLSI) (Clinical and Laboratory Standards Institute, 2012). All assayed samples were two-fold diluted in Müeller-Hinton broth (Biokar Diagnostics, France) and final levofloxacin concentration ranged from 2 mg/L to 0.015 mg/L.

7-week release medium supernatants from plain BC matrices, BC and BC10L, were also tested for antibacterial activity. *S. aureus* (ATCC®25923), *S. epidermidis* (ATCC®12228) and *Escherichia coli* (ATCC®25922) obtained from American Type Culture Collection (ATCC) were first cultured in Tryptic Soy Agar (TSA) (Liofilchem, Italy) and the inoculum was prepared according to CLSI (CLSI, 2012) in Müeller-Hinton broth. Minimum inhibitory concentration (MIC) results were obtained by measurement of absorbance at 595 nm in a Microplate Multimode Detector (Anthos, Zenyth 3100) after 24 h of incubation at 37ºC. All assays were performed with negative controls (not inoculated media) and positive controls (inoculated media). Assays were carried out in three independent experiments.

### *Biofilm inhibition*

Both planktonic bacterial growth and biofilm inhibition were evaluated on BC, BC10L, Lev[BC] and Lev[BC10L] matrices plates (8 × 8 × 0.2 mm) using the method described by Minelli and colleagues (Minelli et al., 2011) and Kwasny and Opperman (Kwasny and Opperman, 2010) with appropriate adjustments. For biofilm experiments, *S. aureus* (ATCC® 25923) was cultured overnight in Brain Heart Infusion (BHI) medium (Liofilchem, Italy), adjusted to a density of 1.0 McFarland units and then diluted 1:100 (V/V) (to $3\times10^6$ CFU/mL) in BHI with 1% (w/V) of glucose medium. This diluted suspension (1 mL) was used to inoculate each well of the 24 well-microtiter plate containing a previously fixed cement plate. Not inoculated medium (blank) was also used in all assays. After incubation at 37ºC for 24 h, bacterial growth was determined and biofilm quantification was performed. In the absence of growth, media was removed and reserved for eventual levofloxacin quantification, new inoculated media was added to those wells and plates were incubated again in the same conditions.

Bacterial growth was achieved by spectrophotometry at 595 nm (Anthos, Zenyth 3100) and biofilm quantification was performed by the crystal violet (CV) staining method (Kwasny et al., 2010, Minelli et al., 2011, Ribeiro et al, 2014). When using CV method attached cells were washed with PBS, fixed with 96% (V/V) ethanol, stained with 0.1% (w/V) crystal violet solution and washed with deionized water (Ribeiro et al, 2014). After drying, each cement 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 Multimode Detector (Anthos, Zenyth 3100). Levofloxacin concentration, on the day before growth observation, was determined using the already described HPLC-UV method to determine biofilm inhibitory concentration (BIC). Assays were performed in three independent experiments.

## 2.9. Biocompatibility assays

### *Cytotoxicity evaluation of the BC extracts*

Release medium supernatants regarding timepoints of 10 min and 24 h, of the Lev[BC10L] matrix, were tested for cytotoxicity. As controls, release medium supernatants from the plain matrices, BC and BC10L, for the same timepoints, were used. The cytotoxicity was assessed using the general cell viability endpoint MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay on L929 (mouse fibroblast cell line, ATCC® CCL-1™) and MG63 (human osteoblast cell line, ATCC® CRL-1427™) (Mosmann, 1983). The day before the experiment, cells were seeded in sterile flat bottom 96 well tissue culture plates (Greiner, Germany), in RPMI 1640 culture medium (Invitrogen, UK) supplemented with 10% (*V/V*) fetal serum bovine, 100 units of penicillin G (sodium salt) (Invitrogen, UK), 100 $\mu$g of streptomycin sulphate (Invitrogen, UK) and 2 mM L-glutamine (Invitrogen, UK), at a concentration that allow cells to grow exponentially during the time of the assay. All samples to be tested and sodium dodecyl sulphate (SDS, used as positive control) were diluted in culture medium. After 24 h and 48 h, the cell media was removed and replaced with fresh medium and the cell viability was assessed. In brief, the MTT dye solution was then added to each well (stock solution 5 mg/mL in 10 mM phosphate buffer solution at pH 7.4). After 3 h of incubation the media was completely removed and the intracellular formazan crystals were solubilised and extracted with 100 μl dimethylsulfoxide. After 15 min at room temperature the absorbance was measured at 570 nm in Microplate Reader (FLUOstar Omega, BMGLabtech, Germany). The relative cell viability (%) compared to control cells was calculated by $[Absorbance_{570nm}]_{sample}/[Absorbance_{570nm}]_{control} \times 100$.

### *Direct contact with BC*

The response of cells (MG63 and L929) in direct contact with BC, BC10L and Lev[BC10L] matrices was evaluated by the observation of the cell culture proliferation by phase contrast after MTT reduction and by fluorescence microscopy.

### *Phase-contrast microscopy*

Cells were cultured at a density of $2.5\times10^4$ cells/cm$^2$ and $5.0\times10^4$ cells/cm$^2$, respectively, in 24 wells tissue culture plates (Greiner, Germany) in direct contact with the BC matrices. Glass slides were used as negative controls and sterile filter paper with SDS (10 mg/mL) were used as positive controls. After 24 h, MTT solution was added to a final concentration of 0.5 mg/mL and incubated for 3 h at 37ºC. Following incubation, culture plates were observed under an inverted microscope coupled with a digital camera and pictures were taken of the interface of the BC matrices and plate.

### *Fluorescence microscopy*

Cells were grown in sterile glass slides or tested Lev[BC10L] matrix (1 cm$^2$) into 24 wells tissue culture plates (Greiner, Germany) for immunocytochemistry assays. After incubation, cells were rinsed three times with 10 mM 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 (Applichem, Germany). After cell fixation, and for actin staining with rhodamine phalloidin, cells were permeabilized with 0.1% (V/V) Triton x-100 for 4 min and then rinsed the same way as described above. The 6.6 mM phalloidin-TRITC (Life Technologies, UK) solution in 10 mM PBS was added to the cells for 30 min at room temperature. Then, and after cells rinsing, cell slides were mounted in fluorescent mounting medium ProLong® Gold antifade reagent with DAPI (Life Technologies, UK) and 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).

## 2.10. Statistical Analysis

All data sets are presented as mean±SD (result of at least three determinations, depending on the assay) and were examined by one-way analysis of variance (ANOVA) with a post-hoc Tukey´s Multiple Comparison Test using GraphPad PRISM 5® (GraphPad Software, Inc., La Jolla, CA). The level of statistical difference was defined at a $p < 0.05$ level.

# 3. Results

## 3.1. Mechanical Assessment

For all the evaluated mechanical parameters, the levofloxacin modified BC matrices showed no statistical difference to the plain BC matrix (Fig. 1). Experimental data was in accordance with the ISO 5833 recommended values, particularly compressive strength ≥70 MPa, flexural strength ≥50 MPa and flexural modulus ≥1800 MPa (International Standard ISO Specification 5833, 2002).

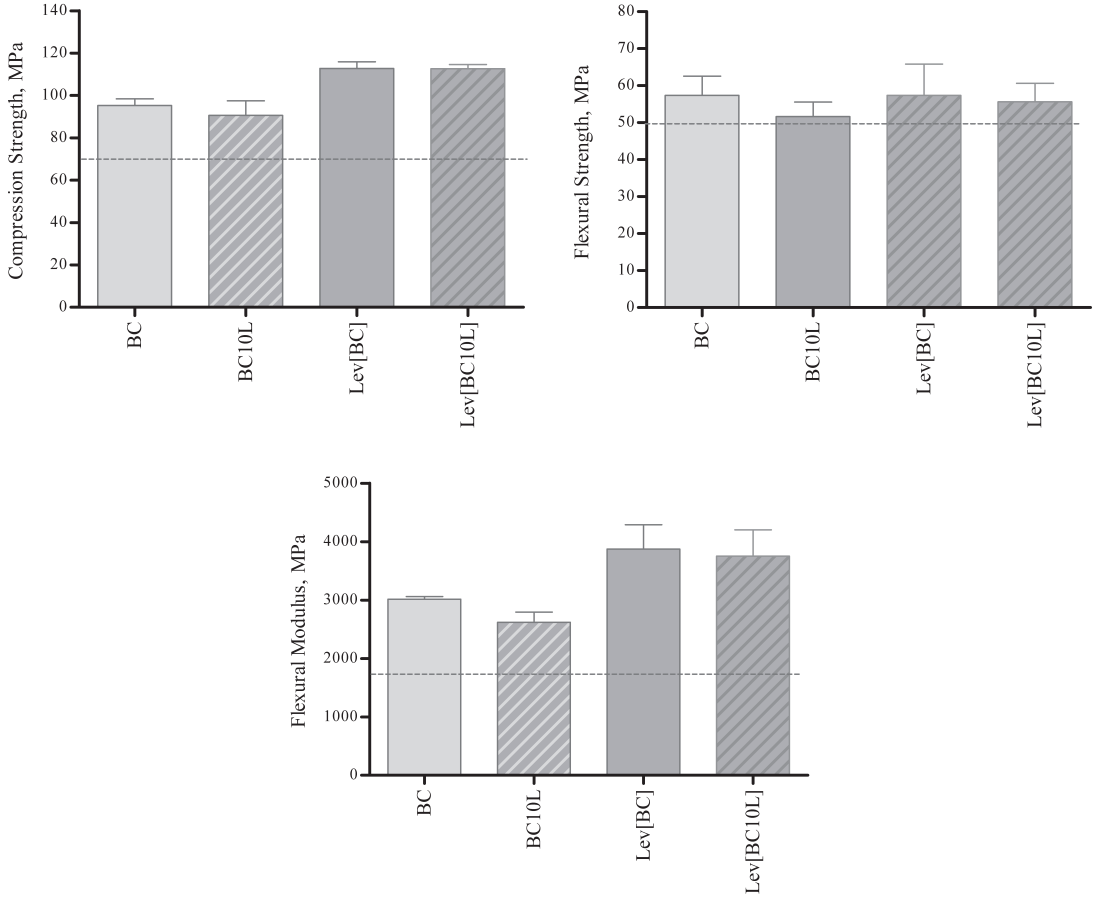



**Fig. 1** Mechanical properties of the BC matrices (mean±SD; n=5). Compressive strength (MPa); Flexural strength (MPa); Flexural modulus (MPa). Dashed lines represent the ISO 5833 recommended values for each mechanical property.

## 3.2. SEM evaluation of the BC inner matrix

Eventual structural changes due to either the loading or the release of levofloxacin and lactose were monitored using SEM analysis.

The BC inner fracture surface matrices revealed a slight increase in the size of cracks and voids with lactose loading, resulting in a rougher surface with visible creation of inner channels in the matrix core (Fig. 2).

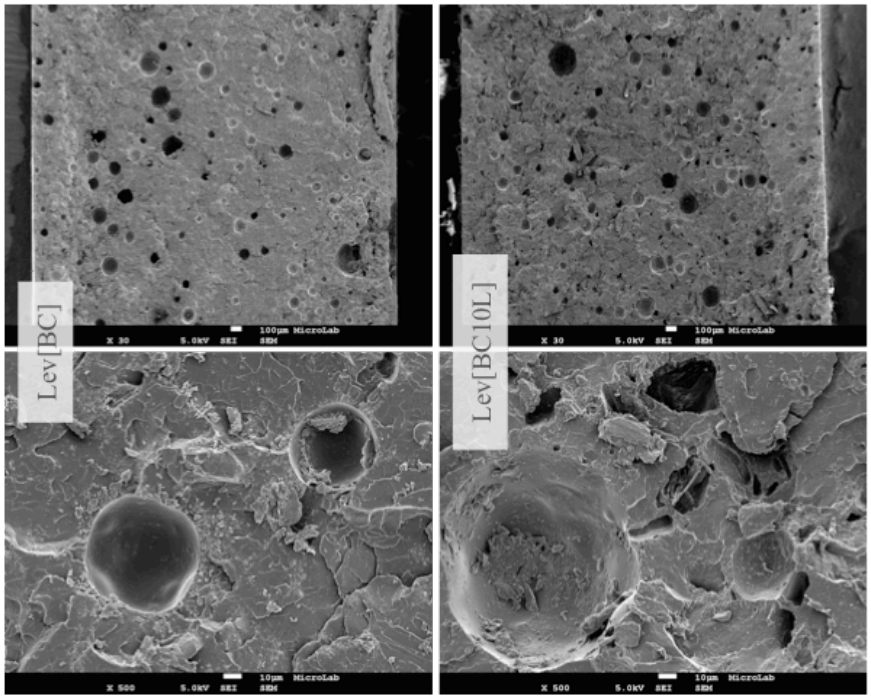



**Fig. 2** FEG-SEM micrographs of the Lev[BC] and Lev[BC10L] matrices rupture surface. Top row shows a (× 30) magnification and bottom row a (× 500) magnification.

## 3.3. Contact angle and surface energy determination

Either levofloxacin or lactose has increased the polar surface energy component ($\gamma^p$) of BC. However, total surface energy ($\gamma$) results did not show any statistical difference ($p > 0.05$) (Table 2) meaning that BC outer surface properties have remained unchanged despite the presence of the loaded components.

**Table 2.** BC experimental values for total *(γ)*, polar *($\gamma^p$)* and dispersive *($\gamma^d$)* surface energy.

| Matrix | $\gamma^d$ (mN/m) | $\gamma^p$ (mN/m) | $\gamma$ (mN/m) |
|---|---|---|---|
| BC | 19.8±1.9 | 9.6±2.7 | 29.4±0.8 |
| BC10L | 18.3±2.4 | 13.2±3.6 | 31.6±1.2 |
| Lev[BC] | 15.1±1.4 | 11.7±2.2 | 26.8±1.6 |
| Lev[BC10L] | 16.0±1.4 | 13.7±1.7 | 29.0±0.7 |

## 3.4. *In vitro* drug release studies

The presence of lactose was crucial for levofloxacin release.

Over 7-weeks drug release was 3.5-fold higher from Lev[BC10L] matrix (35.1±1.7%) when compare to that obtained with Lev[BC] matrix (10.4±0.9%). Both profiles and kinetic fittings are

shown in Fig. 3. In addition, levofloxacin dissolution efficiency (DE) was 4-fold higher for Lev[BC10L] (26.2%) than for Lev[BC] (6.7%).

The release mechanism of levofloxacin from BC matrices was evaluated by fitting the experimental data to the kinetic model known as "coupled mechanism" (Frutos et al., 2010; Matos et al., 2014). Fitted parameters of the Eq. (2) are presented on Table 3.

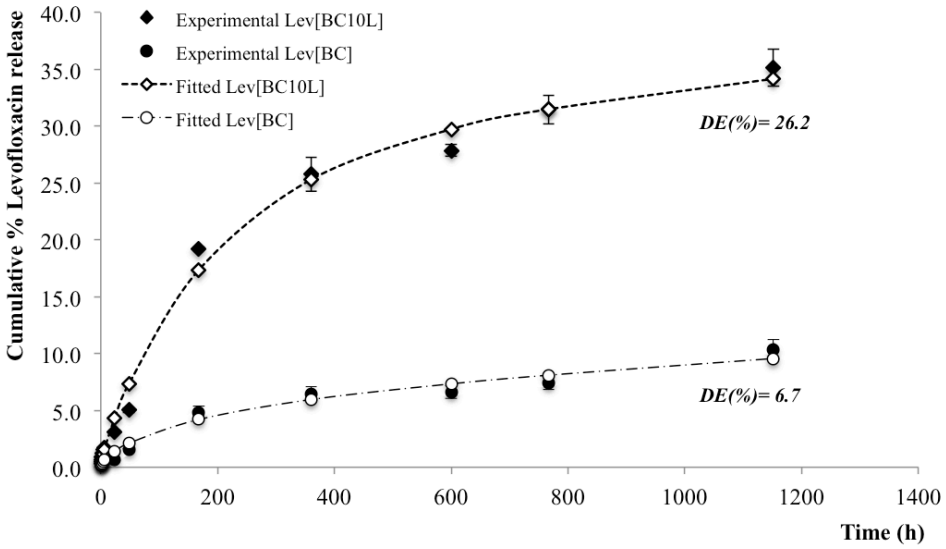



**Fig. 3** *In vitro* release profiles for both Lev[BC] (•) and Lev[BC10L] (•) matrices over a 7-week period (mean±SD; n=9). Dashed lines show the fitting curves obtained from the coupled mechanism kinetic model. *DE* (%) represents dissolution efficiency values.

**Table 3.** Fitted parameter values and $r^2$ for the "coupled mechanism" equation model used to the release kinetic mechanism of levofloxacin from BC matrices.

| Equation Model Parameters | Lev[BC] | | Lev[BC10L] | |
|---|---|---|---|---|
| | 0,5 h ≤ t ≤ 168h | 168 h ≤ t ≤ 1152 h | 0,5 h ≤ t ≤ 168h | 168 h ≤ t ≤ 1152 h |
| $r^2$ | 0.979 | 0.89 | 0.995 | 0.978 |
| a | 0.01 | 1.51 | 0.73 | 10.41 |
| b | 2.00E+04 | 0.00 | 4.2E+04 | 0.00 |
| c ($h^{-1/2}$) | 0.10 | 0.24 | 0.00 | 0.74 |
| $k$($h^{-1}$) | 1.03E-06 | 3.00 | 2.63E-06 | 3.00 |

## 3.5. Computational Study

To explore the possible formation of covalent or non-covalent complexes between levofloxacin and the BC, which could explain the retention of the antibiotic inside the BC, a computational study was performed to investigate the energetics of levofloxacin-BC complexation. With this in

mind, several initial structures representing the PMMA monomer (Fig. 4A), levofloxacin (Fig. 4E), PMMA dimer (Fig. 4C), and respective radicals (Fig. 4B, 4D and 4F) were constructed using MOE software version 2013.10. Also, four potential complexes formed by PMMA monomer and dimer radicals (herein this section referred as PMMAI, to simplify text writing and comprehension) with levofloxacin (covalent and non-covalently bonded) were constructed using the same software (Fig. 4G, 4H, 4I and 4J). All the initial molecular geometries were fully optimized with B3LYP functional using 6-31G(d,p). Fig. 4 presents the minima for each structure and Table 4 the calculated energies of each molecule.

The energies involved on the complexation reactions ($\Delta_{complex}E$) between PMMA and levofloxacin were determined using Equation (3) and are presented at Table 5.

$$\Delta_{complex}E = E_{complex} - (E_{levofloxacin} + E_{PMMAI}) \quad \text{Eq. (3)}$$

where, $\Delta_{complex}E$ represents the complexation energy, $E_{complex}$ represents the corrected energy of the complex (from $E_{th}$ on Table 4), $E_{levofloxacin}$ represents the corrected energy of levofloxacin (from $E_{th}$ on Table 4) and $E_{PMMAI}$ represents the corrected energy of PMMA (from $E_{th}$ on Table 4).

Results show that the covalent complexation between PMMA and levofloxacin is energetically favourable, $\Delta_{complex}E$ = 150 kJ·mol$^{-1}$. Also, the non-covalent complexes optimized showed some interactions between the drug and the BC (PMMA). An hydrogen bond interaction between PMMA carbonyl oxygen and levofloxacin, with $d$(O···H) ~ 2Å, could suggest a favourable interaction. In fact, favourable complexation energy, $\Delta_{complex}E$ ~20 kJ·mol$^{-1}$ was obtained.

The calculated harmonic-vibrational frequencies for the two possible covalent complexes, G and H (from Fig. 4) are presented later on Table 6 along with the frequencies determined by the ATR-FTIR technique.

