Synthesis of polymeric nanocapsules for levofloxacin delivery against *Staphylococcus aureus*

Rodrigo Manuel Nogueira Nobre

Thesis to obtain the Master of Science Degree in

Bioengineering and Nanosystems

Supervisors: José Paulo Farinha, Professor Doutor (Instituto Superior Técnico, Universidade de Lisboa); Ana Francisca Bettencourt, Professora Doutora (Faculdade de Farmácia, Universidade de Lisboa)

Examination committee

Chairperson: Gabriel António Amaro Monteiro , Professor Doutor (Instituto Superior Técnico)

Supervisor: Ana Francisca de Campos Simão Bettencourt, Professora Doutora (Faculdade de Farmácia)

Members of the committee: Ana Clara Lopes Marques, Professora Doutora (Instituto Superior Técnico)

October, 2018

Acknowledgements

To Professor José Paulo Farinha from the Instituto Superior Técnico and to Professor Ana Bettencourt from the Faculdade de Farmacia da Universidade de Lisboa for giving me the chance to work in their laboratories and experience how the life of a researcher is. For all their dedication in helping and teaching me everything I need to know to have a successful life as a researcher.

To all my laboratory partners, especially Laurinda Areias for helping me settle in the laboratory and guide me in my work. Bárbara Casteleiro for all the help she provided in the writing of this thesis as well as guiding me in times of need and being a good friend. Inês Mariz for helping in obtaining the Fluorescence microscopy images.

To Professor Isabel Ribeiro (Faculdade de Farmacia da Universidade de Lisboa) for helping in the microbiological assays.

To Faculdade de Ciências e Tecnologia of Universidade Nova de Lisboa for teaching me all the basics I needed to have a successful life as a researcher during my bachelor's degree.

To all my friends who always cheered me up in sad moments, always tried to help when needed and provided great moments of fun.

To my family, especially my father and my mother for their great efforts to allow me to study what I always wanted and their support in dire moments.

And finally to you. The one reading this thesis for showing interest in my work. I hope this thesis inspires you or helps you in some way to obtain your goals.

To all the people listed above and even more, I thank you all.

Abstract

In recent years, total bone replacement surgeries are becoming more frequent and problems associated to this procedure such as osteomyelitis are getting more common. Currently, therapeutical approaches consist on the administration of large doses of antibiotics for a long period of time. This is often ineffective due to the low vascularization of the prosthesis and the formation of bacterial biofilms. Particularly, infections associated to *Staphylococcus aureus* are specially challenging.

The aim of the present work was to develop novel poly (methyl methacrylate) (PMMA) nanocapsules containing levofloxacin. Nanocapsules were synthesized through nanoprecipitation by solvent evaporation. Two types of nanocapsules were prepared with two different PMMA molecular weight (120000 and 350000 g/mol). The synthesized nanocapsules were characterized by NMR, UV-Visible spectroscopy, fluorescence microscopy and *in vitro* drug release assays. Blank nanocapsules were also prepared to serve as controls. To evaluate the antimicrobial activity of these novel drug delivery systems towards *Staphylococcus aureus* (ATCC®25923), two tests were conducted. The Kirby-Bauer diffusion and the biofilm inhibition on the surface of a PMMA bone cement. With both types of levofloxacin-loaded PMMA nanocapsules, 120000 and 350000 g/mol, the agar-diffusion results showed an inhibition zone diameter of 35 ± 1 mm and 37 ± 2 mm, respectively. Also, the growth of *S. aureus* biofilms was not verified with both types of nanocapsules proving the *in vitro* antimicrobial efficiency of the newly developed drug delivery systems.

Key-words: Levofloxacin, PMMA, Nanoparticulate-systems, Antimicrobial, Osteomyelitis

Resumo

As cirurgias de substituição das articulações são atualmente um procedimento frequente, assim com os problemas associados a estes procedimentos designadamente a osteomielite. Atualmente, o tratamento não cirúrgico consiste na administração de grandes doses de antibióticos durante um longo período de tempo. Este tratamento pode ser ineficaz devido à baixa vascularização das próteses e da formação de biofilmes bacterianos. Particularmente, infeções associadas a *Staphylococcus aureus* são especialmente desafiantes.

O objetivo do trabalho aqui apresentado consistiu em desenvolver uma inovadora nanocápsula de poli(metil metacrilato) contendo levofloxacina. As nanocápsulas foram sintetizadas por nanoprecipitação por evaporação de solvente. Dois tipos de nanocápsulas foram preparadas com PMMA com diferentes massas moleculares (120000 g/mol e 350000 g/mol) e foram caracterizadas por espectroscopia de RMN, de Absorção UV-Visível, microscopia de fluorescência e ensaios de libertação de fármaco. Nanocápsulas vazias foram também preparadas para ser utilizadas como controlos. Para avaliar a atividade antimicrobiana do novo sistema de veiculação de fármacos contra *S. aureus* (ATCC®25923) foram realizados dois tipos de testes. O teste de difusão de Kirby-Bauer e o teste de inibição de biofilme na superfície de um cimento ósseo de PMMA. Com ambos os tipos de nanocápsulas de PMMA carregadas com levofloxacina, 120000 g/mol e 350000 g/mol, os resultados mostraram halos de inibição de 35 ± 1 mm e 37 ± 2 mm, respetivamente. Salienta-se ainda que o desenvolvimento de biofilmes de *S. aureus* não se verificou com ambos os tipos de nanocápsulas o que prova a eficácia antimicrobiana *in vitro* do novo sistema de veiculação de fármacos.