**Table 4.** Optimized electronic energies, Eele, and also Eo corrected with zero point (Eo+ZPVE) and thermal (Eth+Ther) for molecules of Fig. 4. NI represents the number of imaginary frequencies.

| Molecule | $E_{ele}$ (Hartree) | $E_0$ (Hartree) | $E_{th}$ (Hartree) | NI |
|---|---|---|---|---|
| A | -345.7979235 | -345.674351 | -345.666290 | 0 |
| B | -346.3822638 | -346.249465 | -346.240334 | 0 |
| C | -692.8472849 | -692.572340 | -692.555161 | 0 |
| D | -692.2018062 | -691.940259 | -691.923109 | 0 |
| E | -1262.9893669 | -1262.617780 | -1262.595620 | 0 |
| F | -1263.5275904 | -1263.147351 | -1263.124541 | 0 |
| Complex | | | | |
| G (covalent) | -1609.9776380 | -1609.456265 | -1609.424731 | 0 |
| H (covalent) | -1955.7876487 | -1955.137898 | -1955.097838 | 0 |
| I (non-covalent) | -1608.7975058 | -1608.276133 | -1608.244599 | 0 |
| J (non-covalent) | -1955.8509084 | -1955.202511 | -1955.161445 | 0 |

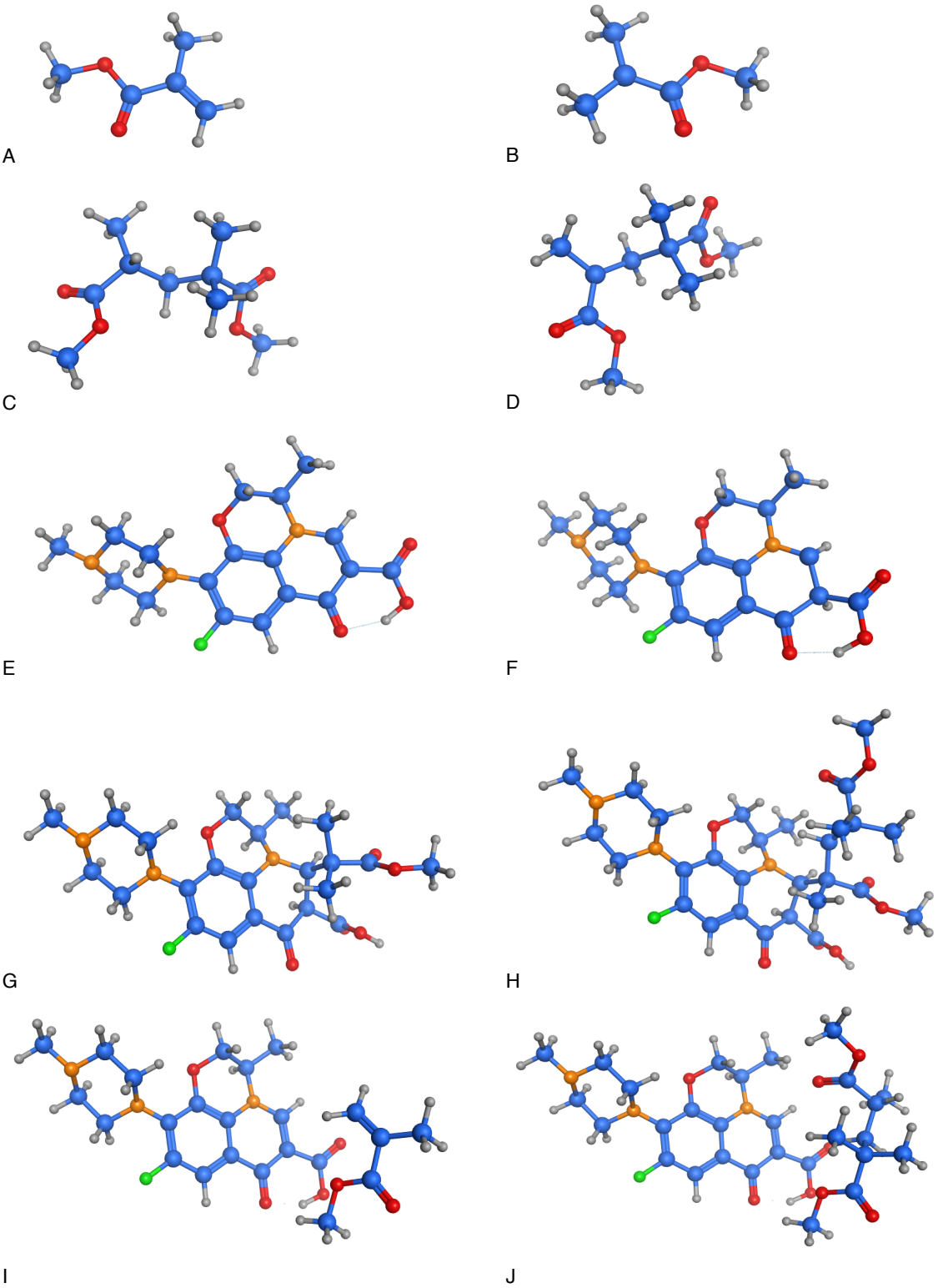



**Fig. 4** Optimised structures of PMMA monomer (A), PMMA dimer (C), levofloxacin (E), and respective radicals (B, D and F), covalently bonded complex of levofloxacin with PMMA monomer (G), and PMMA dimer (H) and non-covalently complexes of levofloxacin with PMMA monomer (I) and PMMA dimer (J).

**Table 5.** Electronic energies, $E_{th}$, of the studied complexes (covalent and non-covalent) calculated and corrected at the B3LYP/6-31G8(d,p) level of theory. $\Delta_{compl}E$ represents the complexation energies.

| Complex | $E_{th}$ (Hartree) | $\Delta_{compl}E$ (kJ·mol$^{-1}$) |
|---|---|---|
| G (covalent) | -1609.424731 | -157.36 |
| H (covalent) | -1955.097838 | -134.34 |
| I (non-covalent) | -1608,264995 | -7.95 |
| J (non-covalent) | -1955.097838 | -28.04 |

## 3.6. ATR-FTIR evaluation of the levofloxacin–BC interactions

The ATR-FTIR spectra for both matrices [BC] and Lev[BC] were obtained to evaluate eventual changes on the inner polymer matrix structure due to levofloxacin presence. For both matrices, the main peak absorptions were observed and were fairly superimposable. Namely, peaks at 2850 cm$^{-1}$ and 2950 cm$^{-1}$ for methylene (-$CH_2$-) and methyl (-$CH_3$) stretches, 1730 cm$^{-1}$ for the C=O stretch, at 1240 cm$^{-1}$ for the C–C–O stretch, at 1140 cm$^{-1}$ for the O–C–C stretch (Ayre et al., 2014). In order to point out slight differences that could exist but not perceived, the spectra of both Lev[BC] and [BC] matrices were subtracted. The obtained subtraction spectrum is presented on Fig. 5 (blue line), where the remained peaks are notorious.

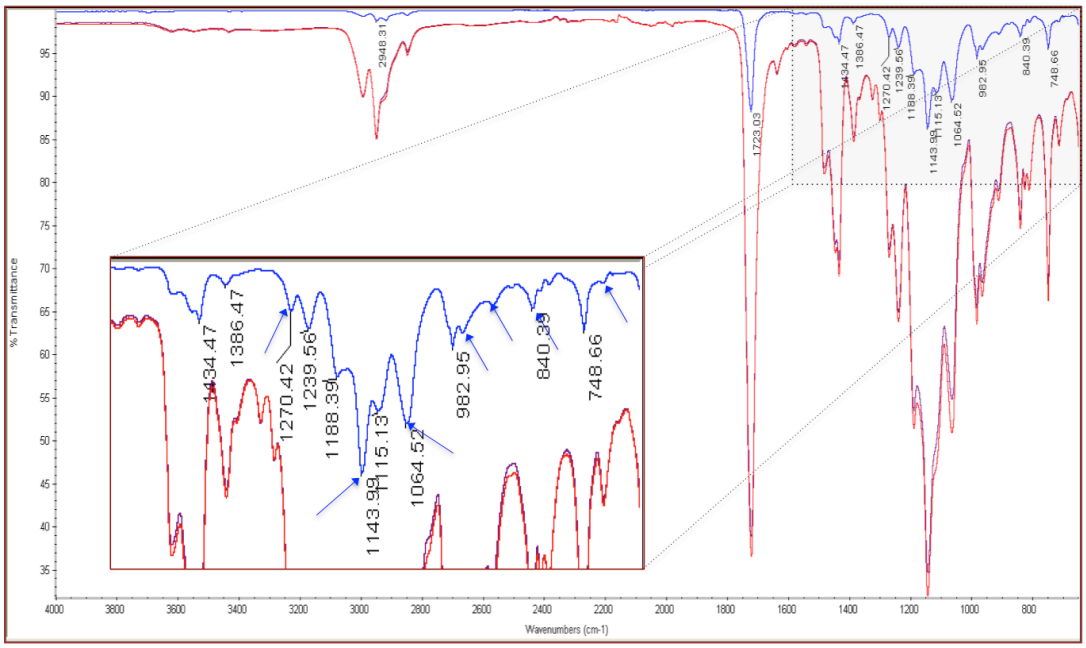



**Fig. 5** ATR-FTIR spectra of both matrices, Lev[BC] (red line) and [BC] (purple line). The blue line represents the resulting subtraction spectra. The zoom window displays the frequencies range 1500-700 cm$^{-1}$. Blue arrows point-out frequencies related to the covalent bond formed during complexation.

Most of these peaks are frequencies of the more characteristic vibrations of levofloxacin: 2950 $cm^{-1}$ and 2830 $cm^{-1}$ for methyl ($CH_3$) stretches, at 1730 $cm^{-1}$ for the C=O stretch, between 1480 $cm^{-1}$ and 1386 $cm^{-1}$ for aromatic ring C–C stretching, scissoring of $CH_2$ and in-plane bending of C–H and O–H, twisting $CH_3$ and in-plane rocking $CH_2$, or at 1270 $cm^{-1}$ for rocking C–H, in-plane bending C–N and ring symmetrical deformation, additionally, between 1300 $cm^{-1}$ and 700 $cm^{-1}$, besides other characteristic peaks of levofloxacin that appear on this interdigital region (Gunasekaran et al., 2003), it is also possible to distinguish peaks coincident with the calculated harmonic frequencies for the covalent bonds formed between levofloxacin and PMMA, as reported on Table 6.

**Table 6.** Infrared vibrational frequencies for levofloxacin (FTIR-ATR) and complexes bond formed between levofloxacin and PMMA (DFT).

| ATR-FTIR ($cm^{-1}$) | DFT frequencies ($cm^{-1}$) | |
|---|---|---|
| | Complex G | Complex H |
| 1270 | 1279 | |
| 1239 | 1217 | |
| 1188 | | 1185 |
| 1144 | | 1133 |
| 1115 | 1077 | |
| 1064 | 1063 | 1071 |
| 983 | 945 | |
| 920 | 939 | 921 |
| 840 | | 839 |
| 748 | 768 | 752 |
| 730 | | 733 |

## 3.7. Assessment of levofloxacin antimicrobial activity

### *Antimicrobial susceptibility testing*

The MIC values obtained represent the lowest concentration of levofloxacin that prevents detectable growth of methicillin-sensitive *S. aureus, S. epidermidis* and *E. coli* (as a control assay). Results for the antimicrobial susceptibility to the levofloxacin released from BC matrices after a 7-week period are shown on Table 7. Levofloxacin free solutions released from BC and BC10L matrices were also tested and no antimicrobial activity was observed (data not shown).

### *Biofilm inhibition*

Concerning *S. aureus* adhesion and attachment to the biomaterial surface, the modified acrylic BC showed a two-fold increase in time needed for biofilm formation as biofilm appeared after 7 days for the Lev[BC] matrix and 13 days for the Lev[BC10L] matrix. At this stage, levofloxacin

concentrations that no longer prevented bacteria growth and biofilm formation were similar in both matrices (Table 7**)**. Moreover, when levofloxacin-free matrices were assayed biofilm formation was observed within the first 24 h.

**Table 7.** Microbiological results, MIC (minimum inhibitory concentration) and BIC (biofilm inhibitory concentration) for the 7-week levofloxacin released from both BC matrices.

| Microorganism Strain | MIC (mg/L) | | | BIC (mg/L) | |
|---|---|---|---|---|---|
| | Levofloxacin | Lev[BC] | Lev[BC10L] | Lev[BC] | Lev[BC10L] |
| *S. aureus* | ≥ 0.25 | ≥ 0.25 | ≥ 0.25 | ≥ 1.84±0.15 | ≥ 1.99±0.07 |
| *S. epidermidis* | ≥ 2 | ≥ 2 | ≥ 2 | n.d | n.d |
| *E. coli* | ≥ 0.03 | ≥ 0.03 | ≥ 0.03 | n.d | n.d |

n.d. – non-determined

## 3.8. Biocompatibility assays

### *Cytotoxicity evaluation of the BC extracts*

The biocompatibility evaluation proceeded with the Lev[BC10L] matrix due to the higher values of levofloxacin released. Results of the 10 min and the 24 h extract solutions showed that levofloxacin released did not cause cytotoxicity, since no statistical difference to plain BC10L was found ($p > 0.05$) (Fig. 6).

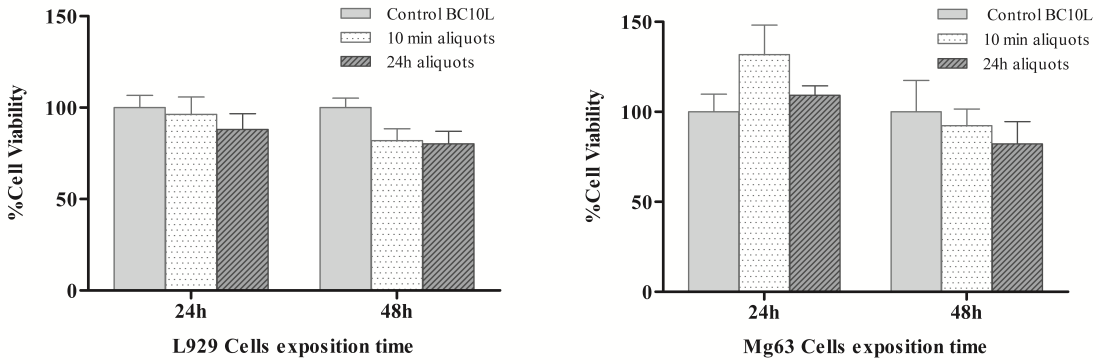



**Fig. 6** Cell viability of the Lev[BC10L] matrix regarding the MG63 and the L929 cells. No significant differences towards BC10L matrix were found, ($p > 0.05$). 10 min and 24 h aliquots represent the release time of levofloxacin extract solutions. Results are expressed as mean ± SD (n = 15).

### *Direct contact with BC*

Regarding direct contact studies, phase-contrast micrographs were taken at the interface of the cell layer with outer contact areas of the BC, BC10L and Lev[BC10L] matrices (Fig. 7). Only the cells in contact with filter paper containing SDS (positive control) did not grow, all the others presented a monolayer of viable cells. Furthermore, the two cell lines, MG63 and L929, grown on

Lev[BC10L] surface and on glass slides were visualized with a fluorescence microscope. The staining of the actin protein was made with fluorescent marks visualised on red channel. The images show the spatial localization of actin on the surfaces (Fig. 8). The fibroblast (L929) and osteoblasts (MG63) fluorescence images show that cells are well spread out on the surface of biomaterial, indicating a good cell adhesion and proliferation. The actin filaments are well highlighted in all cells and evidence the cytoskeleton organization. Therefore, the interaction with the surface of the biomaterial did not alter the osteoblasts and fibroblasts morphology in comparison with the glass surface, thus supporting the material biocompatibility.

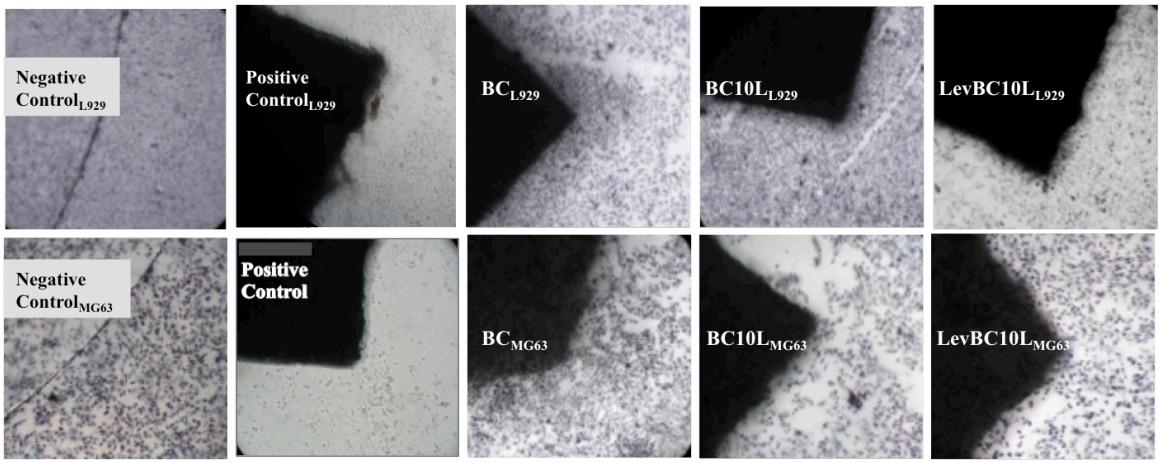



**Fig. 7** Phase-contrast micrographs of the interface of the cell layer, L929 and MG63, with outer contact areas of the Lev[BC10L] matrix.

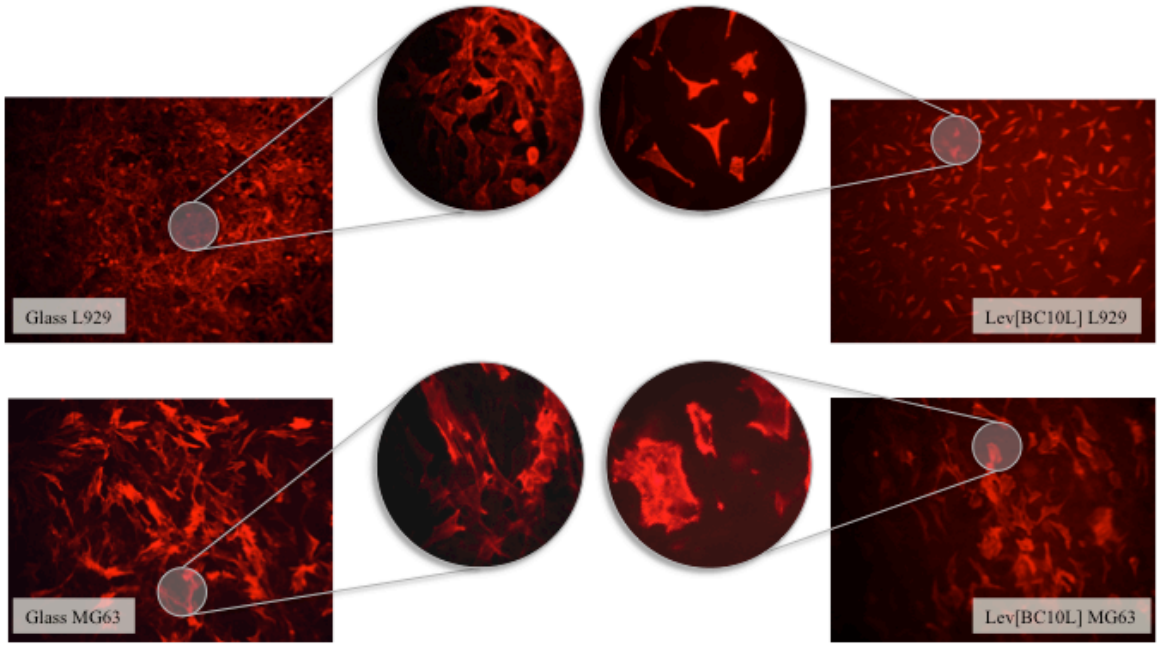



**Fig. 8** Fluorescence images of MG63 and L929 cell lines on glass slides and surface of biomaterial (red staining of actin in cells cultured on the surface after 3 days of culture; ×10 zoomed to ×40 magnification).