Palavras-chave: Levofloxacina, PMMA, Nanosistemas particulados, Antimicrobiano, Osteomielite

Index

Index of Figures

Index of tables

Index of abbreviations

A.U – Arbitrary unit

ATCC – American Type Culture Collection

BHI – Brain heart infusion

$CDCl_3$ – Deuterated chlorophorm

CFU – Colony-forming unit

CP – ciprofloxacin

EE – Encapsulation Efficiency

LF – Levofloxacin

LM – Lomefloxacin

MC – metronidazole

MHz – Megahertz

MHA – Mueller-Hinton Agar

NMR – Nuclear magnetic resonance

PBS - Phosphate-buffered saline

PLGA – Poly(Lactic-co-glycolic acid)

PMMA – Poly(methyl methacrylate)

RNA – Ribonucleic acid

RPA – Registo Português de Artroplastia

St. – Saint

TC – Tetracycline for bone marking

TC-R – Tetracycline resistant

TS-C – Tetracycline susceptible

USA – United States of America

UV – Ultraviolet

UV-Vis – Ultraviolet-visible

Aims and Thesis structure

The treatment of infections related to bone procedures such as arthroplasty and others using medical devices remains one of the most serious complications in orthopaedic surgery. *Staphylococcus aureus* is one of the main pathogens associated to this type of infections, mainly due to its ability to form biofilms on the surface of the biomaterials. In this context, the main purpose of this master's thesis is to produce a novel nanoparticulate drug delivery system for the local delivery of antibiotics and to assess its efficiency against *S. aureus.*

This thesis is divided in 5 chapters: Introduction; Materials and Methods; Results and Discussion; Conclusions and Future work.

The first chapter gives an insight of the problem that this thesis is trying to solve and some theoretical aspects about the method of synthesis of the polymeric nanocapsules and why this type of drug delivery system can be a promising strategy to solve the problem of orthopaedic implant associated infections.

The second chapter explains the procedures performed to synthesize and characterize the polymeric nanocapsules as well as the *in vitro* drug release assays and the microbiological tests conducted to evaluate its efficiency against *S. aureus*. This chapter also lists all the materials and equipment used to perform those tasks.

The third chapter describes and discusses the results related to the synthesis and characterization of the polymeric nanocapsules, as well as the nanocapsules' activity against *S. aureus* including biofilms forms (grown on the surface of a bone cement). Results are discussed focusing on the characterization parameters and microbiological aspects which are relevant to the novel nanoparticulate drug delivery system for targeting bone associated infections.

The fourth and fifth chapter refers to the main conclusions of the master's thesis and gives some suggestions to future work in order to optimise the novel drug delivery system and further improving it, respectively.

1. Introduction

As the world population grows in number, so has the average life expectancy. This has increased the amount of diseases related to aging, among those, bone related diseases. As such the replacement of bone and joints is a common orthopaedic procedure (an example of a total knee arthroplasty is represented in Figure 1.1).

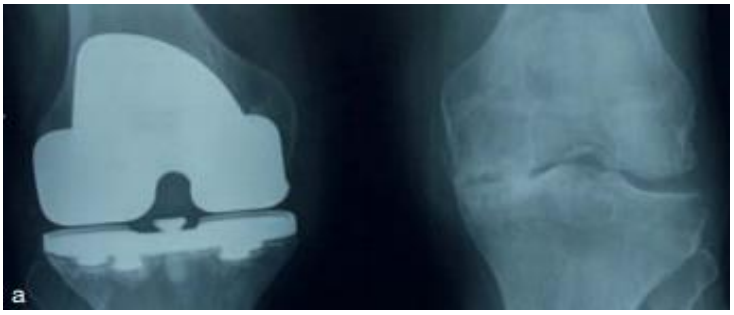

Figure 1.1 - X-ray image of a knee after total knee arthroplasty (arthroplasty is the surgical reconstruction or replacement of a joint) (left); Healthy knee (right) (adapted from Ref. [1]

The number of arthroplasty procedures done in Portugal in 2013 recorded in "Registo Português de Artroplastias (RPA)" were 9223, in which 926 were revision procedures (Table 1.1), with the 61 to 80 years old groups being the most predominant (Figure 1.2). [2]

Table 1.1 – Number of arthroplasty procedures performed in Portugal in 2013 recorded by RPA (Due to registration of the procedure being a voluntary act, these numbers might be higher) (adapted from Ref. [2]*)*

Anatomical area	Primary surgery	Revision surgery
Hip	4440	638
Spine	15	0
Elbow	18	3
Knee	4234	272
Shoulder	224	9
Wrist and hand	59	4
Ankle and foot	7	0
Total	9223	

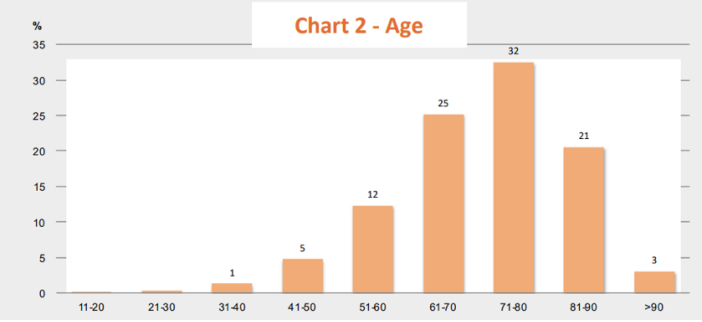

Figure 1.2 – Age groups of the patients in which the arthroplasty procedures were performed in Portugal in 2013 (adapted from Ref. [2]*)*

Often some problems arise with bone replacement and revision of the procedure have to be performed. The infection of prosthetic joints is one of the primary complications of this type of procedures since it can lead to amputation of the infected limb or even death. [3]

1.1. Osteomyelitis and infections associated with medical devices

Osteomyelitis is the infectious inflammation of bone tissue. It is primarily caused by bacteria (particularly *Staphylococcus aureus*)[4] but can also be induced by fungi. [5]

Normally, bone is extremely resistant to infections due to its inaccessibility by infectious agents and in most cases, infection is contracted as a result of deep trauma, bone surgery, bone replacement or the presence of foreign bodies. It can affect every bone of the body and needs a primary source of infection to spread to the exposed bone. [6]

Four types of osteomyelitis have been described: [6]

- Acute osteomyelitis which is described as a fast spreading infection (several days or weeks).
- Chronic osteomyelitis that is a slow, low severity infection of the bone that lasts several months or even years.
- Haematogenous osteomyelitis that affects mainly prepubertal children and elder people which is caused by pathogenic bacteria present in the blood that can nidate on only slightly injured bone.
- Osteomyelitis secondary to vascular insufficiency. In most of the cases, derives from necrotic infections caused by diabetes that spread to the nearby bone.

The propagation of the infection causes the necrosis of the bone tissue and destruction of the bone trabeculae and bone matrix (Figure 1.3). The constriction of the blood vessels due to the inflammation also contributes to the further necrosis of the tissue by limiting the oxygen supply to that area.

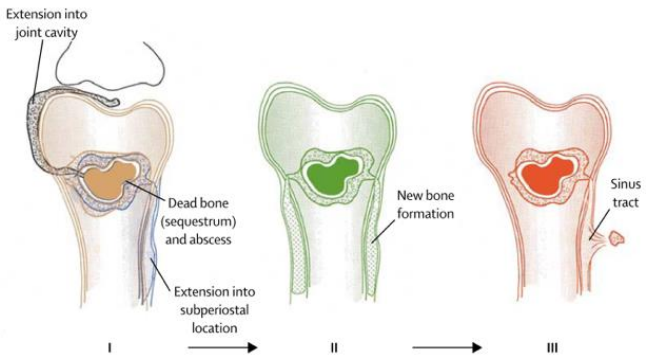

Figure 1.3 - Propagation of chronic osteomyelitis (adapted from Ref. [6]) I – Devascularized dead bone progresses into a intramedullary infection can then spread into a septic arthritis or to a subperiosteal location which can lead to periosteal elevation. II: New bone is formed as a result of massive periosteal elevation. III: Extension of the devascularized dead bone and necrotic material through cortical bone creates a fistula and ultimately, breaks through the skin.

1.2. Difficulty of treatment

Infections associated with medical devices and osteomyelitis are extremely difficult to manage because of the low vascularization of the bone. [6] Moreover, infections on devices are often associated to the presence of bacterial biofilms with increased resistance to antibiotics and to the immune system. [7] Cells in biofilms become 10 to 1000 times more resistant to antimicrobial agents than its planktonic form due to: [8,9]

- Physical and chemical diffusion barriers;
- Slow growth of the biofilm owing to nutrient limitation causing decreased metabolic activity;
- A general stress response corresponding to the development of phenotypes resistant to the antimicrobial agents and a high cell density.

In medical devices, biofilms become more propitious for its formation due to the fact that, in the arthroplasty procedure, extracellular matrix proteins such as collagen, fibronectin and fibrinogen are adsorbed on the surface of the prosthesis to increase biocompatibility, allowing an easier attachment of pathogenic bacteria to the surface and formation of pathogenic biofilms. [6]

Treatment of bone and device associated infections consists on the removal of the dead tissue and/or implant, and intravenous administration of antibiotics for four to six weeks. The treatment is ineffective on several cases because prolonged exposure to antibiotics can cause nephrotoxicity, ototoxicity and gastrointestinal problems. [10] Difficulties in solving these problems reside in: [11]

- The skeletal system is extremely complicated due to several types of cells, complex anatomical nature particularly in the avascular cartilage region;
- Visceral organs consume and eliminate a major part of the bulk antibiotic flowing in the blood stream, causing a small portion of the drug to arrive at the bone tissue;
- Antibiotics are commonly excreted from the body before a significant amount can reach the site of action.

Due to these factors, strategies to counter these situations are being developed, for example based in local drug delivery systems which continuously deliver antibiotics *in situ,* bypassing the need of going through the circulatory system.

1.3. Local drug delivery systems

Drug delivery systems are developed to allow targeting of the drugs to the site of action. [12] For this purpose, several types of drug delivery systems have been developed such as nanoparticles [13], microgels [14], liposomes [15] and microspheres [16], among others. These types of systems can replace the development of new drugs by improving the therapeutical potential of existing drugs. Drug delivery systems allow slow delivery, dose regulation, dose titration (adjustment of the dosage to the patient's response), site-targeting and individualizing the therapy to the patient. [17]

Among local drug delivery systems, the ones with great potential in terms of controlled drug release are particulate systems, such as polymeric nanocapsules (Figure 1.4). By making a polymeric nanocapsule it is possible to have inside any drug intended and control the characteristics of the drug release using different formulations.. [18,19]