# 4. Discussion

The current research on developing innovative materials as drug delivery systems for the prevention and management of orthopaedic infections is unquestionable. Presently, efforts are focusing on developing modified materials by loading suitable additives that either have antibacterial activity, like chitosan and silver nanoparticles (Arora et al., 2013; Tan et al., 2012), as might enhance drug release from the inner BC matrix, as lactose (Frutos et al. 2010; Matos et al., 2014) or xylitol (Salehi et al., 2014; Weiss et al., 2009).

Often these modified materials do not achieve the desirable mechanical performance that BC must exhibit to adequately perform its primary structural function between bone and the prosthetic element (Zhang et al., 2014). For this reason the ALABCs still gathers a general consensus and a recent study has considered cemented implants the procedure with a more suitable cost/effectiveness ratio on orthopaedic surgery such as arthroplasty (Jameson et al., 2015). Therefore, it is of most relevance to find new antibacterial agents to load into BC with an effective mechanism against the microorganisms causing serious bone infection as the *Staphylococci* spp. (*S. aureus* and *S. epidermidis*), which account for more that 50% of the occurrences of periprosthetic hip and joint infection (Ryu and Patel, 2015).

Fluoroquinolones are frequently used in bone infections and show one of the highest median extents of bone penetration of all antibiotic groups partly due to quinolone binding to the calcium in bone (Landersdorfer et al., 2015). Levofloxacin is being referred as the quinolone with the higher values for its group (Metallidis et al., 2007; Rimmelé et al., 2004). Moreover a continued interest for its use in orthopaedic infections was found to be advantageous, since *S. aureus* was shown to penetrate into and survive in osteoblasts *in vitro* (Giacometti et al., 2003; Hart et al., 2010; Holtom et al., 2000; Hudson et al., 1995; Landersdorfer et al., 2009; Lima et al., 2014; Van Bambeke et al., 2005; Wright and Nair, 2010). As such, it was found relevant to load levofloxacin, on a lactose-modified commercial acrylic cement (Matos et al., 2014), to use as prosthesis fixation, and evaluate the overall properties of the resultant BC matrix namely, mechanical, structural, as well as microbiological activity and biocompatibility. To the best of our knowledge, the loading of levofloxacin into commercial acrylic BC, to be used with a load bearing function, has not been assessed or reported, therefore a therapeutic concentration of levofloxacin (2.5% ($w/w_{BC}$)) was loaded into both plain as into 10.0% ($w/w_{BC}$) lactose-loaded BC matrices. The BC mechanical integrity is a key issue due to the important function of the BC to guarantee the implant fixation to bone and load transfer from prosthesis to the bone. Therefore, assessing the mechanical properties of the Lev[BC] and Lev[BC10L] matrices became of highest importance. As a result, and considering the required biomechanical characteristics established by ISO 5833 for evaluation of acrylic cements for surgery (International Standard ISO Specification 5833, 2002), it was concluded that levofloxacin and lactose loading to the commercial plain BC does not interfere with the expected mechanical properties, namely, with the compressive and flexural strength and

modulus (Fig.1). This compliance is highly valuable particularly regarding the flexural strength, which is the more restrictive mechanical property.

The *in vitro* release of levofloxacin held throughout a 7-week time-period, followed a sustained and controlled release either from the Lev[BC10L] as from Lev[BC] (Fig.3). As expected (Frutos et al., 2010; Matos et al., 2014), lactose-loading strongly influenced the amount of levofloxacin released from the BC, which was 3.5-fold higher, ~35%, than levofloxacin released from plain BC matrix, ~10%. This is a very promising value once a different study with BC beads loaded with levofloxacin has reported only 24–28% of the loaded levofloxacin being recovered after 48 h (Anguita-Alonso et al., 2006). Considering kinetic release, from Fig. 3, which evidence the fitting curve of the experimental data, and from Table 3 where fitted parameters of the mathematical model used are presented, it is possible to conclude that levofloxacin release followed the same kinetic mechanism for Lev[BC] and Lev[BC10L] matrices. The burst effect, parameter *a*, was negligible, and the fitted *b* and *c* parameters varied inversely, depending on the time-period of the release study. During the first week (168 h) there was a prevalence of the dissolution process but subsequently, and until the end of the release study, the diffusion process prevailed. The SEM analysis permitted to understand the porosity influence on the *in vitro* levofloxacin release differences between Lev[BC] and Lev[BC10L] matrices. Fig. 2 shows a lower inner porosity for Lev[BC] matrix, therefore, the levofloxacin release started by dissolution of molecules exposed on the BC surface followed by a slowest diffusion from the inner matrix specifically from the voids, pores or imperfections. Lev[BC10L] matrix, with higher pores, due to lactose presence, has revealed a higher capacity to dissolve and diffuse more amount of drug from the inner matrix. During BC setting, lactose acted like a poragen, increasing porosity and creating inner channels paths within the matrix core where later the release medium reached more easily and, initially, promoted levofloxacin dissolution and then permitting its diffusion during the release period (Matos et al., 2014; Tukaram et al., 2010). These suppositions were supported by the *DE* (%) parameter (Khan and Rhodes, 1975). As expected, *DE* increased from 6.7% to 26.2% (Fig. 3) when lactose was loaded in the BC matrix, possibly due to increase in the total surface area exposed to the release medium.

With 35% of the levofloxacin loaded being recovered from the BC inner matrix it became necessary to comprehend what happened to the remainder levofloxacin. During BC setting, the antibiotic molecules become encased in the insoluble polymer matrix and some of the drug molecules might remain isolated or even bonded to the biomaterial, and therefore unable to be released (Landersdorfer etal., 2009). To the best of our knowledge, there are not studies reporting comprehension of these particular interactions, between a commercial PMMA-based BC and the loaded antibiotic that might occur during BC setting. Interestingly, Dizman *et al*. (Dizman et al., 2005) reported the chemical synthesis of methacrylate polymers with another fluoroquinolone (norfloxacin). Although the type of reaction was different from the one observed during BC setting,

it suggests the possibility of reaction between fluoroquinolones and PMMA materials. Therefore, the assessment of the resulting chemical interactions between levofloxacin and BC was of crucial interest to understand the antibiotic interaction with the polymer matrix. For this purpose, a computational study was performed followed by ATR-FTIR analysis. Results suggest that levofloxacin established covalent and non-covalent interactions with PMMA, during polymer setting. The interaction possibilities and the energetics involved on the complexes formation, between levofloxacin and the material (PMMA), have been evaluated by means of density functional theory (DFT) considering two hypotheses. The most stable complexes (Table 4) that resulted from the computational study are presented on Fig. 4. First, during BC setting, it is conceivable that levofloxacin and a free radical (PMMA monomer or other) could interact and form a covalent bond, hindering the antibiotic inside of the cement matrix and inhibiting it from being released, and second, strong interactions could be established between the antibiotic and the cement leading to the formation of a non covalent complex. Covalent (G, H in Fig. 4) and non-covalent complexes (I, J in Fig. 4) might be formed, if a monomer or polymer radical (B, D in Fig. 4) is formed during polymerization, and if a levofloxacin radical (F in Fig. 4) is also present. Both complexation reactions are energetically favourable, with $\Delta_{compl}E < 0$ (Table 5), with the higher interaction energy calculated for the levofloxacin-PMMA covalent complex (150 kJ·mol$^{-1}$) compared to the non-covalent complex (20 kJ·mol$^{-1}$). The formation of important interactions between levofloxacin and PMMA were confirmed by calculations that pose both molecules very close to each other, predicting the establishment of an H-bond between carbonyl oxygen and levofloxacin within a distance of ~2Å. These complexes are most probably responsible for the antibiotic retention inside the BC matrix and consequently release inhibition.

Moreover, the formation of the referred complexes was further confirmed with ATR-FTIR. Fig. 5 shows the obtained spectra for both Lev[BC] and [BC], as well as the resultant subtraction spectrum (blue line), necessary to evidence peaks that were not from the BC matrix. At Fig. 5 a zoom window displays the frequencies range 1500-700 cm$^{-1}$ from which it is possible to locate peaks correspondent to the covalent bond of complexes G and H (Fig. 4) according with Table 6 data, confirming the existence of those complexes. However, one of the limitations of this study relies on the impossibility to assess the stoichiometry of the reaction to better evaluate the amount of levofloxacin involved and better judge whether the remaining 65% of levofloxacin was completely complexed as it was assumed. Nonetheless, the computational study has revealed to be a fundamental tool that should be often applied, to better understand the possible reasons for antibiotic retention inside the BC matrix.

Antimicrobial susceptibility results (Table 7) disclosed that levofloxacin released from the matrices, after a 7-week period, retained the antibacterial properties against *S. aureus*, *S. epidermidis*, in agreement to the MIC values of a levofloxacin standard solution. *E coli* ATCC® 25922 was used as quality control strain to guarantee that the test system performed as expected

and lead to results that fall within specified limits listed in CLSI M100-S17 (Clinical and Laboratory Standards Institute, 2007). The obtained MIC values for *S. aureus, S. epidermidis* and *E. coli* were also in agreement with literature, namely 0.25 – 0.5 mg/L, 0.5 – 7.8 mg/L and 0.008 – 0.06 mg/L respectively (Boyd et al., 2009; Hurst et al., 2002; Lin et al., 2014). These results suggest that the freely dispersed levofloxacin in the inner matrix was not affected during the BC setting or by the acidic release medium (pH 5.4). This is an important result considering that localized acidity occurs after surgery, with the pH value near the implant varying typically from 5.3 to 5.6 (Manivasagam et al., 2010), and these conditions have been described to reduce drastically the potency of antibiotics in its pure form (Uskokovic and Desai, 2014).

As already mentioned, orthopaedic infections are frequently caused by *S. aureus*, and occasionally became very difficult to eradicate due to biofilm formation (Murillo et al., 2006). With this in mind, further microbiological testing was considered specifically for evaluating the Lev[BC10L] effect on the growth of *S. aureus* biofilm. *S. aureus* ATCC® 25923 was chosen for biofilm studies for being referenced by several authors as a strong biofilm producer. Also this strain has been stated to produce biofilm in hostile conditions such as in the presence of $H_2O_2$ (3%) (Zmantar et al., 2010). Additionally, in order to maximize biofilm production, culture medium for biofilm inhibition study was supplemented with glucose (1%). A highly relevant result was obtained: the inclusion of lactose on the BC matrix induced a delay on the biofilm formation, most probably due to the higher levofloxacin concentration achieved on BC surface during release.

Lastly, the biocompatibility evaluation was of utmost importance as the BC is directly connecting with the bone cells. The biomaterial outer surface characteristics strongly influence the biocompatibility behaviour, as the surface energy is related to the hydrophilic degree of the surface, which is responsible for the cells liaison (Menzies and Jones, 2010). Our results of BC matrices surface energy assessment have shown that none of the loaded components has significantly changed the total surface energy and, therefore, the outer surface properties of the BC (Table 2), thus contributing to the biocompatibility of Lev[BC] and Lev[BC10L] matrices. Considering the high levels of levofloxacin released from Lev[BC10L], and in spite of levofloxacin being referred as the fluoroquinolone with lower toxicity (Holtom et al., 2000), cytotoxicity assays with fibroblasts and osteoblasts incubated with extracts or in direct contact were conducted with this matrix. Our study revealed no significant increase of cytotoxicity, regarding the MG63 (osteoblasts) and L929 (fibroblasts), when the modified matrices were compared with plain BC10L (Fig. 6), which means that the complexation bonds formed during polymerization do not contribute to cytoxicity, as do not residual monomers, additives or other polymerization by-products (Bettencourt et al., 2007; 2002; 2000; Matos et al., 2014). Moreover, the observed maintenance of the cytoskeleton organization, of either osteoblasts and fibroblast cell lines, on the surface of the Lev[BC10L] is a strong evidence of this matrix biocompatibility (Figs. 7 and 8).

# 5. Conclusion

Acrylic BC is considered a valuable material for local antibiotic delivery in the context of orthopaedic implant-associated-infections. The comprehensive evaluation performed on the novel BC matrix, Lev[BC10L], showed improved antibiotic release (during a 7-week period) with antibacterial activity against *S. aureus* and *S. epidermidis*. Furthermore, improved inhibition of *S. aureus* biofilm by the lactose-modified BC matrix was observed, when compared to the unloaded commercial acrylic BC matrix. For the first time an original *in silico* approach provided an insight of the drug-biomaterial interaction and demonstrated the existence of both covalent and non-covalent interactions between levofloxacin and BC.

The obtained results clearly show that novel levofloxacin BC matrix is a valuable approach for local antibiotic delivery in bone infections. Hereafter, assessing other doses of levofloxacin and lactose will enrich the withdrawn conclusions.

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# Chapter 4

# NOVEL DOPED CALCIUM PHOSPHATE—PMMA BONE CEMENT COMPOSITES AS LEVOFLOXACIN DELIVERY SYSTEMS

This Section was adapted from the published paper in:


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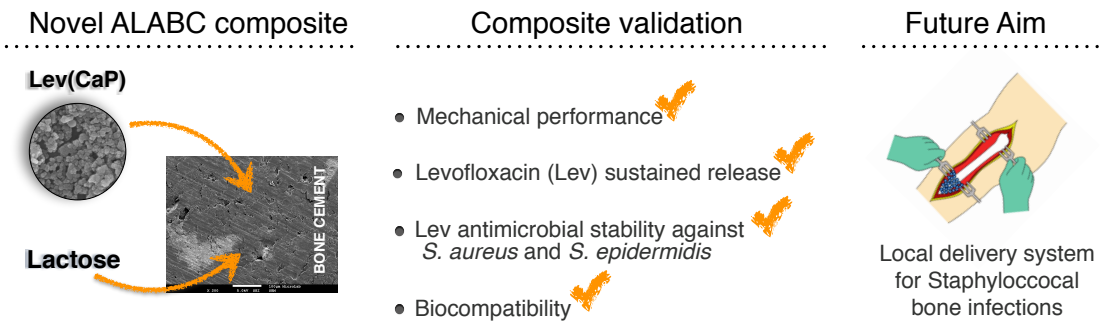
# Graphical Abstract




## Highlights

- Novel BC composites with improved mechanical integrity.
- Sustained *in vitro* release of the antibiotic (levofloxacin) from the BC composites over an 8-week period.
- Levofloxacin retained the antimicrobial activity against *Staphylococcus aureus* and *Staphylococcus epidermidis* over an 8-week *in vitro* release period.
- Biocompatible BC composites.

# Abstract

Antibiotic-loaded acrylic bone cements (ALABCs) are well-established and cost-effective materials to control the occurrence of bone and joint infections. However, the inexistence of alternative antibiotics other than those already commercially available and the poor ability to bind to bone tissue hampering its biological function are still major drawbacks of ALABCs clinical application. The concept of this research work is to develop a novel bone cement (BC) drug delivery system composed by Mg- and Sr-doped calcium phosphate (CaP) particles as drug carriers loaded into a lactose-modified acrylic BC, which, to the best of our knowledge, has never been reported. CaP particles are known to promote bone ingrowth and current research is focused on using these carriers as antibiotic delivery systems for the treatment of bone infections, like osteomyelitis. Levofloxacin is a fluoroquinolone with anti-staphylococcal activity and adequate penetration into osteoarticular tissues and increasingly being recommended to manage bone-related infections. Also, the lactose-modified BC matrix, with a more porous structure, has already proved to enhance antibiotic release from the BC inner matrix. This novel BC composite biomaterial has shown improved mechanical integrity, biocompatibility maintenance, and sustained release of levofloxacin, with concentrations over the minimum inhibitory concentration values after a 48 h while maintaining antibacterial activity over an 8-week period against *Staphyloccocus aureus* and *Staphyloccocus epidermidis*, common pathogens associated with bone infections.

**Keywords:** Poly(methylmethacrylate), Biphasic calcium phosphate, Fluoroquinolone, Controlled release, Osteomyelitis, Biocompatibility, Drug delivery system.

# 1. Introduction

The use of antibiotic-loaded acrylic bone cement (ALABC) is still considered to be the standard of care for patients with chronic bone and joint infection, providing local delivery of high levels of antibiotics for an extended period of time without exceeding systemic toxicity, while being a more cost-effective procedure when compared to cementless implants (Jameson et al., 2015; Zilberman and Elsner, 2008). However these ALABCs present major drawbacks, such as the incomplete and inadequate kinetic release of the drug, the limited number of antibiotics available in commercially premixed formulations (Jiranek et al., 2006; Lewis, 2009; Shi et al., 2010) and the poor bone tissue integration. Regarding the latter, one of the most described strategies to improve the biological performance of ALABCs, which are polymethylmethacrylate (PMMA) based cements, is the inclusion of osteoconductive materials aiming to enhance fixation at bony sites. PMMA-based BCs incorporating calcium phosphate (CaP) ceramics to improve biological fixation between bone and cement have been one of the most recently reported materials in the field (Sa et al., 2015). Additionally, the properties of CaPs are not modified when incorporated in the PMMA matrix, maintaining its ability to promote bone ingrowth while the cement stays mechanically stable (Canul-Chuil et al., 2003; Lopez-Heredia et al., 2012). Therefore, loading CaPs into PMMA-based BC allows establishing a compromise between the desired mechanical and biological properties, combining the vast clinical experience of using PMMA, and the biological potential of CaP materials (Lopez-Heredia et al., 2012). Particularly, hydroxyapatite (HA) is an example of those CaPs due to the chemical and structural similarities with the inorganic phase of human bone, along with its biocompatibility, osteoconductive and osteophilic nature (Kim et al., 2004). More recently, doping of CaPs through the inclusion of foreign ions into the CaPs crystal lattice is described as an effective approach for improving CaPs osteointegration and mechanical properties (Boanini et al., 2010; Lima et al., 2011). Among others, magnesium ($Mg^{2+}$) is a particularly desired substitution ion for HA because it is associated with the mineralization of calcified tissues and indirectly influences mineral metabolism (LeGeros, 1991; Ren et al., 2010). Another interesting substitution ion is strontium ($Sr^{2+}$), which increases osteoclast apoptosis and enhances preosteoblastic cell proliferation and collagen synthesis, with consequent depression in bone resorption and increase bone formation (Guo et al., 2005; Pina et al., 2010).

The current study is intended at exploring important key-properties for the future development of a suitable system for the controlled release of antibiotics, circumventing the above-referred ALABCs handicaps. Hence, a lactose-modified acrylic BC matrix was chosen to favour drug release due to its more porous structure, when compared to plain BC, and proved mechanical and biocompatibility compliance (Frutos et al., 2010; Matos et al., 2014, 2015). The selected antibiotic was levofloxacin, a fluoroquinolone with high anti-staphylococcal activity and low toxicity, besides an adequate penetration into osteoarticular tissues above the minimum inhibitory concentration (MIC) for susceptible pathogens generally present in bone and joint infections (Zimmerli, 2015).