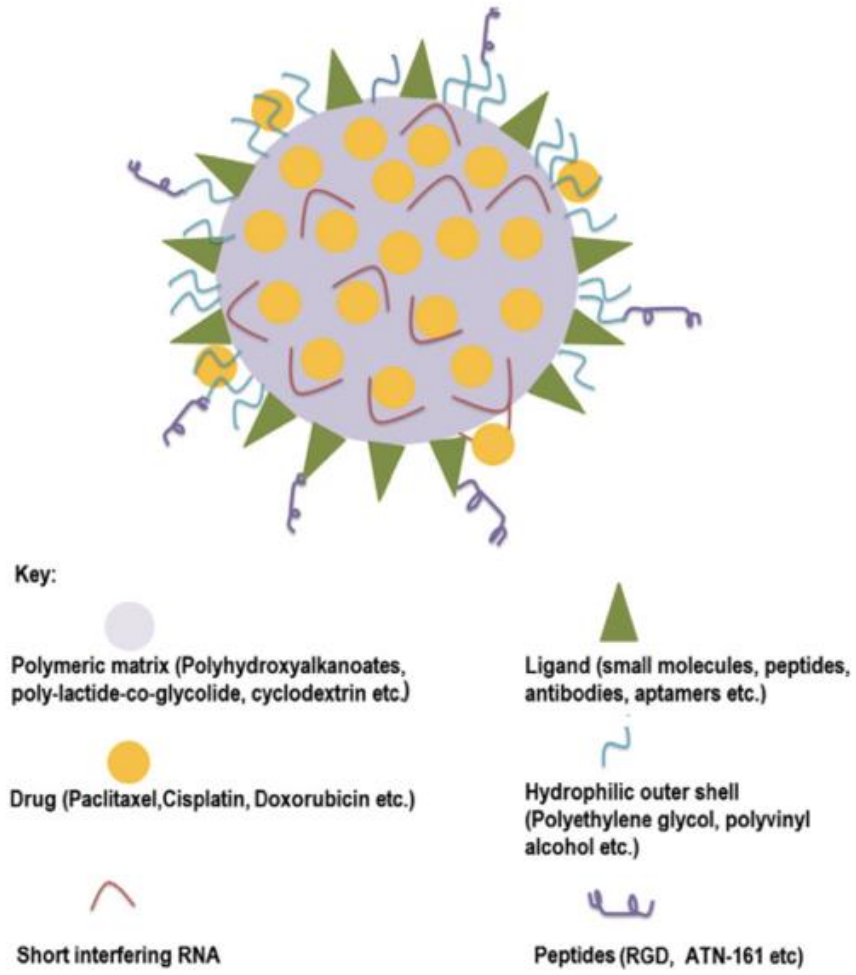

Figure 1.4 – Schematic of a polymeric nanocapsule (adapted from Ref. [20]). Polymeric nanocapsules and nanoparticles consist on a polymeric shell or matrix in which the molecule of interest is encapsulated or incorporated in that matrix (a drug or short interfering RNA for example). Other types of molecules can be absorbed on the surface to allow easier recognition, act on a specific site (for example recognition molecules such as antibodies or small peptides) or just be more hydrophilic and allow extra protection or solubility in the medium.

The controlled release of drugs from polymer capsules can be grouped in three categories: [21]

i) Delayed dissolution in which the drug is release due to the slow degradation of the polymer capsule.
ii) Diffusion controlled in which the drug molecules slowly diffuse through pores in the polymer shell but are unable to freely diffuse due to the insolubility of the polymer capsule.
iii) Drug solution flow control in which water molecules cross the semi-permeable polymeric shell due to high osmotic gradient and solubilize the drug allowing it to flow through the pores in a controlled way.

1.4. Polymer carriers

As for all medical devices, to design polymeric nanocapsules for medical purposes the main characteristic of the polymer is its safety for human use. [22] Multiple types of polymers are currently being used for biomedical applications (Table 1.2).

Table 1.2 - List of polymers commonly used for biomedical applications (adapted from Ref. [23]).

Polymers	**Biomedical applications**
Poly(methyl methacrylate)	Rigid contact lenses, intra-ocular lenses
Polymeric compounds based on methyl methacrylate	Acrylic cements for orthopedy and odontology, facial prostheses, joint surgeries, filling of bone cavities and porous bony tissues
Poly(2-hydroxyethyl methacrylate)	Flexible contact lenses, plastic surgery, hemocompatability of surfaces
Nylon-type polyamides	Sutures
Poly(vinyl chloride)	Blood pushes, catheters
Poly(ethylene terephthalate)	Vascular prostheses, cardiac valves
Polytetrafluoroethylene	Orthopedy, vascular clips
Polyurethanes	Catheters, cardiac pumps
Silicones	Plastic surgery, tubes, oxygenators

One of the most used polymers for nanoparticle design is poly(methyl methacrylate) (PMMA) (Figure 1.5) due to its stability, biocompatibility and ease of manipulation, being inexpensive and versatile. [24] It is also widely used as a prosthetic material for dental applications and in the orthopaedics field as a bone cement. [25]

A bone cement is used for implant fixation by acting as a space-filler creating a tight seal between the implant and the pre-existing bone [26]. PMMA has no registered adverse effects such as oncogenicity and it has been used in local delivery systems of antibiotics since 1970 and some patents have been approved for the use of PMMA as a drug carrier. [24] Due to such characteristics, this polymer was chosen as the carrier for this work.

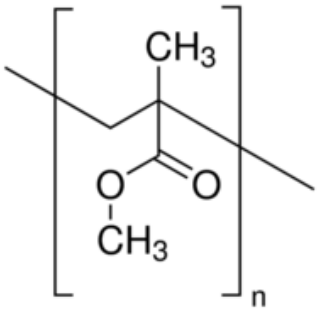

Figure 1.5 – Chemical structure of poly(methyl methacrylate).

The preparation of PMMA particles also influences their characteristics and safety to use in medical applications. Common problems are excess of monomer and radicals (which can cause toxicological problems), the scalability of the process, and type of solvents used (in some techniques, organic solvents can be avoided). Advantages and disadvantages of several PMMA particle preparation techniques are listed in Table 1.3

Table 1.3 – General advantages and disadvantages of different preparation techniques for the synthesis of PMMA particles (adapted from Ref. [24])

Technique	Advantages	Disadvantages
	Polymerization	
Conventional	Fast and easily scale-up method, low polydispersity	Toxicological issues (use of organic solvents, surfactants and initiators; liberation of residual monomers, oligomers and free radicals during polymerization)
SFEP[a)]	Simple technique, low polydispersity, surfactant-free method	Toxicological issues (initiator and residual molecules in polymerization medium)
Micro and mini-emulsions	Possible use of low amount of surfactant; preparation of small size particles (less than 20 nm) with very low polydispersity	Toxicological issues as in conventional method
	Preformed polymer	
Solvent/extraction	Simple and inexpensive technique, use of accessible equipment, possible encapsulation of different types of drugs	Use of organic solvents and surfactants
Nanoprecipitation	Possible encapsulation of hydrophilic drugs, simple technique and easy scale-up	Limited to the use of water-miscible organic solvents, use of organic solvents
Spray-drying	Possible encapsulation of hydrophilic drugs, surfactant free method, easy scale-up	Use of organic solvents
Crystallization	High precision control over particle size, no additives are needed.	Use of organic solvents, expensive equipment.
SCF[b)]	Organic solvent-free method, easily scalable, allows purification of PMMA matrix and loads the drug simultaneously.	Poor yield of production requires high initial capital investment for equipment, complex high-pressure apparatus
a) SFEP – surfactant-free emulsion polymerization; b) SCF – supercritical fluid.		

Nanocapsules with other polymers have also been tested such as nafcillin loaded poly(lactic-co-glycolic acid) or teicoplanin loaded poly(ethylene glycol), poly(lactic-co-glycolic acid) for the treatment of osteomyelitis [10,13] and several more are listed in Table 1.4.

Table 1.4 – Example of different polymeric particulate systems under evaluation for targeting bone infections.

Polymer	Type of system	Antimicrobial agent	Size (nm)	Technique of synthesis	Target microbial agent	Reference
PLGA	Nanoparticle	Levofloxacin	≈200	Nanoprecipitation	*Staphylococcus aureus*	27
Chitosan	Nanoparticle	Levofloxacin	317 -501	Ionic gelation	*None*	28
PLGA	Nanocapsule	Carvacrol	≈210	Solvent displacement	*Staphylococcus epidermidis*	29
PLGA in chitosan gel	Nanoparticle	Sparfloxacin	≈180	Nanoprecipitation	*None*	30
Poly(Ɛ- caprolactone)	Nanocapsule	Tioconazole	≈155	Interfacial deposition	*Candida albicans*	31
PMMA/PMMA+Eudragit RL100	Microcapsule	Daptomycin	1000-1600	Double emulsion solvent evaporation	*Methicilin resistant Staphylococcus aureus*	32
PMMA	Nanofiber	Silver nanoparticles	Not specified, Nanometer range	radical-mediated dispersion polymerization	*Escherichia coli, Staphylococcus aureus*	33

In this context, the aim of the present work was to develop novel antibiotic loaded PMMA nanocapsules. Due to the reasons previously mentioned, the usage of PMMA as a carrier can be an interesting strategy to deliver antibiotics such as levofloxacin to target bone infections.

Nanoprecipitation technique allows to easily form different sizes of particles just by altering parameters such as the amount of surfactant, organic-to-aqueous phase ratio and the stirring rate. [34] Thus, this technique allows us to obtain different sizes of capsules with different characteristics resulting in a possible therapy individualization. Since it does not rely on polymerization, toxicological problems such as excess monomer or radicals are avoided. [24]

1.5. Preparation of nanocapsules

One of the main steps to produce nanocapsules is the miniemulsion's formation (Figure 1.6). To obtain a miniemulsion, two steps must occur: [35]

i. Shearing of the droplets using high energy ultrasounds to obtain smaller droplets with increased surface area;
ii. Stabilization of the newly created droplets using surfactants and co-stabilizers.

This two steps create a miniemulsion of thermodynamically stable droplets that can be used to obtain nanoparticles. [35]

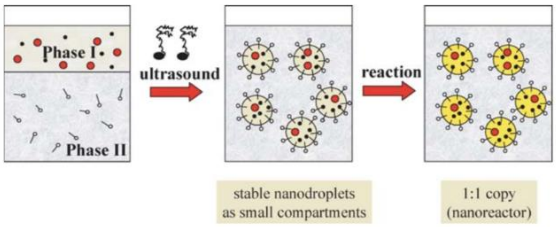

Figure 1.6 - Generic process of miniemulsion. Two separate phases first suffer ultrasonication to shear one of the phases in droplets. These droplets are stabilized by a surfactant and an emulsion is formed. After the formation of the emulsion a reaction can occur to obtain the final product. In the case of the figure, a small reaction occurred inside the droplets resulting in a nanoreactor. In our case we will precipitate PMMA at the interface of the droplets to form a polymer shell (adapted from Ref. [35]*).*

These stable nanodroplets can be designed to have a certain function, by modifying the contents of each phase of the miniemulsion, which grants great versatility to this technique. [35]

The direct miniemulsion (oil-in-water) process consists on having a large volume of aqueous phase when compared with the organic phase. This process obtains droplets of the organic phase emulsified on the aqueous phase. In this type of processes, the droplet size is controlled through the ratio of organic and aqueous phase, density and solubility of the organic phase and the amount of surfactant. Direct miniemulsions are mainly used to obtain droplets with hydrophobic molecules inside. [35]

In inverse miniemulsions (water-in-oil) the larger volume is of organic phase, with a small volume of aqueous phase. It is mainly used to trap in the droplets lipophobic molecules such as ionic compounds, simple salts or sugars. Due to the change of the continuous phase from hydrophilic to hydrophobic, the surfactant must have a low hydrophilic-lipophilic balance to allow droplet stabilization. [35] Figure 1.7 illustrates the differences between both processes of miniemulsion.