Lastly, CaP particles, plain and doped with two different cations, $Mg^{2+}$ and $Sr^{2+}$, were used as the carriers of levofloxacin to be loaded into the lactose-modified acrylic BC matrix.

In short, a novel BC composite was developed and tested, aiming at an *in vitro* release of the antibiotic, with concentrations above the MIC for the *Staphylococcal* spp., along with mechanical and biocompatibility compliance, for the potential application in bone and joint associated infections.

# 2. Materials and Methods

## 2.1. Materials

Commercial acrylic BC CMW1® Radiopaque (high viscosity BC intended for digital application) was purchased from DePuy Synthes Portugal. Levofloxacin (Lev, on specimens designation), polysorbate 20 (Tween20®), sodium chloride, lactose monohydrate (L, on specimens designation) and the analytical grade solvents, acetonitrile, triethylamine and ortho-phosphoric acid 85% were commercially purchased. Calcium nitrate tetrahydrate ($Ca(NO_3)_2{\cdot}4H_2O$, Quality Chemicals, Spain), diammonium hydrogen phosphate ($(NH_4)_2HPO_4$, Quality Chemicals, Spain), magnesium nitrate hexahydrate ($Mg(NO_3)_2{\cdot}6H_2O$, Sigma-Aldrich), strontium nitrate ($Sr(NO_3)_2$, Sigma-Aldrich) and ammonium hydroxide ($NH_4OH$, Sigma-Aldrich).

## 2.2. CaP particles preparation

Strontium and magnesium doped CaP particles were obtained by aqueous precipitation and then loaded with levofloxacin (Lev, on specimens designation) (Sigma-Aldrich). Briefly, the synthesis was accomplished by the slow addition of $(NH_4)_2HPO_4$ (Quality Chemicals, Spain) solution to a continuously stirred mixed solution of $Ca(NO_3)_2{\cdot}4H_2O$ (Quality Chemicals, Spain) and $Mg(NO_3)_2{\cdot}6H_2O$ or $Sr(NO_3)_2$ (Sigma-Aldrich). For all compositions the precursor's concentrations were designed to achieve a [Ca + (Sr or Mg)]/P molar ratio of 1.67. The concentration of each doping element was fixed at 5 mol% (*i.e.*, 0.95 × 1.67 mol of Ca + 0.05 × 1.67 mol of Sr, or similarly, 0.95 × 1.67 mol of Ca + 0.05 × 1.67 mol of Mg). Pure HA was also prepared and use as a standard control material. The synthesized particles were calcined at 800ºC (Pt30%Rh/Pt6%Rh thermocouple, Thermolab) and dry milled for 30 min in a high energetic ball mill, using a porcelain mill (weight ratio of alumina balls to powder of 3:1). The obtained mean particle size was ~300 nm.

A levofloxacin aqueous solution was prepared with a suitable concentration of ~3 wt.% ($w_{Lev}/w_{Particles}$) to impregnate an estimated amount of 2.5 wt.% of drug in CaP particles. The impregnation process was similar for the three CaP particles and consisted in immersing particles in the Lev solution for 24 h, in the absence of light, followed by freezing particles at –80ºC (Hettich, Germany) for 4 h, and freeze-drying at –51ºC under a pressure of 1.5 Pa (Labconco, USA) for 48 h. According to their compositions, the levofloxacin impregnated powders will be hereinafter referred to as Lev[HA], Lev[Sr-HA] and Lev[Mg-HA]. The non-loaded and levofloxacin loaded particles will be also generically referred to along the text as CaPs and Lev(CaPs), respectively.

## 2.3. Preparation of the BC composite specimens

Commercial acrylic BC CMW1® Radiopaque (DePuy Synthes Portugal), a high viscosity BC intended for digital application, was used to prepare the BC composite specimens. Lactose monohydrate (Merck Millipore, Portugal), L on specimens' designation, was added as the poragen additive. Parallelepiped and cylindrical specimens were obtained according to the ISO specifications (ISO 5833, 2002), at room temperature (23±1ºC) and atmospheric pressure, maintaining the commercial supplier recommended proportion [CMW1® powder]:[Monomer liquid]. Lactose, CaPs and Lev(CaPs) were added over the CMW1® powder. After careful mixing of all components, the monomer was added up to obtain a dough with the desired consistency. The dough was then manually cast into aluminium moulds. Cure proceeded for 1 h at room temperature. All specimens were finished to careful polishing, measured with a digital micrometer (Mitutoyo Digimatic, Painesville, Ohio, USA) with an accuracy of 0.01 mm, and stored in a vacuum desiccator (at 23±1ºC for 24±2 h) before use.

Cylindrical specimens were used for compressive strength determination and *in vitro* release studies and parallelepiped specimens were used for flexural strength and modulus determination. Table 1 presents the acronyms of the different samples.

**Table 1.** Sample codes and composition of the BC composite specimens, expressed as wt.% of CMW1® powder.

| BC Composite | Lactose | CaP particles | Lev(CaP) particles |
|---|---|---|---|
| $BC_L$ | 10.0 | | |
| [HA]$BC_L$ | 10.0 | 2.5 | |
| [Sr-HA]$BC_L$ | 10.0 | 2.5 | |
| [Mg-HA]$BC_L$ | 10.0 | 2.5 | |
| [Lev(HA)]$BC_L$ | 10.0 | | 2.5 |
| [Lev(Sr-HA)]$BC_L$ | 10.0 | | 2.5 |
| [Lev(Mg-HA)]$BC_L$ | 10.0 | | 2.5 |

## 2.4. Mechanical Assessment of the BC composites

Tests were performed at room temperature in a servo-hydraulic universal machine (TIRAtest® 2705). Assay parameters used for the compressive strength, flexural modulus and flexural strength determination were in strictly accordance with ISO 5833 specifications (ISO 5833, 2002). At least five specimens of each BC composite were tested and results were expressed as mean±SD.

## 2.5. BC composites inner structure and outer surface analysis

### *Scanning electron microscopy (SEM) and energy dispersive spectroscopy (EDS) analysis*

The inner structure of the cylindrical BC specimens was analysed and photographed through a thermal field emission scanning electron microscopy, FEG-SEM, model JSM7001F (JEOL, Japan) operated at 5 kV. Samples 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. Analysed specimens were ~1 mm slices of a selected group of representative specimens, obtained using a cut-off machine (Struers Accutom-5®, Struers, Denmark), 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).

### *X-ray diffraction studies*

X-ray diffraction studies of BC composites were performed to evaluate any eventual change in crystallinity due to CaPs particles loading. These studies were carried out using a PANalytical X'Pert Pro, with Cu-Kα radiation ($\lambda$ = 1.541874 Å) produced at 45 kV and 40 mA. Data sets were recorded in the 2θ range of 20–80º with a step size of 0.0260º 2θ/s and the sample *spinning* at 0.4 s.

### *Contact angle and surface energy*

Outer surface evaluation was performed according with our teamwork-established procedure using BC composite samples cut in adequate dimensions (Bettencourt et al., 2002; Matos et al., 2014, 2015). Assays were assessed with a Kruss K100 tensiometer (Kruss GMBH, Hamburg, Germany) using the Wilhelmy Plate method by immersing plates into the test liquids, water and 1,2-propanediol, at a speed of 3 mm.min$^{-1}$, at 25±0.1ºC.

Advancing contact angles were used for surface energy ($\gamma$) estimation of the BC matrices, as well as its dispersive ($\gamma^d$) and polar components ($\gamma^p$) based on the harmonic mean method proposed by Wu (1971). At least three plates were independently tested. Equations for surface tension estimation (Bettencourt et al., 2002; Matos et al., 2014) were solved using the equation handling KRUSS-software program: contact angle measuring system K100 (version 2.05).

## 2.6. *In vitro* release studies

*In vitro* levofloxacin release was featured from cylindrical specimens (⌀ 6 × 12 mm) with ~400 mg weight, incubated in eppendorfs tubes containing a 1:5 (w/V) proportional volume of a release medium with pH 5.4, composed by NaCl 0.9%(w/V) (AppliChem GmbH, Germany) with 0.05%(V/V) Tween20® (Sigma-Aldrich, Spain), in a shaking water-bath at 37°C. At predetermined timepoints, throughout 8 weeks, 250 μL aliquots of the supernatant were collected and analysed in triplicate. The withdrawn aliquots were always replaced with equal volume of fresh release medium and sink conditions were guaranteed during the whole study. At least three independent assays were performed.

Levofloxacin content was determined by HPLC-UV (Shimadzu LC-6A and SPD-6A, Kyoto Japan), using an adjusted method described in literature (*see* Annex, Hart et al., 2010). Briefly, the chromatographic analysis was performed using a 125-4, 5 μm, LiChrosphere® 100 RP-18 (Merck, Darmstadt, Germany) column, a degassed mobile phase of water:acetonitrile and triethylamine (85:15(V/V), 0.6%(V/V)) adjusted to pH 3 using ortho-phosphoric acid (Sigma Aldrich and Panreac Quimica, Spain), a 1.2 mL/min flow rate and UV detection at 284 nm. All chromatographic separations were carried out at 25ºC.

The drug release mechanism was evaluated through experimental data fitting to the "coupled mechanism" represented by the Eq. (1) (Frutos et al., 2010; Matos et al., 2014, 2015),

$$M_t = a + b\left(1-e^{-kt}\right) + c\sqrt{t} \qquad \text{Eq. (1)}$$

where $M_t$ denotes the fraction of drug released up to time $t$, $k$ is a constant of the mathematical model, $a$, represents the burst effect on drug release, $b$, is related with the drug dissolution process and $c$ with the drug diffusion process.

## 2.7. Antimicrobial susceptibility testing

The antimicrobial susceptibilities of selected strains to levofloxacin standard solution and to 8-week release medium supernatants, were obtained by the Broth Microdilution Method described by the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2012). All assayed samples were two-fold diluted in Müeller-Hinton broth (Biokar Diagnostics, France) and final levofloxacin concentration ranged from 2 mg/L to 0.015 mg/L.

8-week release medium supernatants from $BC_L$, [HA]$BC_L$, [Sr-HA]$BC_L$ and [Mg-HA]$BC_L$, were also tested for antibacterial activity. *Staphylococcus aureus* (ATCC®25923), *Staphylococcus epidermidis* (ATCC®12228) and *Escherichia coli* (ATCC®25922) were first cultured in Tryptic Soy Agar (TSA) (Liofilchem, Italy) and the inoculum was prepared according to CLSI (CLSI, 2012) in Müeller-Hinton broth.

MIC results were obtained by measurement of absorbance at 595 nm in a Microplate Multimode

Detector (Anthos, Zenyth 3100) after 24 h of incubation at 37ºC. All assays were performed with negative controls (not inoculated media) and positive controls (inoculated media).

*E coli* ATCC®25922 was used as a quality control strain to guarantee that the test system performed as expected (CLSI, 2007).

Assays were carried out in three independent experiments.

## 2.8. Biocompatibility assays

### *Cytotoxicity evaluation of the BC composites release extracts*

Release medium supernatants collected at 30 min and 24 h, from the [Lev(HA)]$BC_L$, [Lev(Sr-HA)]$BC_L$ and [Lev(Mg-HA)]$BC_L$ in vitro release assays, were tested for cytotoxicity. Release medium supernatants from the correspondent levofloxacin-free composites, were used as controls, for the same time-points. The cytotoxicity was assessed using the general cell viability endpoint MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay on MG63 (human osteoblast cell line, ATCC®CRL-1427™) and L929 (mouse fibroblast cell line, ATCC®CCL-1™) (Mosmann, 1983).

The day before the experiment, cells were seeded in sterile flat bottom 96 well tissue culture plates (Greiner, Germany), in RPMI 1640 culture medium (Invitrogen, UK) supplemented with 10% (V/V) fetal serum bovine, 100 units of penicillin G (sodium salt) (Invitrogen, UK), 100 μg of streptomycin sulphate (Invitrogen, UK) and 2 mM L-glutamine (Invitrogen, UK), at a concentration that allow cells to grow exponentially during the time of the assay. All samples to be tested and sodium dodecyl sulphate (SDS, used as positive control) were diluted in culture medium. After 24 h and 48 h, the cell media was removed and replaced with fresh medium and the cell viability was assessed.

In brief, the MTT dye solution was then added to each well (stock solution 5 mg/mL in 10 mM phosphate buffer solution at pH 7.4). After 3 h of incubation, the media was completely removed and the intracellular formazan crystals were solubilised and extracted with 100 μL dimethylsulfoxide. After 15 min at room temperature, the absorbance was measured at 570 nm in Microplate Reader (FLUOstar Omega, BMGLabtech, Germany). The relative cell viability (%) compared to control cells was calculated by $[Absorbance_{570nm}]_{sample}/[Absorbance_{570nm}]_{control}\times100$. Two independent experiments were performed, each one comprising six replicate cultures.

### *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.

### *Phase-contrast microscopy*

Cells were cultured at a density of $2.5\times10^4$ cells/cm$^2$ and $5.0\times10^4$ cells/cm$^2$, respectively, in 24 wells tissue culture plates (Greiner, Germany) in direct contact with the BC composite matrices. Glass slides were used as negative controls and sterile filter paper with SDS (10 mg/mL) were used as positive controls. After 24 h, MTT solution was added to a final concentration of 0.5 mg/mL and incubated for 3 h at 37ºC. Following incubation, culture plates were observed under an inverted microscope coupled with a digital camera and pictures were taken of the interface between matrices and plate.

### *Fluorescence microscopy*

Cells were grown in sterile glass slides or tested matrices (1 cm$^2$) into 24 wells tissue culture plates (Greiner, Germany) for immunocytochemistry assays. After incubation, cells were rinsed three times with 10 mM 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 (Applichem, Germany). After cell fixation, and for actin staining with rhodamine phalloidin, cells were permeabilized with 0.1% (V/V) Triton X-100 for 4 min and then rinsed the same way as described above. The 6.6 mM phalloidin-TRITC (Life Technologies, UK) 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 (Life Technologies, UK) and 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).

## 2.9. Statistical Analysis

All data sets are presented as mean±SD (result of at least three determinations, depending on the assay) and were examined by one-way analysis of variance (ANOVA) with post-hoc Dunn's, for the contact angle assay, and Tukey's Multiple Comparison Test, to other methods, using GraphPad PRISM 5® (GraphPad Software, Inc., La Jolla, CA). The level of statistical difference was defined at a $p < 0.05$ level.

# 3. Results

## 3.1. Mechanical Assessment of the BC composites

For all the evaluated mechanical parameters, loading the Lev(CaPs) particles did not impair the BC composites mechanical performance. Experimental data was in total agreement with the ISO 5833 recommended values, particularly compressive strength ≥70 MPa, flexural strength ≥50 MPa and flexural modulus ≥1800 MPa (Fig. 1). Furthermore, compressive strength and flexural modulus have significantly increased with CaPs loading, when comparing to plain $BC_L$, with exception to compressive strength of the [Lev(Sr-HA)]$BC_L$ ($p > 0.05$). Regarding flexural strength statistical analysis showed no significant difference ($p > 0.05$).

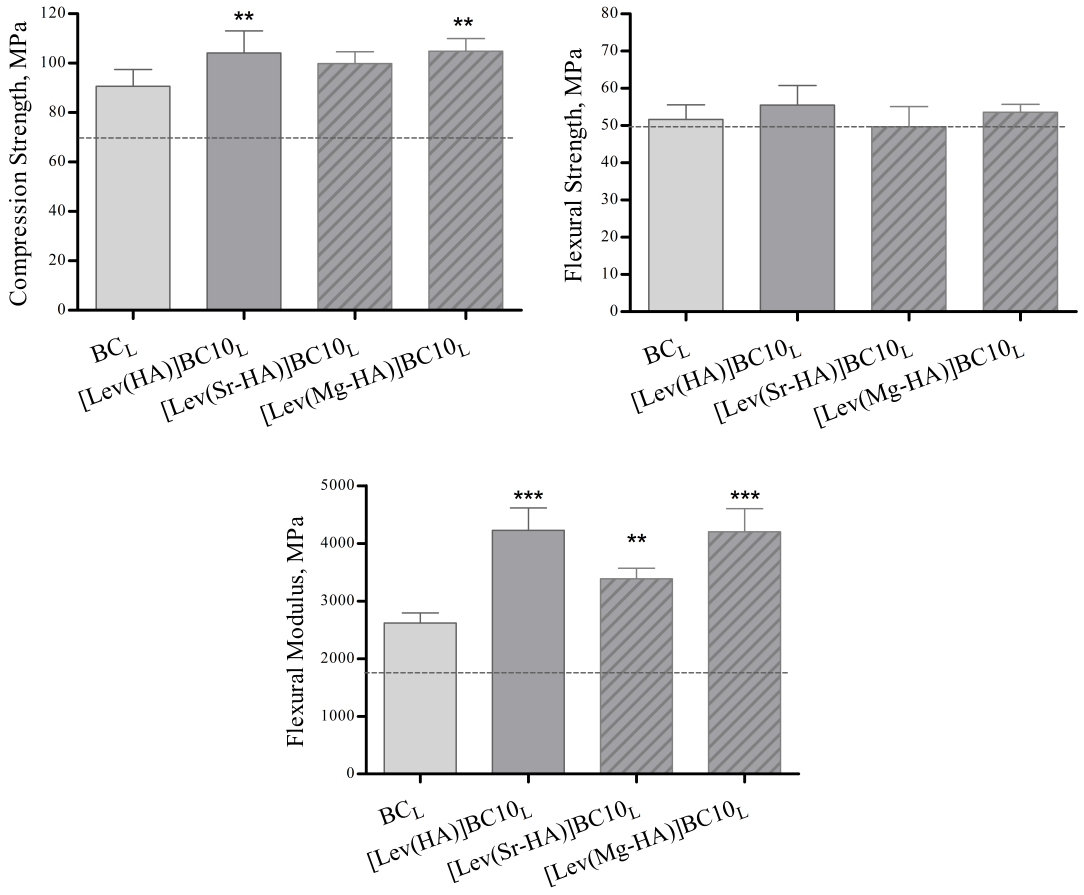



**Fig. 1** Mechanical properties of the BC composites (mean±SD; n=5), compressive strength, flexural strength and flexural modulus. Dashed lines represent the ISO 5833 recommended values for each mechanical property.

## 3.2. BC composite inner structure and outer surface analysis

### *FEG-SEM and EDS analysis of the inner matrix*

Eventual differences in the inner structure between the Lev(CaPs)-loaded $BC_L$ were monitored

using FEG-SEM analysis. Cut-off slices of the compression specimens were used to locate and evaluate the dispersion of the Lev(CaPs) particles on the BC composites matrices, using both FEG-SEM and BSE images (Fig. 2). FEG-SEM images revealed a porous surface with cracks and pores distributed along the matrix core, predicting the existence of inner-channels (upper row of Fig. 2). The stripes evident along the three surfaces are resultant from the specimens cut-off process. Also visible were light-grey spots randomly dispersed in the BC composites matrix. Complementary BSE and EDS analysis attested those spots to be the CaP particles through the acquisition of the elemental chemical composition (middle and bottom rows on Fig. 2, respectively), which confirmed the presence of the characteristics elements of each of the CaPs, used in this study, namely Ca, P, Sr and Mg.