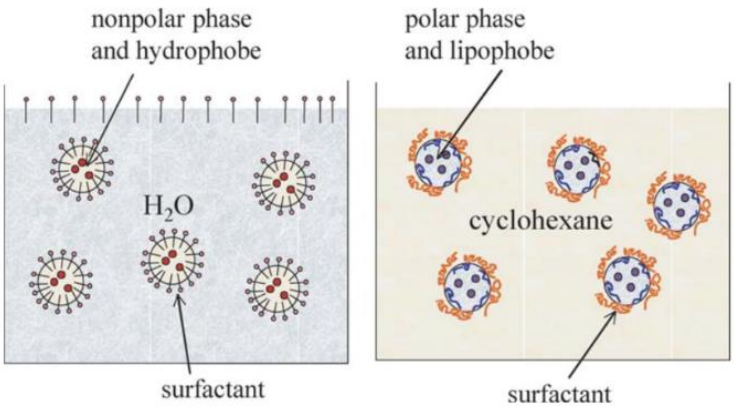

Figure 1.7 - Comparison between direct miniemulsion (left) and inverse miniemulsion (right)(adapted from Ref. [35]*).*

After the miniemulsion preparation, PMMA must precipitate at the interface of the droplets. This is achieved by nanoprecipitation. [34] This technique was chosen since it is fast and easy to reproduce.

Nanoprecipitation occurs when the solvent no longer has the capacity to solubilize the polymer, leading to its precipitation. This can be achieved through variations in the pH, salt concentration or addition of a non-solvent phase. Through the addition of a non-solvent phase, four major steps occur: [34]

1. Supersaturation;
2. Nucleation;
3. Growth;
4. Coagulation.

Supersaturation occurs because the solution no longer has the capability to solubilize the polymer. This can be performed by adding non-solvent phase or evaporating of the solvent phase. After this step, nucleation starts to happen to achieve thermodynamic stability. As the precipitation of the polymer causes the supersaturation state to fluctuate, the primary nuclei increase in size and agglomerates to improve thermodynamic stability. This stops when the supersaturation state is no longer verified. Growth is the next step, happening by condensation or coagulation. Condensation occurs by adsorption of the polymer in solution to the nuclei formed, increasing further in size. Coagulation is the agglomeration of the particles to each other when the attractive interactions are stronger than the repulsive interactions. If the particles grow in size too much, coagulation is always inevitable. [34]

In the present work, this technique was adapted to allow the precipitation at the interface of the water droplets formed by miniemulsion, which generates nanocapsules loaded with the antibiotic present in the aqueous phase.

1.6. Levofloxacin-loaded PMMA nanocapsules

In this work, the nanoparticles are PMMA nanocapsules loaded with levofloxacin (Figure 1.8). It allows a high local concentration of the drug by applying them directly *in situ*, which is optimal for the treatment of osteomyelitis and other infections associated with medical devices. It can help bypass the problems of traditional methods of treatment. [36,37]

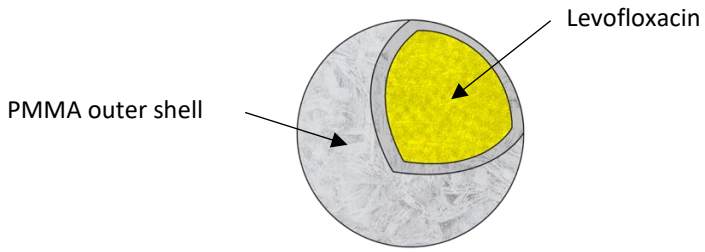

Figure 1.8 - Schematic of the Levofloxacin-loaded PMMA nanocapsules

Levofloxacin is a third-generation fluoroquinolone (Figure 1.9) and it is the L-isomer of ofloxacin with activity against gram-negative and gram-positive bacteria. The potential of levofloxacin as a treatment for various infections such as conjunctivitis, osteomyelitis or keratitis has already been proven. [38]

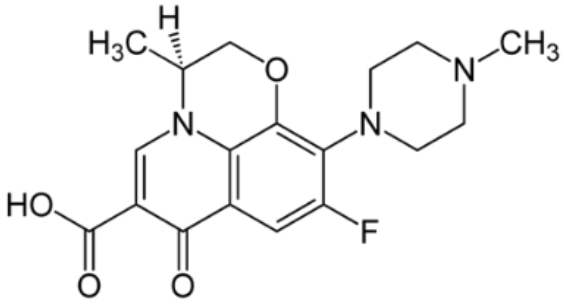

Figure 1.9 - Chemical structure of levofloxacin.

This drug was the selected to be encapsulated since it's a powerful antibiotic in the treatment of osteomyelitis, [39] commonly caused by *Staphylococcus aureus.* [4,6] In Table 1.5, we can verify the efficiency of levofloxacin among other fluoroquinolones for the treatment of osteomyelitis.