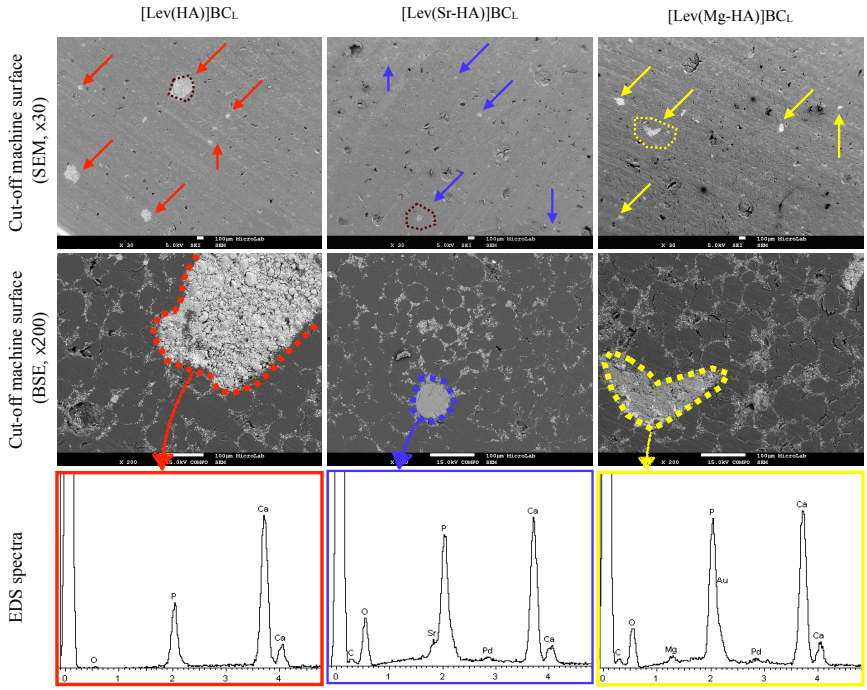



**Fig. 2** FEG-SEM micrographs, with ×30 magnification, of the BC compression specimens cut-off surface (upper-row); BSE images, with ×200 magnification, of the same area but with higher magnification (middle-row); and EDS spectra presenting the elemental composition of each CaPs particles outlined on the previous micrographs (bottom-row).

### *X-ray diffraction studies*

The X-ray diffraction (XRD) patterns of the different compositions displayed in Fig. 3 reveal the presence of all expected major component phases including BC, barium sulphate (04-002-9537) and lactose hydrate (00-030-1716). Although being crystalline, calcium phosphates could not be detected, probably due to the small-added amount (2.5 wt.%) and to the embedding effect of the $BC_L$ matrix. The crystallinity of the $BC_L$ matrix was not affected by the incorporation of CaPs or Lev[CaPs] particles.

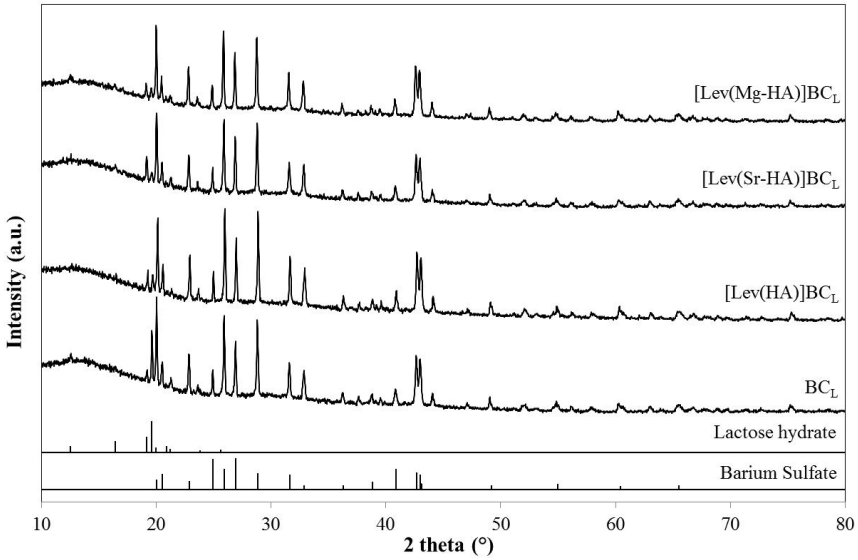



**Fig. 3** XRD patterns for comparison of the BC composites loaded with Lev[CaPs] particles.

### *Contact angle and surface energy*

Regarding the polar ($\gamma^p$) disperse ($\gamma^d$) and total surface energy ($\gamma$) results showed that outer surface properties of [Lev(CaPs)]BC$_L$ composites have remained unchanged when compared to the unloaded BC$_L$ matrix (Figure 2).

**Table 2.** BC composites results for total ($\gamma$), polar ($\gamma^p$) and dispersive ($\gamma^d$) surface energy.

| BC composite | $\gamma$ (mN/m) | $\gamma^p$ (mN/m) | $\gamma^d$ (mN/m) |
|---|---|---|---|
| BC$_L$ | 31.6±1.2 | 13.2±3.6 | 18.3±2.4 |
| [Lev(HA)]BC$_L$ | 28.1±1.5 | 12.0±2.7 | 16.1±1.9 |
| [Lev(Sr-HA)]BC$_L$ | 32.1±0.7 | 14.7±0.8 | 17.5±0.6 |
| [Lev(Mg-HA)]BC$_L$ | 29.3±0.9 | 13.9±0.5 | 15.5±0.9 |

## 3.3. *In vitro* release studies

Release profiles of levofloxacin release over 8-weeks are shown in Fig. 4. All [Lev(CaP)]BC$_L$ composites achieved a maximum levofloxacin release after a 4-week period. In percentage terms all matrices released comparable values of levofloxacin comprised between 4 and 6% (solid markers on Fig. 4). It is noteworthy that after a 6 h period time of release, all composites delivered a levofloxacin concentration above the MIC value for the *S. aureus* (0.25 μg/mL) (Fig. 5). Moreover, after 48 h the concentration of levofloxacin delivered from all composites was also above the MIC value for *S. epidermidis* (1.0 μg/mL) bacteria (Fig. 5). The kinetics of levofloxacin

release from BC composite matrices was evaluated by fitting the experimental data to the coupled-mechanism kinetic model (Frutos et al., 2010). Kinetic fittings of levofloxacin release are shown in Fig. 4 and fitted parameters of the model equation (Eq. (1)) are presented on Table 3. For all composites the dissolution process (parameter *b* in Table 3) prevailed over the diffusion process (parameter *c* in Table 3) and the burst effect was not significant (parameter *a* in Table 3).

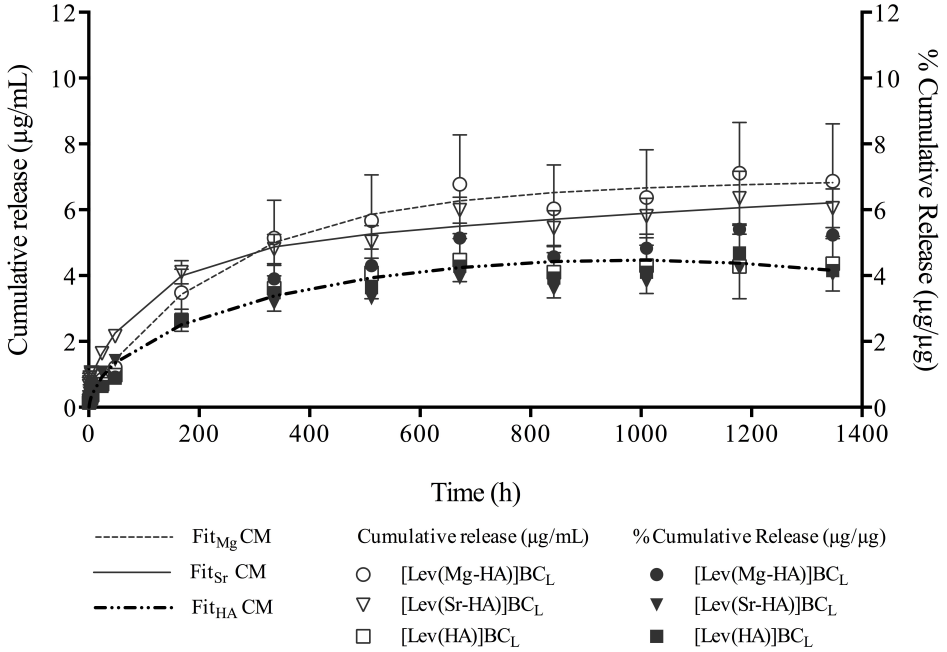



**Fig. 4** *In vitro* release profiles for [Lev(HA)]BC$_L$, [Lev(Sr-HA)]BC$_L$ and [Lev(Mg-HA)]BC$_L$ matrices over a 8-week period (mean±SD; n=9). Dashed curved lines show the fitting curves obtained from the coupled mechanism kinetic model. Dashed straight lines represent the MIC values for *S. aureus* and *S. epidermidis*.

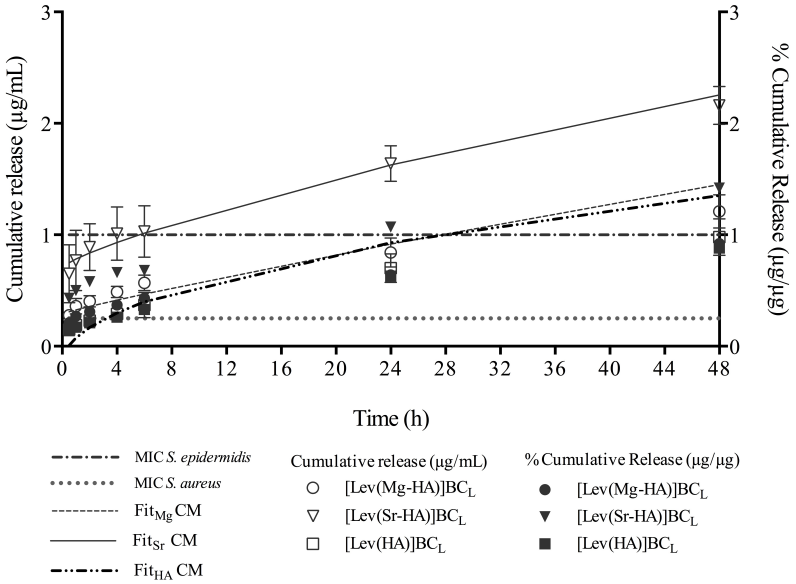



**Fig. 5** Zoom of the time-period [0 h ; 48 h] from Fig. 4, evidencing the time-points to which each matrix delivered an amount of levofloxacin above the MIC values for *S. aureus* and *S. epidermidis.*

**Table 3.** Fitted parameter values and $r^2$ for the coupled mechanism equation model for the release kinetic mechanism characterisation.

| Equation model parameters | [Lev(HA)]$BC_L$ | [Lev(Sr-HA)]$BC_L$ | [Lev(Mg-HA)]$BC_L$ |
|---|---|---|---|
| $k_{cm}(h^{-1})$ | 0.00E+00 | 9.16E-03 | 4.04E-03 |
| a | 0.00 | 0,69 | 0.28 |
| b | 2.49 | 3,13 | 5.94 |
| c ($h^{-1/2}$) | 2.30E-01 | 6,52E-02 | 1.71E-02 |
| $r^2$ | 0.986 | 0,993 | 0.993 |

$M_t$ denotes fraction of drug released up to time t. *k* is the constant of the mathematical model. *a*, represents the burst effect, *b*, is related with the dissolution process, and *c* with the diffusion process of the "coupled mechanism" model.

## 3.4. Antimicrobial susceptibility testing

The MIC values obtained represent the lowest concentration of levofloxacin that prevents detectable growth of methicillin-sensitive *S. aureus, S. epidermidis* and *E. coli* (as a control assay). Results for the antimicrobial activity of the levofloxacin released from BC composite matrices after an 8-week period, was retained against *S. aureus* and *S. epidermidis* (Table 4). Solutions released from plain $BC_L$, (HA)$BC_L$, (Sr-HA)$BC_L$ and (Mg-HA)$BC_L$ matrices were also tested and no antimicrobial activity was observed.

**Table 4.** Microbiological results, MIC, for the 8-week levofloxacin released from BC composite matrices.

| Microorganism strain | MIC (mg/L) | | | |
|---|---|---|---|---|
| | Levofloxacin | [Lev(HA)]$BC_L$ | [Lev(Sr-HA)]$BC_L$ | Lev(Mg-HA)]$BC_L$ |
| *S. aureus* | ≥ 0.25 | ≥ 0.25 | ≥ 0.25 | ≥ 0.25 |
| *S. epidermidis* | ≥ 2 | ≥ 1 | ≥ 1 | ≥ 1 |
| *E. coli* | ≥ 0.03 | ≥ 0.03 | ≥ 0.03 | ≥ 0.03 |

## 3.5. Biocompatibility assays

### *Cytotoxicity evaluation of the BC release extracts*

The biocompatibility evaluation of all three [Lev(CaPs)]$BC_L$ composites proceeded with the 30 min ($t_1$) and the 24 h ($t_2$) extract solutions and showed that levofloxacin released, from any of the three BC composites matrices, did not reduced cell viability by more than 30% and therefore did not cause cytotoxicity (ISO 10993-5, 2009)(Fig. 6) after 24 h or 48 h of cells exposure.

### *Direct contact with BC composites*

Regarding direct contact studies, phase-contrast micrographs were taken to the interface of the cell layer with outer contact areas of all [CaPs]$BC_L$ and [Lev(CaPs)]$BC_L$ composites (Fig. 7).

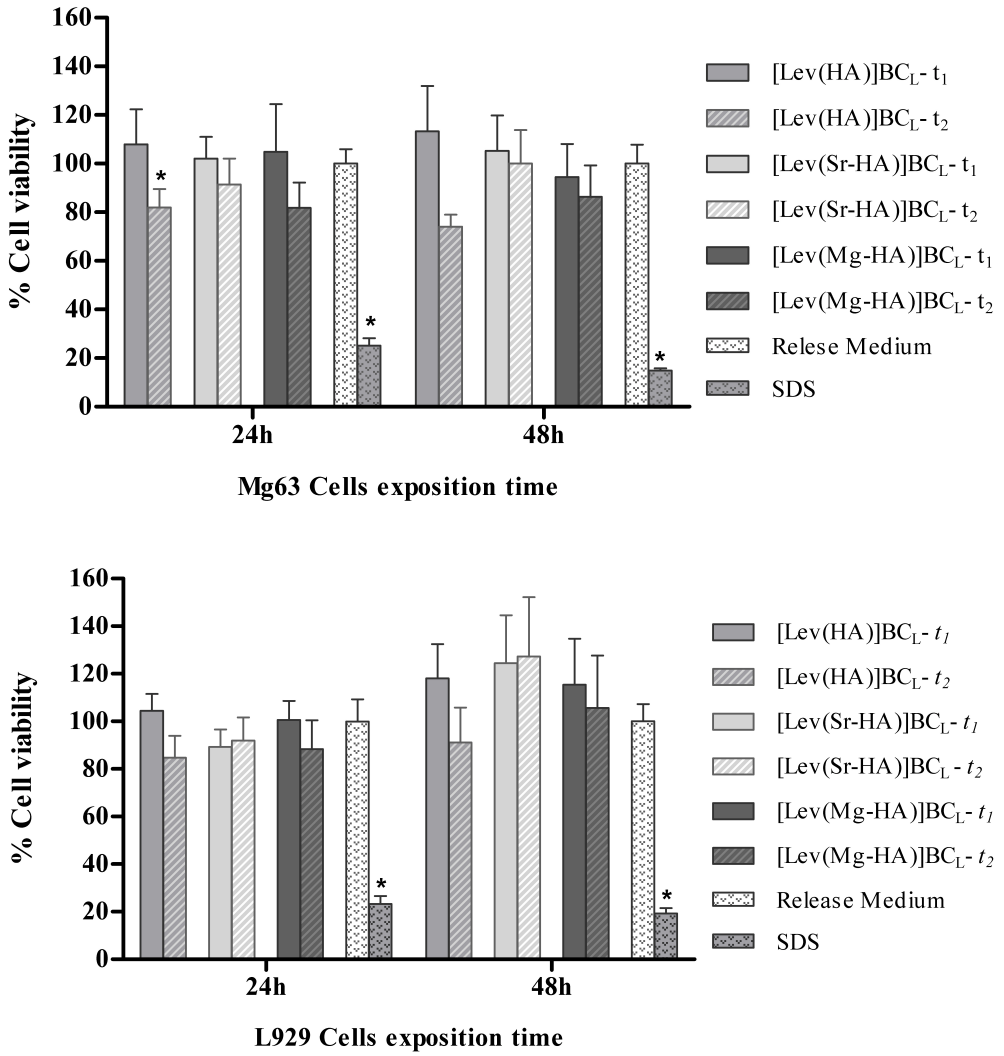



**Fig. 6** Cell viability of the BC composites regarding the MG63 (up) and the L929 (down) cells. ($t_1$) and ($t_2$) represent the release time of levofloxacin extract solutions, 30 min and 24h, respectively (mean±SD;n = 12).

Only the cells in contact with filter paper containing SDS (positive control) did not grow, all the others presented a monolayer of viable cells. Furthermore, the two cell lines, MG63 and L929, grown on all surfaces and on glass slides were visualized with a fluorescence microscope.

The staining of the actin protein was made with fluorescent marks visualised on red channel. Fig. 8 images show the spatial localization of actin on the surfaces for the [Lev(CaPs)]BC$_L$ composites.

The fluorescence images show that cells are well spread out on the surface of biomaterial, indicating a good cell adhesion and proliferation. The actin filaments are well highlighted in all cells and evidence the cytoskeleton organization. Therefore, the interaction with the surface of the biomaterial did not alter the osteoblasts and fibroblasts morphology in comparison with the glass surface, thus supporting the levofloxacin-loaded BC composite biocompatibility.

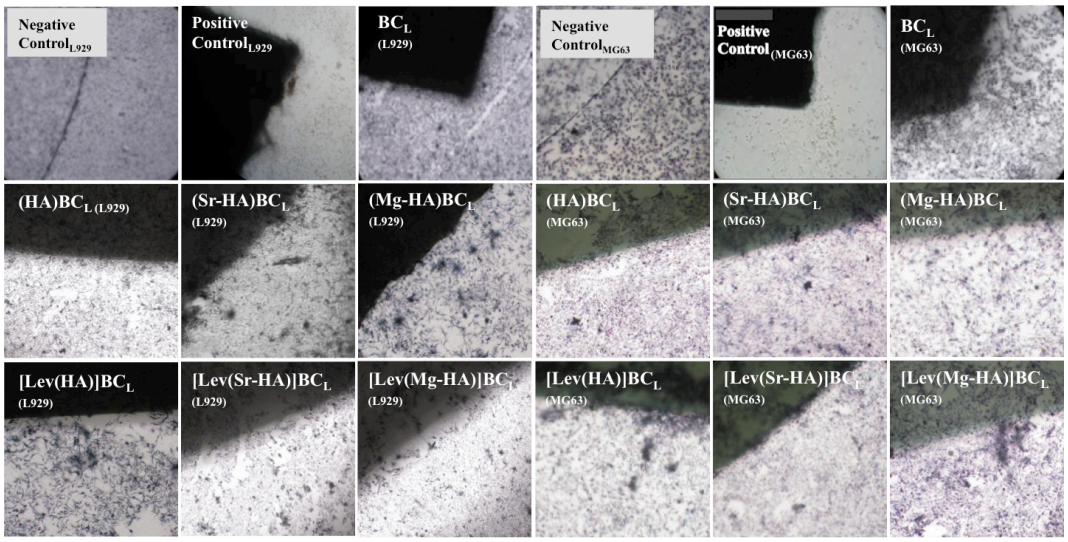



**Fig. 7** Phase-contrast micrographs of the interface of the cell layer, L929 and MG63, with outer contact areas of the different BC composites and controls.

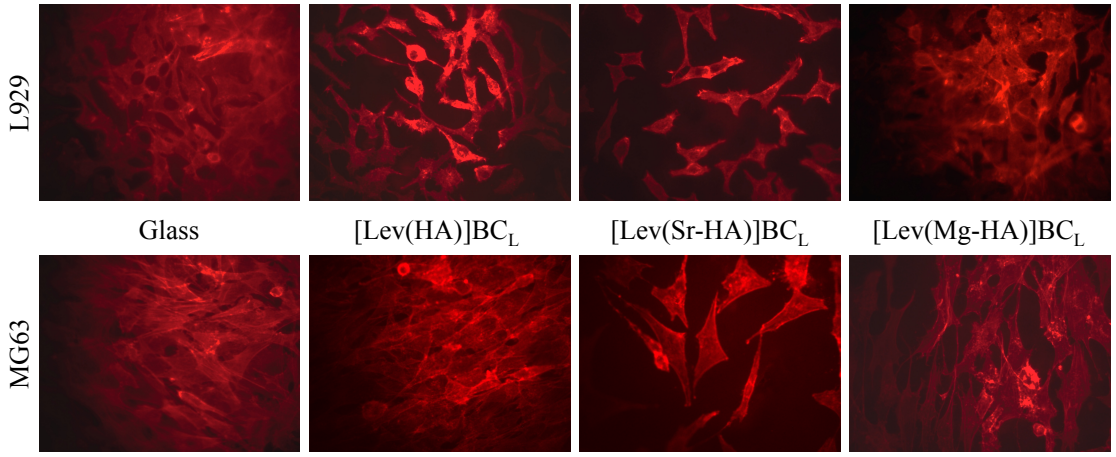



**Fig. 8** Fluorescence images of MG63 and L929 cell lines on glass slides and surface of BC composites (red staining of actin in cells cultured on the surface of the materials; ×40 magnification).

# 4. Discussion

The use of either CaPs or PMMA-based BC as drug delivery systems is well established and comparisons of both systems performance on delivering the same drugs has been often reported (Ginebra et al., 2006; Nandi et al., 2009; Uskokovic', 2015; Uskokovic' and Desai, 2014). Also, the inclusion of polymers into the CaPs structures to delay drug release is being reported (Bohner et al., 2000; Takechi et al., 2002). However, and to the best of our knowledge, the inclusion of antibiotic-loaded CaP particles into acrylic BC has never been reported, being that the explored concept within this research work. The rational was to develop a novel antibiotic delivery system merging the upsides of both the acrylic BC (mechanical resistance and porous structure) and CaPs (osteogenicity and biodegradability) and, simultaneously, obviate their downsides (the inadequate biological performance of the BC and the low mechanical resistance and high resorption rate of CaPs) (Dall'Oca et al., 2014).

Therefore, a new BC composite, containing levofloxacin-adsorbed CaP particles loaded into a lactose-modified acrylic BC, was assessed regarding structure, mechanical performance, biocompatibility, kinetics of levofloxacin release and antimicrobial activity against *Staphylococcal* spp. $Mg^{2+}$ or $Sr^{2+}$ doped CaP particles were used due to the well-known therapeutic effects on the mineralization of calcified tissues (LeGeros, 1991; Ren et al., 2010) and on depressing bone resorption, while maintaining bone formation (Guo et al., 2005; Pina et al., 2010).

On deciding the percentages of each component to add to the commercial BC powder it was taken into consideration the level of the radiopacifier (barium sulphate) that should not be lowered to a value that could compromise the radiopacity of the final BC composite. The CMW1® Radiopaque, contains 9.1% (w/w) barium sulphate, and the reported values on commercial BCs are comprised between 8.0% and 15.0% (Hosseinzadeh et al., 2013). Therefore, the barium sulphate concentration in the composite could not decrease more than 1.0%. Hence, the maximum amount of additives allowed to be added to the CMW1® Radiopaque powder would be 12.5% ($w/w_{BCpowder}$). Previous studies, including our own publications, have already described that 10.0% ($w/w_{BCpowder}$) of lactose loaded into BC significantly improve the amount of antibiotic delivered without hampering the BC mechanical integrity (Frutos et al., 2010; Matos et al., 2014, 2015). So, the amounts chosen to produce the composite were 10.0% ($w/w_{BCpowder}$) of lactose and 2.5% ($w/w_{BCpowder}$) of CaP particles.

The BC composite mechanical properties assessment was of utmost importance due to the crucial function of guaranteeing the implant fixation and load transfer from prosthesis to the bone. Considering that the composite under study is mainly acrylic BC, the mechanical properties evaluation followed the standard ISO 5833 for acrylic cements for surgery (ISO 5833, 2002), to which the obtained results were in fully agreement, namely, the compressive and flexural strength and flexural modulus (Fig.1). This compliance is particularly valuable regarding the flexural strength, the most restrictive mechanical property. Also, these results were in agreement with

other reported studies about CaPs loaded into porous acrylic BC (Dall'Oca et al., 2014; Sa et al., 2015; Zerbajad et al., 2011).

The FEG-SEM images and EDS data of the inner structure of the BC composites revealed CaP particles dispersed and encased in the composite matrix (Fig. 2). According to Sa et al. (2015), small CaP particles tend to fill the pores of the BC composite as an agglomerated phase. In fact, surface energy results have shown that loading Lev(CaP) particles have not significantly changed the total surface energy and, therefore, the outer surface properties of the materials (Table 2) as the particles were mainly confined to the inner matrix (Fig.2). Moreover, according to XRD results (Fig. 3) this confinement of the CaP particles to the inner BC matrix has not changed the BC composites structure crystallinity.

Release of an antibiotic from the inner BC monolithic and hydrophobic matrix depends on the availability of cracks and pores through which the medium can diffuse in and simultaneously allow antibiotic dissolution and release. Moreover, factors like pores form, length and diameter, and the tortuosity of available pathways, often leading to "dead ends", are also referred as being responsible for delay and eventually obstruct antibiotic release (Siepmann et al., 2012). Therefore, and considering SEM images, a moderate *in vitro* levofloxacin release was expected. In fact, the release of levofloxacin from the BC composites throughout an 8-week time period resulted in comparable values comprised between 4 and 6% (solid markers on Fig. 4) with all composites achieving a maximum and stable concentration of levofloxacin after a 4-week period. However these percentage values are correspondent to a meaningful levofloxacin concentration once all composites after a 6 h period time of release have delivered a levofloxacin concentration above the MIC value for the *S. aureus* (0.25 μg/mL) and after 48 h a concentration above the MIC value for *S. epidermidis* (1.0 μg/mL) bacteria (Fig. 5).

The obtained kinetic parameters evidenced low rate constants and a prevalence of the dissolution process over diffusion, which along with SEM images (Fig.2) suggest that levofloxacin release occurred mostly by dissolution from the CaPs particles closer to the surface and then continued more slowly by diffusion through the fine inner pores generated by lactose.

Antimicrobial susceptibility results (**Table 4**) disclosed that after an 8-week period, levofloxacin retained the antibacterial properties against *S. aureus* and *S. epidermidis* in agreement to the MIC values of a levofloxacin standard solution. *E. coli* ATCC®25922, used as quality control strain to guarantee that the test system performed as expected, lead to results that fell within specified limits listed in CLSI M100-S17 (CLSI, 2007). The obtained MIC values for *S. aureus*, *S. epidermidis* and *E. coli* were also in agreement with literature (Boyd et al., 2009; Hurst et al., 2002; Lin et al., 2014). These results suggest that the levofloxacin adsorbed in the CaPs particles surface was not affected during the BC composite setting or by the acidic release medium (pH 5.4). This is an important result considering that: (i) often after surgery localised acidity occurs, with the pH value near the implant varying typically from 5.3 to 5.6 (Manivasagam et al., 2010); (ii)

acidic conditions have been described to reduce drastically the potency of antibiotics in its pure form (Uskokovic' and Desai, 2014).

Considering the moderate levels of antibiotic released from the composites, and in spite of levofloxacin being referred as the fluoroquinolone with lower toxicity (Zimmerli, 2015), cytotoxicity assays with fibroblasts and osteoblasts incubated with extracts or in direct contact with BC composites were conducted with this matrices. These are common tests aimed to have a first look at materials biocompatibility as recommend by international guidelines (ISO 10993-5) and assessed by researchers in the field (Lin et al., 2014; Zhou et al., 2014). Results revealed no cytotoxicity regarding the MG63 (osteoblasts) and L929 (fibroblasts) even after 48 h of exposure (Fig. 6). This means that polymerisation, residual monomers, additives or other polymerisation by-products did not contribute to cytoxicity (Bettencourt et al., 2000, 2002, 2007; Matos et al., 2014, 2015). Moreover, the observed maintenance of the cytoskeleton organisation of both osteoblasts and fibroblast cell lines, on the surface of the composites, is a strong evidence of biocompatibility of these materials (Figs. 7 and 8).

## 5. Conclusion

A strong effort on evolving currently available ALABCs is leading the clinical technology research and development. The objective is to address ALABCs major disadvantages, which are the inadequate kinetic and often incomplete release of the drug, the limited options of antibiotics available in commercially premixed formulations and the poor bone tissue integration.

An original approach of using antibiotic-containing CaP particles included in acrylic BC for the control of bone and joint infections was presented. Results revealed composites with mechanical integrity and biocompatibility compliance, sustained release of the antibiotic (levofloxacin) and maintenance of antimicrobial activity against *S. aureus* and *S. epidermidis* over an 8-week release period.

Overall, reported results represent a valuable step forward to develop novel BC composites with doped CaP particles as antibiotic carriers for the control of bone and joint infections. Hereafter, assessing other CaP particle size and doses of adsorbed levofloxacin could be the next step towards this BC composite technological development.

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# Chapter 5

## CONCLUSIONS AND FINAL REMARKS

Currently the use of antibiotic-loaded acrylic bone cement (ALABC) is inevitable either for prophylaxis as for treatment of prosthetic and bone infections. For long literature reported ALABC implications on the:

- increasing of bacteria resistance due to both the incomplete release of the drug and the poor diversity of antibiotics loaded into the approved commercial ALABC (namely by the FDA), which is limited to gentamicin or tobramycin alone or combined with vancomycin or clindamycin;
- high rates of early implant failure due to the poor interaction of bone cement (BC) with bone tissue, which is mainly an anchorage interaction rather than biological.

Given the undeniable clinic use of ALABC a plethora of studies emerges proposing solutions to address these drawbacks. However, those often imply the addition of other substances over the base-components of the BC, which directly affect its mechanical performance, hampering its use for prophylaxis.

This thesis aims at contributing to the development of a novel modified ALABCs matrix with improved antibiotic delivery by adding a poragen ingredient, lactose, into a commercial BC without jeopardising the mechanical behaviour or the biocompatibility. To test this matrix, two unforeseen antibiotics, minocycline and levofloxacin, were loaded as antibiotic model-drugs, resulting in important contributions to the field.

# 1. Conclusions and Near-Future Research

- **Lactose was successfully used as a poragen**

Loading 10% lactose ($w/w_{BC}$) into BC did not compromise either its mechanical properties or biocompatibility. This water-soluble excipient allowed a significant increase of minocycline and levofloxacin release from BC. Considering that release of an antibiotic from the inner BC monolithic and hydrophobic matrix depends mainly on the availability of cracks and pores through which the medium can diffuse in and simultaneously allow antibiotic to dissolve and be released, the observed increase of porosity due to lactose loading was of utmost importance. Pore distribution along the matrix was revealed as uniform, with increasing number of both small and large pores when compared to plain BC matrix. This suggests the pathways and inner channel network have also increased, allowing a better diffusion in and out of the aqueous release medium through the ALABC matrix. Another important feature was that lactose did not change the mechanism of release based on dissolution and subsequent diffusion of the antibiotic from matrix.

In further research work the effect of loading lower amounts of lactose into BC should be studied to determine the lower amount of lactose that can be loaded into BC without changing the

required release profile or threating the compliance with the international standards for mechanical properties. It would be also interesting to investigate whether the effect of lactose on release is independent of the antibiotic molecular size (or molecular weight), thus allowing to load larger antibiotic molecules, such as daptomycin, or even antibiotic combinations.

- **Both antibiotics demonstrated to be suitable for incorporation into BC**

Minocycline and levofloxacin are both broad-spectrum antimicrobials, active against the main microorganisms causative of bone infections as *S. aureus* and *S. epidermidis,* maintaining their antibacterial activity throughout harsh experimental conditions. Both antibiotics demonstrated to be resistant to thermal conditions involved in the PMMA polymerisation reaction. In addition, they were not affected by the acidic release medium (pH 5.4) used during *in vitro* release studies, which has been described to drastically reduce the potency of free antibiotics. Regarding minocycline no chemical bonding to BC matrix was detected, being completely released within a one-week period and suggesting it will not contribute to the development of bacterial resistance. As for levofloxacin the *in silico* study revealed covalent and non-covalent bonding to BC matrix, which limited the percentage of release. This fact was seen as an advantage whereas bonded levofloxacin would not contribute with a sub-therapeutic or sub-inhibitory concentration that could induce resistance. Still as the stoichiometry was not determined, it was not possible to conclude if some of the levofloxacin entrapped in the matrix core was still active. Should this happen, it might contribute to the development of bacterial resistance.

Therefore, it will be crucial to address the referred stoichiometry.

On the other hand, as antibiotic concentrations achieved during release were relatively high, it can be hypothesised that lower amounts of antibiotics might be sufficient to achieve the same microbiological results.

Also, as minocycline and levofloxacin present protein bonding levels of at least 70% and 40% respectively, release studies under different biomimetic conditions should be performed, *e.g.* using human serum.

Finally, it would be interesting to load BC with larger antibiotic molecules (*e.g.* daptomycin), which is currently used for local treatment of bone infection due to its activity against multi-resistant staphylococci. Some studies have been performed but only for biofilm inhibition study. Additional studies on the mechanical properties and the antibiotic release profile could be performed and enrich available data.

- **Antibiotics antimicrobial activity maintenance**

Both minocycline and levofloxacin maintained their antimicrobial effectiveness against *S. aureus* and *S. epidermidis*, after being released from the lactose-modified matrix. Moreover, the inclusion of lactose on the BC matrix induced a delay on the *S. aureus* biofilm formation when levofloxacin was loaded.

Thus, near future work shall evaluate the BC antimicrobial activity, particularly the interaction between *Staphylococcus* biofilms and the BC surface, using for example flow chamber systems and confocal laser scanning microscopy for the study of biofilms.

- **Promising particulate systems as drug carriers**

Plain and antibiotic-loaded PMMA particles were successfully prepared, with suitable surface morphology, yield of preparation, stability and size, but the inclusion into BC hindered setting, most probably due to the high amount loaded. This drawback precluded any further developments into this line of research.

As an alternative, bioactive doped-calcium phosphate (CaP) particles containing levofloxacin adsorbed onto the surface were used. These particles were dispersed and incorporated in the composite matrix, which is predictive of bone ingrowth promotion into bioactive-BC. A sustained release of levofloxacin was observed over 8 weeks, reaching concentrations over the *Staphylococcus* spp. MIC after 48h. Moreover, the inclusion of CaPs has not changed the release mechanism meaning that the structure of ALABC matrix was maintained. Regarding the mechanical properties there was an increase of all *quasi-static* properties.

Further investigation may include:

Encapsulation of other antibiotics in PMMA particles, with higher drug loading and more effective antibiotic release, envisaging preservation of the mechanical properties of PMMA;

Biomineralisation studies to evaluate the bone ingrowth extension. For example, quantification of calcium liberation could be an important indicator;

Loading nanoCaPs ceramics into BC to find out the better compromise between the biological response and mechanical impairment. NanoHA (70 – 100 μm) are being tested as injectable material antibiotic carrier to impair growth of *Staphylococcus* spp. biofilm; this would be also promising alternative regarding infection prophylaxis;

- ***In silico* studies provided important insight of the drug-biomaterial interaction.**

The computational study herein presented has revealed as a fundamental tool that should be often applied, to understand better the possible reasons for antibiotic retention inside the BC matrix due to bonding or chelate liaisons.

The extension of bonding or chelate formation needs to be further assessed by varying the amount of the loaded antibiotic and, ultimately, combined with ATR-FTIR analysis.

- **Biocompatibility maintenance**

All matrices studied were non-cytotoxic according with the standard specifications, *i.e.* standard ISO 10993-5. Tests carried out using a murine (mouse fibroblasts) and a human (human osteoblasts) cell lines successfully proved biocompatibility. Surface energy results revealed an unchanged surface with minocycline, levofloxacin and lactose loading, indicating the interaction between BC and biological tissues was not modified.

Additional cell function tests should be performed, including cell cycle analysis and apoptosis detection, quantification of bone-specific alkaline phosphatase involved in the calcification of bone matrix, lactate dehydrogenase (LDH) measurement, which measures the plasma membrane integrity, to evaluate the biological effects of the novel ALABC matrices.

- ***Quasi-Static* mechanical properties compliance with ISO 5833**

Under static conditions, the compressive strength, the flexural modulus and flexural strength fulfilled the requirements of the standard ISO 5833.

In the future, dynamic mechanical properties should be assessed, such as, fatigue life testing and estimation of fatigue limit, before and after *in vitro* aging conditions. In this context, the ISO 5833 and/or ISO 16402:2008 “Implants for surgery – Acrylic resin cement – Flexural fatigue testing of acrylic resin cements used in orthopaedics” should be followed. This is an important test regarding the ability of long-term implant failure in the form of aseptic loosening when ALABC is used prophylactically.

## 2. Final Remark

The research work presented in this thesis led to the development of a mechanically compliant and biocompatible matrix to improve the delivery of antibiotics loaded into acrylic BCs.

The ALABCs are widely used to prevent or treat the occurrence of bone infections in cemented arthroplasties, being considered as a more cost-effective procedure when compared to cementless implants. However, considering the challenge of treating device-associated infections, the number of formulations available in the market is significantly low. The response from the industry to medical needs is still too slow considering the rapid change in the infecting microbial profile and the emergence of multiresistant strains. The impact of prosthetic-infections is devastating, often leading to higher mortality, prolonged hospitalisations, frequent readmissions, and increased costs and overall healthcare burden. Clinicians' awareness of the possible recourse of a versatile and quick platform to allow testing the feasibility of loading approved antibiotics in BC might be useful when deciding about a treatment approach on specific cases of bone-related infections.

Future research on this field must aim at developing safe customisable acrylic BCs, almost on demand for each patient, "providing the right patient with the right drug at the right dose at the right time".

*"… it is preferable not to insist that*
*we always know how and why something works."*

David F. Williams
in "On the nature of Biomaterials"

# Annex

## DEVELOPMENT AND VALIDATION OF SIMILAR HPLC-UV METHODS FOR FLUOROQUINOLONE AND TETRACYCLINE MONITORING IN RELEVANT BIOMIMETIC MEDIA

# Highlights

- Levofloxacin and minocycline quantification in NaCl, PBS and Müeller-Hinton media.
- Simple, economic and fast HPLC methods, only differing in the wavelength setting.
- Validated methods with acceptable linearity, precision, accuracy and recovery.
- Rigorous stability studies of both antibiotics, including stability at 37ºC.
- Useful for antibiotics monitoring thru *in vitro* release and microbiological assays.