Table 1.5 - Results of quinolone treatments performed on patients with osteomyelitis (adapted from Ref. [39]*)*

Patient no.	Antimicrobial drug	Duration of quinolone treatment (days)	Infecting organism[b]	Site of infection	Outcome	Duration of follow-up (mo)	Adverse reaction
81	LF	66	*Peptostreptococcus magnus,*[c] *Streptococcus epidermidis,*[c] *Staphylococcus simulans*[c]	Left tibia	Cure	9	None
82	LF	89	*Enterobacter cloacae,*[c] *Staphylococcus aureus*[c]	Right femur	Cure	18	None
83	LF	42	*Peptostreptococcus species,*[c] *Staphylococcus saprophyticus*[a]	Right third metetarsal head	Cure	24	None
84	LF	38	*Group B streptococcus,*[c] *Pseudomonas aeruginosa,*[c] *Flavobacterium adoratum*[c]	Right calcaneous	Relapse (inadequate debridement)	12	None
85	LF	60	*Staphylococcus hemolytica*[c]	Right femur	Cure	10	None
86	LF, TC	51	*Staphylococcus aureus (TC-S),*[c] *Group B streptococcus (TC-R)*[c]	Left tibia	Cure	3	None
87	LF	86	*Staphylococcus aureus,*[c] *Proteus mirabilis, Enterobacter cloacae, Group B streptococcus, Klebsiella oxytoca (aspirate)*	Left calcaneous	Failure (inadequate debridement)		None
88	LF, MC	56	*Staphylococcus epidermidis (aspirate)*	Left fifth metatarsal	Relapse (inadequate debridement)	5	None
89	LF	52	*Escherichia coli,*[c] *Pseudomonas aeruginosa,*[c] *Morganella morganii,*[c] *Bacteroides species*[c]	Right tibia	Cure	21	None
90	LF	2	*Staphylococcus aureus*[c]	Right media	Treatment stopped due		

				malleolus Left second finger	to tongue swelling and rash		
91	LF, MN	90	*Bacteroides (aspirate)*	Left second finger	Cure	18	None
92	LF	75	*Staphylococcus aureus,*[c] *Pseudomonas aeruginosa*[c]	Right Femur	Cure	12	None
93	LF	44	*Escherichia coli*[c]	Left calcaneous	Cure	14	None
94	LF	39	*Staphylococcus aureus,*[c] *Enterococcus faecalis*[c]	Right great toe	Relapse	5	None
95	LF	60	*Staphylococcus aureus*[c]	Right malleolus	Relapse (inadequate debridement)	3	None
96	LM	28	*Serratia marcescens*[c]	Right femur	Cure	1	None
97	LM	55	*Staphylococcus aureus*[c]	Left femur	Cure	17	Photophobia, dyspepsia
98	LM, MN	16	*Staphylococcus aureus*[c]	Left tibia	Improving when MN started and LM stopped		Clostridium difficile asso-ciated with diarrhea
99	LM	2	*Staphylococcus aureus*[c]	Right knee	Stopped on day 2		Dizziness, nausea
100	LM	44	*Serratia marcescens*[c]	Left ulna	Cure	8	Photosensitivity
101	LM	28	*Staphylococcus aureus,*[c] *Eikenella corrodens, Klebsiella odytoca (aspirate)*	Right second finger	Cure	14	None
102	LM	59	*Escherichia coli*[c]	L4-L5 disc	Cure	0	None
103	CP	52	*Staphylococcus aureus*[c]	Right tibia	Failure		None
104	CP	98	*Staphylococcus aureus*[c]	Left tibia	Failure		None
105	CP	110	*Staphylococcus aureus*[c]	Left fibula	Cure	14	None
106	CP	78	*Staphylococcus aureus*[c]	Left tibia	Relapse	5	None
107	CP	96	*Staphylococcus epidermidis*	Right femur	Cure	36	None

[a] Abbreviations: LF, levofloxacin; CP, ciprofloxacin; LM, lomefloxacin; MC, macrodantin; MN, metronidazole; TC, tetracycline for bone marking; TC-R, tetracycline resistant; TC-S, tetracycline susceptible.
[b] The infecting organism was obtained by culture of a clinical specimen.
[c] Obtained by surgical debridement and biopsy.

Fluoroquinolones are frequently used in bone infections due to their high bone penetration associated with the binding of the quinolone to calcium in the bones. Levofloxacin has the highest value for the median extents in bone penetration of all the fluoroquinolones. [40,41] It has several characteristics that can be advantageous such as not creating mutants resistant to fluoroquinolones in animal models and being effective in monotherapy. Also, levofloxacin has a lower minimum inhibitory concentration for gram positive pathogens when compared with previous generations fluoroquinolones as ciprofloxacin and ofloxacin [42]. Due to levofloxacin also being extremely sensible to light exposure [43], encapsulation of this antibiotic might lead to extra protection overcoming the mentioned problems.

1.7. Characterization of the nanocapsules

Nanocapsule characterization was assessed by size distribution and encapsulation efficiency. Size distribution needs to be evaluated to verify if our system was synthesised as planned. Encapsulation efficiency must be measured to know how much antibiotic our samples contain and if in fact the encapsulation process was successful. The size distribution can be evaluated by using dynamic light scattering and fluorescence microscopy. Encapsulation efficiency and quantification of entrapped drug can be obtained using several spectroscopic techniques such as Fourier-transform infrared spectroscopy [44], nuclear magnetic resonance spectroscopy [45], UV-Visible absorption spectroscopy [46], and liquid chromatography. [47]

To further characterize the nanocapsules, *in vitro* drug release studies were performed. With this type of tests, the drug release profile of the nanocapsules was obtained. Drug release profiles can give some insight on how the nanocapsules will behave in the biological environment. [46,48,49] This type of studies can provide information about the kinetic release rates allowing further improvement of the nanocapsules formulations [49].