# Abstract

Monitoring of antibiotics in biomimetic biological media, during *in vitro* assays, as microbiological and drug release tests, is of paramount importance in drug-delivery-systems development. Simple and rapid high-performance liquid chromatography (HPLC) methods for levofloxacin and minocycline quantification in NaCl at 0.9%, PBS and Müeller-Hinton media were developed and validated. Both methods are similar only differing in the wavelength setting, *i.e.*, for levofloxacin and minocycline quantification, the UV detection was set at 284 nm and at 273 nm, respectively. The separation of both antibiotics was achieved using a reversed-phase column (LiChrospher 100 RP-18) and a mobile phase consisting of acetonitrile and water (15:85) and 0.6% (*V*/*V*) triethylamine, adjusted to pH 3. As an internal standard for levofloxacin quantification, minocycline was used and *vice versa*. The average calibration curves for both methods were linear ($r$ = 0.99) over a concentrations range of 0.3 –16 $\mu$g/mL and 0.5 – 16 $\mu$g/mL for levofloxacin and minocycline quantification, respectively, with acceptable intra- and inter-day precision, accuracy and recovery. Levofloxacin revealed short-term and long-term stability in all media, except when it was submitted to more than one freeze-thaw cycle. Minocycline presented a more accentuated degradation profile over prolonged time courses, mainly in alkaline media.

**Keywords:** Levofloxacin; minocycline; liquid chromatography; validation methods; physiological temperature stability, biological biomimetic media

# 1. Introduction

With the emerging growth of resistance to antibiotics, a tremendous economic and social burden on the healthcare system has been imposed, leading to the need for new therapeutic approaches (Brooks and Brooks, 2014). The use of local drug-delivery-systems has been one of the most promising alternatives against bacterial antibiotic resistance mechanisms, promoting a correct drug dose delivery in the target site over extended periods, if necessary (Wu and Grainger, 2006). In order to find an ideal delivery system, a wide range of *in vitro* drug release and microbiological tests are primarily required. Such systems need careful evaluation of the effective dosage and drug release kinetics profiles, as well as the evaluation of the system matrix performance, into which the antibiotic is loaded, and an appropriate selection of the drug. As a first step in drug-delivery-systems development, *in vitro* assays like drug release and microbiological testing are primordial. These assays allow the evaluation of the continuous bactericidal activity efficacy *in vitro* by calculating the ratio of maximum drug concentrations to the specific minimum inhibitory concentration (MIC) (Sousa et al., 2013; Watabe et al., 2010). Also, it is possible to obtain these values over time, which is important when monitoring drug concentrations to attain the optimal drug dosage regimens and to prevent bacterial resistance.

Levofloxacin and minocycline are fluoroquinolone and tetracycline antibiotics, respectively, both with a broad spectrum of activity against Gram-positive and Gram-negative bacteria, being used to treat a variety of infections, including skin, respiratory tract, bone and soft tissues (Bertino and Fish, 2000; Brogden et al., 1975; Chopra and Roberts, 2001; Fish and Chow, 1997; Matos et al., 2014; Noel, 2009; Senneville et al., 2007). As such, both are considered relevant antibiotics to be loaded in different drug-delivery-systems (Orti et al., 2000; Unsal and Akkaya, 1994; Zhang et al., 2009). However, the actually available HPLC methods to quantify levofloxacin or minocycline, are mostly designed for the evaluation of samples in complex biological fluids, such as plasma, parotid saliva and urine (Colovic and Caccia, 2003; Djabarouti et al., 2004; Fang et al., 2010; Kamruzzaman et al., 2011; Masher, 1998; Neckel et al., 2002; Nguyen et al., 2004; Orti et al., 2000; Watabe et al., 2010; Wrightson et al., 1998), which are not often used *in vitro* assessment assays.

Moreover, to the best of our knowledge, the different HPLC techniques described for minocycline or levofloxacin quantification (Aoyagi et al., 2007; Hart et al., 2010; Kashi et al., 2012) do not endeavour to validate the analytical methodologies in biological biomimetic fluids with interest for *in vitro* assays.

As so, the aim of this study was to develop simple and fully validated HPLC-UV methods for the quantification of these antibiotics in three aqueous biomimetic fluids often used in *in vitro* drug release and microbiological assays, namely NaCl at 0.9% (w/V) with Tween20® at 0.05% (V/V) (hereinafter referred as NaCl medium) a saline solution with pH 5, which mimics the acidic

environment in the infection site (Radovic-Moreno et al., 2012); phosphate buffered saline, PBS, a buffer solution with osmolarity, ion concentrations and pH 7.4 similar to human serum (Das and Dash, 2015) and Müeller-Hinton broth, a microbiological growth medium commonly used for antibiotic susceptibility testing, with pH 7.3 (Ochei and Kolhatkar, 2000). The procedures involved a unique mobile phase, easy and quick to prepare, required a minimum sample volume and the use of an internal standard, which allows enhanced precision (Wong, 1985). Special attention has been drawn to the stability of the antibiotics in different conditions and stages of analysis, according to international guidelines (GI-BMV-FDA, 2001; ICH Q2(R1), 2005). Furthermore, the stability of both antibiotics at 37ºC was studied, as it is the temperature typically used for *in vitro* studies, since it simulates the body temperature (Xian, 2009).

# 2. Experimental

## 2.1. Chemicals and reagents

Levofloxacin, HPLC-grade acetonitrile, PBS (pH 7.4) and polysorbate 20 (Tween 20®) were all obtained from Sigma-Aldrich (Spain). Minocycline hydrochloride was kindly provided by Atral-Cipan (Castanheira do Ribatejo, Portugal). Sodium chloride was obtained from AppliChem ppliChem GmbH, Darmstadt, Germany). Orthophosphoric acid (analytical grade) and triethylamine were purchased from Panreac (Spain). Müeller-Hinton broth was obtained from Biokar (Biokar Diagnostics, France). The deionized water used for solutions and mobile phase preparation was obtained from a Millipore analytical deionization system (F9KN225218) and further filtered under vacuum (Vacuum pump V-700, Büchi) with 0.45 $\mu$m hydrophilic cellulose filters. All substances and solvents have been used without any further purification.

## 2.2. Chromatographic conditions

Both development and validation of the analytical methods were performed on HPLC system (Shimadzu system LC-6A, Shimadzu Corporation) with LiChrospher® 100 RP-18, 5 µm i.d. particle, 125-4 analytical column (LiChroCART®, Merck, Darmstadt, Germany). HPLC was equipped with an autosampler (Waters 717plus Autosampler) and a thermostatic column compartment (Dionex STH 585 Column Oven). The autosampler was programmed with an injection volume of 20 $\mu$L, a carousel temperature of 20°C and a time run of 5 min. The mobile phase consisted in a mixture of acetonitrile and water with a volume ratio of 15:85, respectively, and 0.6% (*V*/*V*) triethylamine (TEA), adjusted to pH 3 with orthophosphoric acid (85-90%). The isocratic separation was performed at 1.2 mL/min constant flow-rate with column temperature maintained at 25°C. The detection wavelength was set at 284 nm for levofloxacin and at 273 nm for minocycline. The Class LC10 software (Shimadzu Corporation, Kyoto, Japan) was used to process the data acquisition. Samples under study were identified by chromatographic comparison with reference standards.

## 2.3. Stock solutions

Stock solutions of levofloxacin at an exactly-known concentration of ~1000 $\mu$g/mL were prepared by dissolving with water an exactly-weighed amount of levofloxacin, ~25 mg, in a 25 mL volumetric flask. Following, a 100 $\mu$g/mL working solution was prepared by water-dilution of the stock solution, in a 25 mL volumetric flask. This working solution was subdivided in 2 mL Eppendorf® tubes and each was stored protected from light at −20ºC for no longer than 3 weeks. Also, a stock and working solutions of minocycline were prepared and stored in the same way.

When minocycline was used as internal standard (IS), the concentration was the same as the working solution. When levofloxacin was used as the IS, the concentration used was 75 $\mu$g/mL prepared by appropriate dilution of the working solution with water.

## 2.4. Calibration standard solutions

The calibration standard solutions of levofloxacin and minocycline were prepared daily, diluting adequate volumes of each respective working solution with water. Water was the medium used to perform the linearity assay once it was the main matrix component of all media under study (earlier described on Introduction).

For levofloxacin quantification, calibration curves were obtained using standard solutions at concentrations of 0.3 – 16 $\mu$g /mL (n=10). As for minocycline quantification, calibration curves were obtained with standards at concentrations of 0.5 – 16 $\mu$g/mL (n=9). 1 mL of each standard solution was spiked with 10 $\mu$L of the respective IS.

Furthermore, three quality control standard solutions (hereinafter referred as $QC_W$) (definition described in FDA guidelines ((GI-BMV-FDA, 2001)) and three calibrators (standards with concentrations equal to those of the calibration curves) were prepared at low, medium and high concentrations according with Table 1.

**Table 1.** QC standard solutions and calibrators used for the linearity assay.

| | Calibrators | $QC_W$ | $QC_M^*$ |
|---|---|---|---|
| **Levofloxacin** | 0.3 | 0.4 | 0.4 |
| | 4 | 5 | 5 |
| | 10 | 12 | 12 |
| **Minocycline** | 0.5 | 0.7 | 0.7 |
| | 4 | 5 | 5 |
| | 10 | 12 | 12 |

*for Müeller-Hinton broth these concentrations are a result of a previous 1:2 dilution on acetonitrile due to the precipitation method procedure.

## 2.5. Samples

Both levofloxacin and minocycline samples were studied in NaCl, PBS and Müeller-Hinton broth media. For each antibiotic, assays carried either in NaCl or PBS media, samples were directly introduced (or previous diluted, when needed) in 1 mL autosampler vials and spiked with 10 $\mu$L of the respective IS. However, for assays carried in Müeller-Hinton broth, a specific protein precipitation method was necessary. Briefly, 1 mL of acetonitrile was added to 1 mL of Müeller-

Hinton sample and poured into a 2 mL Eppendorf® tube, resulting in a mixture of 50:50 (*V*/*V*). After vortexing during 10 s and subsequently centrifugation for 10 min, at 4°C and 12000 rpm, 1 mL of the obtained supernatant was transferred into an autosampler vial and spiked with 10 $\mu$L of the respective IS.

Regarding the QC solutions for samples quantification (hereinafter referred as $QC_M$), for NaCl and PBS media, they were prepared by simple dilution of the working solution. Yet, for the Müeller-Hinton broth, $QC_M$ preparation was done by dilution of the working solution followed by the precipitation method, earlier described.

## 2.6. Calculation procedure and assay validation

Calibration curves, obtained by direct linear regression, relate the ratio of standard peak-area to the IS peak area (*yy*') with the standard concentration (*xx*'). All the QC solutions, calibrators and unknown concentrations of the analytes of interest were calculated by interpolation of these daily calibration curves.

The assay was validated in terms of linearity, precision, accuracy, recovery, limit of detection (LOD), lower limit of quantification (LLOD) and stability (short-term, long-term and freeze-thaw cycles) according to internationally accepted criteria described in FDA and ICH guidelines for bioanalytical methods validation (GI-BMV-FDA, 2001). Additionally, the stability of both antibiotics at 37ºC in the three media was evaluated over time until 10% degradation was observed. Experiments in NaCl and PBS were assessed up to a maximum of one month. For Müeller-Hinton, the stability at 37ºC was performed just until two days, since this medium easily becomes contaminated when it is not in sterile conditions.

# 3. Results and Discussion

## 3.1. Chromatographic methods development

The proposed methods aimed at a simple levofloxacin and minocycline quantification in three relevant biomimetic media. Development of the analytical methods started by the selection of the mobile phase composition in order to separate both analytes. A simple mobile phase consisting in acetonitrile, deionized water and TEA at acidic pH was found to be optimal. The ideal working temperature of the column was found to be 25°C and the flow rate of 1.2 mL/min, resulting in a short run time of analyses of 5 min, except for Müeller-Hinton broth samples, for which the run time increased up to 13 min due to its inherent complexity. Using the mentioned chromatographic conditions, it was possible to compensate the high hydrophobicity effect of minocycline, which usually increases the retention time in most of the methods, making them morose and consequently more expensive (Jain et al., 2007).

The selection of the maximum absorption wavelength of levofloxacin and minocycline was based on both UV–absorption spectrums. Levofloxacin featured a spectrum with absorption maximum in the range of 282-290 nm (Fig. 1A) and the minocycline spectrum showed absorption maxima at 273 and 350 nm (Fig. 1B). Hence, for the levofloxacin quantification, the HPLC detection wavelength was set at 284 nm, whereas for minocycline quantification was set at 273 nm. Thus, due to the proximity, but sufficiently separated from the two maxima absorption wavelengths, it was possible to improve the sensitivity for the desired quantification and also use minocycline as IS for levofloxacin quantification and *vice versa*.

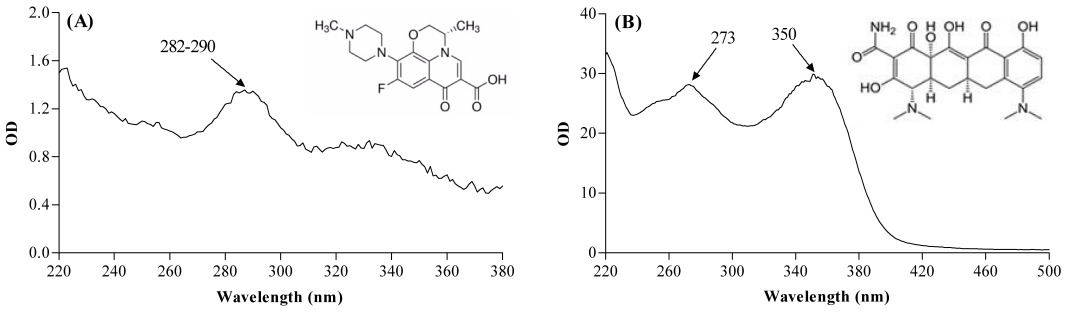


**Fig. 1** UV-absorption spectra of levofloxacin (A) and minocycline (B) with the respective molecular structure representation. The maxima absorption wavelengths are pointed out in each UV-spectra.

As so, two methods only differing on the wavelength setting, were developed using a simple mobile phase, inexpensive and quick to prepare, an affordable and accessible column, featuring a short time run with a minor injection volume, very useful when sample amounts are sparse.

Representative chromatograms obtained for levofloxacin and minocycline quantification in the

different media are shown in Fig. 2 and Fig. 3, respectively. On both figures, it is possible to observe completely separated peaks with symmetrical shape. Also, it is noticeable that matrix components do not interfere with the region of interest where the antibiotic peaks are detected. At 284 nm for levofloxacin quantification, the observed retention times were 2.4 min and 3.7 min for IS and levofloxacin, respectively. At 273 nm for minocycline quantification, retention times were 2.7 min and 4.1 min for minocycline and IS, respectively.

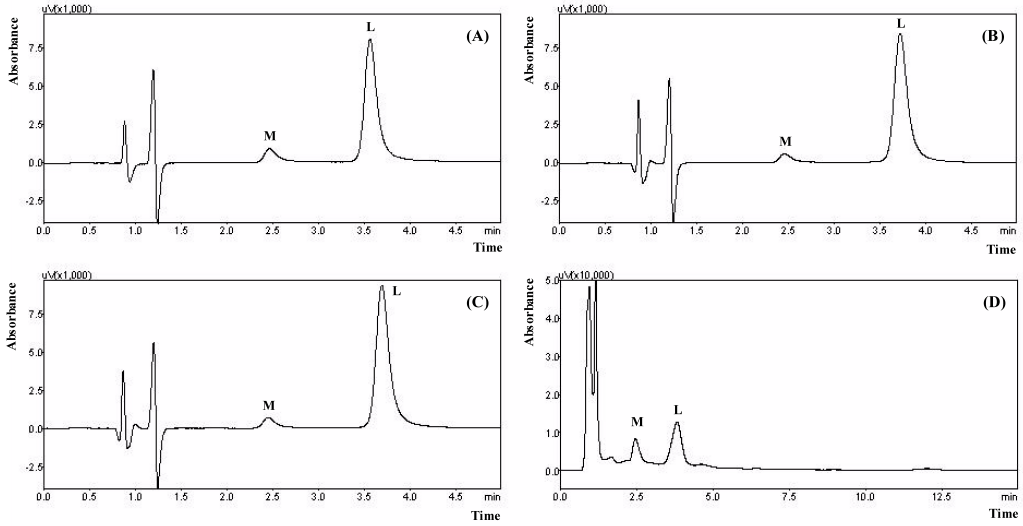


**Fig. 2** HPLC chromatograms at 284 nm of a levofloxacin (L) solution at 2 $\mu$g/mL and IS (minocycline, M) in H2O (A), NaCl (B), PBS (C) and Müeller-Hinton broth (D).

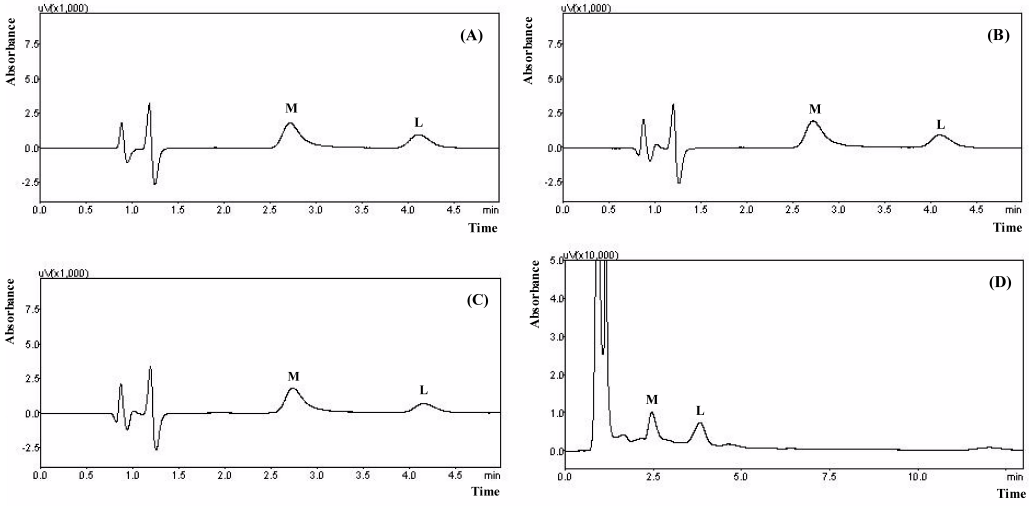


**Fig. 3** HPLC chromatograms at 273 nm of a minocycline (M) solution at 2 $\mu$g/mL and IS (levofloxacin, L) in $H_2O$ (A), NaCl (B), PBS (C) and Müeller-Hinton broth (D).

## 3.2. Calibration curves range and linearity

The lowest standard on the calibration curve should be assumed as the LLOQ. For each antibiotic, LLOQ was assessed in water samples and was defined as the lowest concentration of the analyte that could be determined with an inter-day precision below 20% (bias within ±20%) ((GI-BMV-FDA, 2001). LOD was the lowest concentration of analyte in a sample that could be detected, based on visual evaluation (ICH Q2(R1), 2005).

The obtained values for LLOQ and LOD for both antibiotics are given in Table 2. To the best of our knowledge, there are no values described in literature for LLOQ or LOD of levofloxacin or minocycline in any of the media studied. However, similar results were described in other biological media. For example, HPLC-UV studies of levofloxacin in plasma, bronchoalveolar lavage (BAL) and bone tissues refer a LLOQ and LOD of 0.20 and 0.05 μg/mL for plasma samples, 0.4 and 0.1 μg/mL for BAL samples and 0.5 and 0.2 μg/mL for bone samples, respectively, using the same injection volume of 20 μL (Djabarouti et al., 2004). As for minocycline, HPLC-UV studies for quantification in human plasma and parotid saliva featured a LLOQ and LOD of 0.1 μg/mL and 0.05 μg/mL, respectively, using also 20 μL as the injection volume (Orti et al., 2000).

**Table 2.** Analytical and statistical parameters for the average calibration curves.

| Parameter | Levofloxacin | Minocycline |
|---|---|---|
| Calibration equation | $y = 6.072x - 1.221$ | $y = 1.035x - 0.280$ |
| Intercept | -1.221 | -0.280 |
| Slope | 6.072 | 1.035 |
| Standard error of the intercept | 0.803 | 0.126 |
| Standard error of the slope | 0.115 | 0.017 |
| Correlation coefficient *(r)* | 0.988 | 0.991 |
| Range (μg/mL) | 0.3 – 16 | 0.5 – 16 |
| Number of points | 10 | 9 |
| LOD (μg/mL) | 0.03 | 0.05 |
| LLOQ (μg/mL) | 0.3 | 0.5 |

Both antibiotics under study present a broad-spectrum activity against *Staphylococcus* spp., which are the main microorganisms responsible for bone and joint infections (Bishburg, K. Bishburg, 2005; Zimmerli, 2014). The MIC of minocycline described in literature for methicillin-resistant S. *aureus* (MRSA) range between 0.5 – 1 μg/mL (Goff and Dowzicky, 2007; Hoban et al., 2005; Waites et al., 2006). As for levofloxacin, the MIC values for methicillin-susceptible S. aureus (MSSA) range between 0.5 – 8 μg/mL (Goff and Dowzicky, 2007; Hoban et al., 2005; Horii et al., 2003; Waites et al., 2006).

Therefore, the calibration curves concentration range was chosen in accordance with those MICs values, that is, 0.3 – 16 μg/mL for levofloxacin quantification and 0.5 – 16 μg/mL for minocycline quantification, in order to be able to compare with the microbiological and in vitro drug release results of specific delivery systems with potential application for treatment of *S. aureus* infections.

The proposed ranges should be evaluated in terms of their linear relationship. ICH defines linearity of an analytical procedure as its ability (within a given range) to obtain test results that are directly proportional to the concentration (amount) of analyte in the sample (ICH Q2(R1), 2005).

So, to assess the two methods linearity, it was assayed one calibration curve, in duplicate, during seven independent days (n=7). An average calibration curve was obtained (Table 2).

Calibrators and $QC_W$ used for linearity assay were analysed at six concentrations (three concentrations each) in order to analyse if its concentrations did not span 80 – 120% of the expected concentration range (ICH Q2(R1), 2005). The accuracy of these calibrators and $QC_W$ (mean relative error (bias) between measured and nominal concentrations) did not exceed the nominal concentration value for all concentration levels (±15%) (GI-BMV-FDA, 2001).

Linear regression was used to determine the slope and intercept of the average calibration curve. Analytical and statistical parameters of calibration are listed in Table 2. All statistical parameters of the equations were calculated through linear regression using Microsoft Excel 2010.

Moreover, regarding the obtained limits of quantification and despite the advantage of the proposed methods of a low injection volume, both could be decreased with the increase of injection volume if needed.

## 3.3. Precision, accuracy and recovery

The precision, accuracy and recovery of both methods are given in Table 3 and in Table 4 for levofloxacin and minocycline, respectively.

### *Precision and accuracy*

Precision and accuracy of the proposed HPLC analytical method, expressed in terms of the coefficient of variation (CV) and bias, respectively, were evaluated at four concentration levels in the range of the respective calibration curves: 0.4, 2, 5 and 12 μg/mL for levofloxacin and 0.7, 2, 5 and 12 μg/mL for minocycline. Intra-day repeatability was evaluated through the analyses of five replicates for each concentration in the same day. The inter-day repeatability was determined assaying six replicates for each concentration in different days.

In summary, precision and accuracy did not exceed the acceptable criteria: coefficients of variation lower than 15% and bias within ±15% (GI-BMV-FDA, 2001), demonstrating that methods

are reproducible, accurate and suitable for the quantitative determination of levofloxacin and minocycline in the different media.

**Table 3.** Intra- and inter-day precision (CV%); accuracy (bias,%) and recovery (%) of levofloxacin method.

| Medium / Theoretical Concentrations (μg/mL) | Intra-day reproducibility (mean; n=5) | | | Inter-day reproducibility (mean; n=6) | | | |
|---|---|---|---|---|---|---|---|
| | Experimental concentration (μg/mL) | CV (%) | Bias (%) | Experimental concentration (μg/mL) | CV (%) | Bias (%) | Recovery (%) |
| **$H_2O$** | | | | | | | |
| **0.4** | 0.41 | 8.93 | 2.04 | 0.43 | 6.68 | 6.35 | 106.35 |
| **2.0** | 1.87 | 3.15 | -6.56 | 1.79 | 5.05 | -10.27 | 89.73 |
| **5.0** | 4.62 | 6.78 | -7.50 | 4.50 | 5.65 | -10.27 | 89.73 |
| **12** | 10.98 | 5.74 | -8.53 | 10.68 | 5.09 | -10.03 | 89.97 |
| **NaCl** | | | | | | | |
| **0.4** | 0.45 | 1.54 | 13.70 | 0.45 | 1.44 | 13.03 | 113.03 |
| **2.0** | 2.07 | 4.43 | 3.58 | 2.10 | 4.24 | 4.86 | 104.86 |
| **5.0** | 4.92 | 8.15 | -1.54 | 5.21 | 8.13 | 4.24 | 104.24 |
| **12.0** | 11.05 | 8.26 | -7.90 | 10.79 | 6.23 | -10.09 | 89.91 |
| **PBS** | | | | | | | |
| **0.4** | 0.44 | 3.99 | 12.34 | 0.45 | 2.65 | 12.04 | 112.04 |
| **2.0** | 1.80 | 7.27 | -10.01 | 2.00 | 14.57 | 0.09 | 100.09 |
| **5.0** | 4.92 | 6.60 | -1.51 | 4.97 | 6.50 | -0.51 | 99.49 |
| **12.0** | 11.09 | 1.22 | -7.61 | 11.70 | 6.96 | -2.53 | 97.47 |
| **Müeller-Hinton** | | | | | | | |
| **0.3** | 0.34 | 2.31 | 12.66 | 0.31 | 9.87 | -3.59 | 103.59 |
| **2.0** | 1.85 | 0.89 | -7.18 | 1.85 | 6.02 | 7.61 | 92.38 |
| **5.0** | 4.92 | 10.13 | -1.56 | 4.68 | 8.90 | 6.46 | 93.54 |
| **12.0** | 11.39 | 4.17 | -5.05 | 11.42 | 3.94 | 4.85 | 95.15 |

### *Recovery*

Values of the extraction recovery for each antibiotic in all media were expressed as percent recovery obtained from the ratio of “mean of experimental concentration” to “theoretical concentration”, using six determinations for each concentration. Results showed that the recovery intra-day varied less than 13% for all media under study.

**Table 4.** Intra- and inter-day precision (CV%); accuracy (bias,%) and recovery (%) of minocycline method.

| Medium / Theoretical Concentrations (μg/mL) | Intra-day reproducibility (mean; n=5) | | | Inter-day reproducibility (mean; n=6) | | | |
|---|---|---|---|---|---|---|---|
| | Experimental concentration (μg/mL) | CV (%) | Bias (%) | Experimental concentration (μg/mL) | CV (%) | Bias (%) | Recovery (%) |
| **$H_2O$** | | | | | | | |
| **0.7** | 0.74 | 5.65 | 6.19 | 0.69 | 9.14 | -1.24 | 98.76 |
| **2.0** | 1.88 | 4.73 | -6.14 | 1.85 | 4.82 | -7.25 | 92.75 |
| **5.0** | 4.74 | 3.68 | -5.26 | 4.75 | 11.77 | -5.07 | 94.93 |
| **12.0** | 12.62 | 4.83 | 5.16 | 103.45 | 8.49 | 3.79 | 103.79 |
| **NaCl** | | | | | | | |
| **0.7** | 0.74 | 5.10 | 6.35 | 0.75 | 4.37 | 6.73 | 106.73 |
| **2.0** | 2.07 | 3.87 | 3.32 | 2.01 | 8.65 | 0.63 | 100.63 |
| **5.0** | 4.71 | 4.74 | -5.83 | 4.85 | 8.83 | -3.07 | 96.93 |
| **12.0** | 11.74 | 3.79 | -2.14 | 12.47 | 7.23 | 3.89 | 103.89 |
| **PBS** | | | | | | | |
| **0.7** | 0.71 | 2.03 | 1.91 | 0.76 | 7.64 | 9.13 | 109.13 |
| **2.0** | 2.08 | 14.53 | 4.18 | 2.06 | 9.42 | 3.03 | 103.03 |
| **5.0** | 4.40 | 0.97 | -11.90 | 4.68 | 6.53 | -6.44 | 94.56 |
| **12.0** | 10.97 | 4.25 | -8.59 | 11.34 | 11.30 | -5.47 | 94.53 |
| **Müeller-Hinton** | | | | | | | |
| **0.7** | 0.76 | 2.43 | 9.00 | 0.76 | 3.31 | -9.14 | 109.14 |
| **2.0** | 2.14 | 3.86 | 6.90 | 2.05 | 5.25 | 2.61 | 102.61 |
| **5.0** | 4.43 | 0.23 | -11.48 | 4.38 | 1.50 | -12.32 | 87.67 |
| **12.0** | 10.72 | 3.66 | -10.64 | 10.86 | 4.00 | -9.49 | 90.51 |

## 3.4. Stability

Levofloxacin and minocycline stability was evaluated in water (stock solutions) and in the three tested biomimetic media, regarding short-term, long-term, freeze-thaw and 37ºC stability.

### *Stock solutions stability*

The stock solutions stability in water was evaluated using two $QC_W$ at low and high concentrations, 0.4 and 12 μg/mL for levofloxacin quantification and 0.7 and 12 μg/mL for minocycline quantification, analysed three times each, all prepared from freshly stock solutions, and compared with the same solutions stored at room temperature during 6 h and over a period of one month at –20°C. The obtained results are given in Table 5 and showed that the levofloxacin stock solution presented good stability under these conditions, with losses lower than 8%. Also,

the minocycline stock solution stability was also satisfactory after one month frozen, however it is not recommended to use the same stock solution if stored at room temperature (20°C) for over a period of several hours.

**Table 5.** Stability results of levofloxacin and minocycline stock solutions under different storage conditions.

| Storage conditions | Percent Recovery (mean ± SD) (n=3) | | | |
|---|---|---|---|---|
| | Levofloxacin stock solution ($H_20$) | | Minocycline stock solution ($H_20$) | |
| | $QC_W$, 0.4 | $QC_W$, 12 | $QC_W$, 0.4 | $QC_W$, 12 |
| 6 h, 20°C | 97.89 ± 4.15 | 92.49 ± 3.76 | 91.52 ± 4.68 | 86.66 ± 2.00 |
| 1 month, –20°C | 99.80 ± 0.34 | 103.6 ± 1.40 | 92.00 ± 4.58 | 95.13 ±1.63 |

### *Short-term and long-term stability*

Short-term and long-term stability of levofloxacin and minocycline in the different media were also evaluated using the same concentrations used for stock solutions stability, also with three replicates each. $QC_M$ samples were analysed immediately after preparation (used as reference values) and under different storage conditions.

Short-term stability was assessed at three different conditions: 6 h at room temperature; overnight into the autosampler; and after storage at 4°C for 24 h. Long-term stability was evaluated at –20°C for one month. Short-term and long-term results are presented in Table 6 and Table 7 for levofloxacin and minocycline, respectively.

The short-term stability study of levofloxacin showed that there was no relevant degradation, with losses lower than 7% in all the evaluated media. As for long-term stability, an acceptable deterioration of the drug in NaCl and PBS (< 9%) was found. However, a more pronounced degradation in Müeller-Hinton media (~19%) was observed.

The same study with minocycline revealed a decline in drug concentration, detected under various short-term storage conditions. It is possible to storage NaCl samples 6 h at room temperature and 24 h at 4°C, without special precautions. Still, in overnight measurements of NaCl samples, a loss in minocycline concentration averaging 20% was observed. Short-term stability results of PBS and Müeller-Hinton medium showed a significant degradation of the antibiotic (between 8% – 25%).

Also, the stability at long term at –20°C revealed a substantial degradation of minocycline, mainly in PBS medium.

**Table 6.** Stability results of levofloxacin under various storage conditions.

| Storage conditions | Percent Recovery (mean ± SD) (n=3) | | | | | |
|---|---|---|---|---|---|---|
| | NaCl | | PBS | | Müeller-Hinton | |
| | $QC_M$, 0.4 | $QC_M$, 12 | $QC_M$, 0.4 | $QC_M$, 12 | $QC_M$, 0.4 | $QC_M$, 12 |
| **6 h, 20°C** | 96.19±6.94 | 99.32±0.59 | 99.57±3.87 | 95.95±3.63 | 100.10±3.41 | 97.87±2.69 |
| **Overnight, 20°C** | 91.80±4.65 | 94.57±2.26 | 103.23±2.20 | 99.87±1.07 | 97.24±2.74 | 97.77±3.98 |
| **24 h, 4°C** | 96.51±3.21 | 96.55±2.01 | 96.72±3.45 | 98.69±2.46 | 94.88±6.64 | 93.86±0.91 |
| **Freeze–thaw 1st cycle** | 91.78±5.71 | 94.06±3.14 | 90.17±4.00 | 94.40±5.14 | 91.06±4.23 | 79.4±1.75 |
| **Freeze–thaw 2nd cycle** | 85.86±8.25 | 84.50±2.56 | 87.43±2.51 | 80.96±7.20 | 95.27±9.61 | 69.95±7.88 |
| **Freeze–thaw 3rd cycle** | 81.30±9.15 | 81.83±7.07 | 87.25±2.54 | 76.91±6.97 | 87.81±1.67 | 52.65±5.84 |
| **1 month, –20°C** | 94.69±8.05 | 91.62±3.13 | 99.82±1.79 | 91.20±5.27 | 81.98±1.41 | 97.59±2.40 |

**Table 7.** Stability results of minocycline under various storage conditions.

| Storage conditions | Percent Recovery (mean ±SD) (n=3) | | | | | |
|---|---|---|---|---|---|---|
| | NaCl | | PBS | | Müeller-Hinton | |
| | **$QC_M$, 0.7** | **$QC_M$, 12** | **$QC_M$, 0.7** | **$QC_M$, 12** | **$QC_M$, 0.7** | **$QC_M$, 12** |
| **6h, 20°C** | 94.30±1.98 | 99.09±2.39 | 92.51±2.67 | 75.65±2.84 | 82.47±10.18 | 90.21±1.90 |
| **Overnight, 20°C** | 80.90±8.24 | 88.90±4.24 | 83.72±2.65 | 64.54±3.47 | 81.98±6.52 | 88.58±4.59 |
| **24h, 4°C** | 100.53±4.80 | 100.78±1.25 | 69.89±4.30 | 68.45±0.75 | 94.74±4.79 | 90.42±1.93 |
| **Freeze–thaw 1st cycle** | 93.59±4.48 | 101.92±1.85 | 82.56±4.04 | 72.14±1.35 | 79.84±7.92 | 70.37±3.56 |
| **Freeze–thaw 2nd cycle** | 89.15±0.29 | 101.38±4.11 | 73.81±6.31 | 70.67±5.32 | 70.89±1.50 | 67.79±4.42 |
| **Freeze–thaw 3rd cycle** | 89.29±3.33 | 102.37±2.48 | 73.49±5.69 | 67.04±3.99 | 71.82±1.42 | 66.18±1.64 |
| **1 month, –20°C** | 73.71±3.77 | 78.51±8.02 | 64.48±14.31 | 68.32±4.29 | 86.70±6.93 | 90.21±1.91 |

### *Freeze-thaw stability*

The stability during three freeze-thaw cycles from –20 °C to room temperature was also determined. The correspondent levofloxacin results are shown in Table 6 and Table 7 shows the results of minocycline.

For levofloxacin, after three freeze-thaws, a high degradation in NaCl and PBS was observed, indicating that it is not recommended to freeze samples more than once. After one freeze-thaw cycle with Müeller-Hinton samples, a high degradation of the antibiotic occurred, suggesting that samples should be analysed in a period no longer than 24 h.

Freeze-thaw stability of minocycline revealed that at least three freeze-thaw cycles of NaCl

samples could be tolerated without losses higher than 11%. However, it is not recommended to freeze PBS and Müeller-Hinton samples.

### *37ºC stability*

Additionally, the stability of levofloxacin and minocycline in the different media at 37ºC was also evaluated over time until 10% degradation was obtained. Acquaintance of this data is extremely important when monitoring antibiotics during *in vitro* studies, which normally proceed at 37ºC. Table 8 shows the maximum time stability of both antibiotics spiked in all media.

Levofloxacin revealed good stability at all time periods analysed and in all media. However, minocycline remains stable just until 6 h in NaCl, 1 h in PBS and 24 h in Müeller-Hinton. These results suggest that the *in vitro* assays should be performed with fresh medium replacement at the end of each time period of the study, thus preventing further degradation.

In sum, all the stability tests showed that minocycline is more unstable than levofloxacin. The degradation behaviour of minocycline is due to the fact that tetracyclines are easily degraded under conditions like alkaline pH, chelation and photodegradation (Halling-Sørensen, 2002). In fact, it was observed that minocycline showed less degradation when spiked in NaCl medium, certainly due to its more acidic pH. Degradation studies of minocycline developed by Jain et al. (2007) also suggest that this drug is more susceptible to alkaline than acidic degradation.

Finally, it is recommended, for both antibiotic samples, that protection from external agents is guaranteed, keeping them stored at low temperature and darkened environment until the chromatographic analysis. It is not advisable to submit the samples to more than one freeze-thaw cycle. In case of using minocycline as IS, it should be added immediately before analysis.

**Table 8.** Maximum time stability of levofloxacin and minocycline on the three relevant media at 37ºC.

| **Medium** | **Percent Recovery (mean ± SD) (n=3)** | | | |
|---|---|---|---|---|
| | **Levofloxacin** | | **Minocycline** | |
| | **$QC_M$, 0.4** | **$QC_M$, 12** | **$QC_M$, 0.7** | **$QC_M$, 12** |
| **NaCl** | 4 weeks | | 6 h | |
| | 98.84 ± 2.39 | 99.97 ± 3.90 | 98.00 ± 4.23 | 99.15 ± 2.99 |
| **PBS** | 4 weeks | | 1 h | |
| | 98.85 ± 2.21 | 91.21 ± 1.61 | 90.45 ± 3.02 | 91.12 ±1.33 |
| **Müeller-Hinton** | 48 h | | 24 h | |
| | 97.39 ± 3.48 | 97.30 ± 3.95 | 94.43 ± 6.69 | 97.29 ± 2.22 |

## 4. Conclusions

In the present work, two similar HPLC-UV methods were developed and characterized to quantify minocycline or levofloxacin in three different relevant biomimetic media. These methods demonstrated to be simple, with the advantage of only differing in the wavelength setting, and with a performance satisfying the recruitments for good precision, accuracy and recovery according to international accepted criteria. Furthermore, levofloxacin showed acceptable short-term and long-term stabilities in all media, except when it was submitted to more than one freeze-thaw cycle. Minocycline presented a more accentuated degradation profile over prolonged time courses, mainly in media with pH over 6 (PBS and Müeller-Hinton broth). Hence, to get reliable results, it is advisable to analyse samples with minocycline as quickly as possible. Overall, the proposed methods only require minor laboratory work and are considerably cost-effective for being a small time run and a mobile phase mainly water-based.

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