1.8. Microbiological assays

A biofilm is an agglomerate of microbial cells irreversibly associated with a surface and enclosed in a matrix of polysaccharides (Figure 1.10) [50]. Biofilms are complex systems. The microbial cells in this type of environments transcribe different genes than their planktonic form which can influence the effect of the antimicrobial agent. [50]

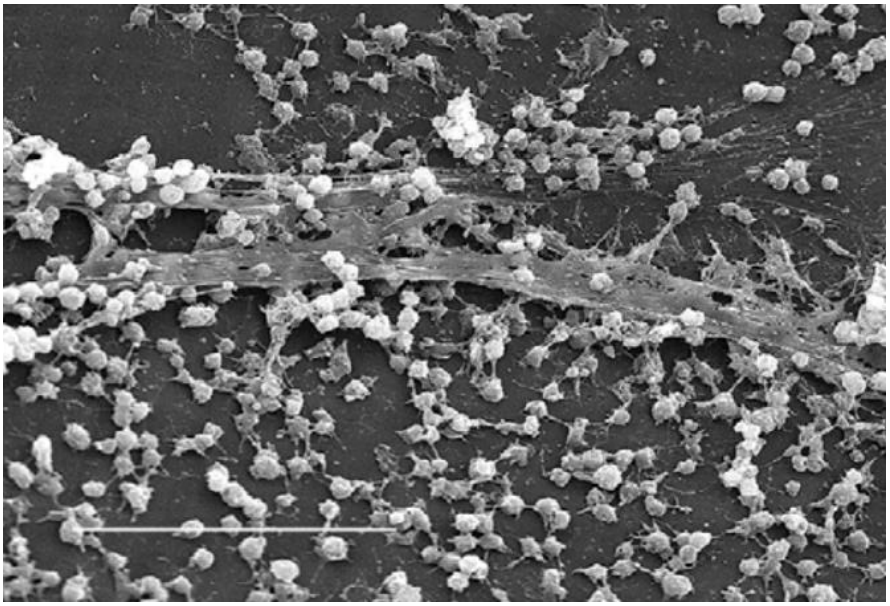

Figure 1.10 - Scanning electron micrograph of a Staphylococcus biofilm (adapted from Ref. [50])

Biofilms are often observed attached to a surface and in the case of osteomyelitis they can either be formed on bone tissue or on an implanted prosthesis. PMMA bone cement is widely used to fill gaps between the existing bone and the prosthesis in order to fix it in place. [26] Since biofilms tend to form on these surfaces, infections are common in bone cement and as such, treatment must be targeted to those biomaterials.

To evaluate the efficiency of the nanocapsules, microbiological assays must be performed. These assays consist on evaluating the effects of the nanocapsules on microbial cultures. Particularly, the effect against biofilms grown on bone cement surfaces is important. The antimicrobial assay against biofilms was necessary due to the fact that *S. aureus* tends to adhere and form biofilms on implanted bone cements. [41] The usage of bone cement for the tests was to resemble as closely as possible an *in vivo* experiment due to the fact that *S. aureus* tends to form biofilms in these biomaterials.

2. Materials and Methods

2.1. Materials and instrumentation

Cyclohexane (spectroscopic grade), poly(methyl metacrylate) (by gel permeation chromatography) (120000 g/mol and 350000 g/mol), dichloromethane (spectroscopic grade) and levofloxacin (analytical standard) were purchased from Sigma Aldrich, St. Louis, Missouri, USA. Pluronic® PE10400 (muster) was purchased from BASF, Ludwigshafen am Rhein, Germany. Each compound was used as received. Sonicator used was a Branson Sonifier 450, Danbury, Connecticut, USA. Centrifuge was a Sigma 2k15 refrigerated laboratory centrifuge, Osterode am Harz, Germany.

The UV-Visible spectrophotometer used was a Jasco UV-660 UV-Vis spectrophotometer with double monochromator and photon multiplier detector and a Peltier temperature control.

Fluorescence Spectroscopy was done in a Horiba Jobin Yvon Fluorolog® 3-22 Spectrofluorometer.

NMR spectroscopy was performed on a Spectro BRUKER AVANCE US+ 300MHz with a probe 5 mm BBFO+ Inverse detection multinuclear dual-broadband with Z-gradients.

Dynamic light scattering measurements was performed on a Zetasizer® Nano ZS model ZFN36000 with 173^{o} detector – 0.3 nm to 10 µm using a 633 nm laser, Malvern Panalytical

The microscope images were recorded with a Leica TCS SP5 confocal fluorescence microscope (DMI6000, Leica Microsystems CMS GmbH) equipped with a CW Argon ion laser (available excitation lines at 458, 465, 488, 496 and 514 nm) and a pulsed Ti:Sapphire (Spectra-Physics Mai Tai BB, 710-990 nm, 100 fs, 82 MHz). A coverslip-corrected water-immersion objective with numerical aperture (NA) = 1.2 and 63x magnification (HCX PL APO CS 63.0×WATER UV) was used.

For microbiological assays, *Staphylococcus aureus* ATCC®25923 strain was obtained from American Type Culture Collection. The diameters were measured using a caliper from Vernier, Beaverton, Oregon, USA

The orbital shaker used in the biofilm experiments was an IKA KS130 BASIC (Merck & Co, Kenilworth, New Jersey, USA). Images of the bone cement to verify the development of the biofilm were obtained by Scanning Electron Microscopy (JSM7001F from JEOL, Ltd., Akishima, Tokyo, Japan).

For the drug release assays, Phosphate-Buffered Saline (PBS) (10x concentrated, BioPerformance certified) (Sigma Aldrich, St. Louis, Missouri, USA) and Tween®20 (For molecular biology) (PanReac AppliChem, Barcelona, Spain) were used as received. For reading the microplates, a FluoStar Omega from BMG Labtech, Ortenberg, Germany, was used. For the thermostatic bath a waterbath from Memmert GmbH + Co. KG, Schwabach, Germany was used.

2.2. Synthesis of nanocapsules

For the synthesis of the PMMA nanocapsules, 12 g of cyclohexane, 9.5 g of dichloromethane, 75 mg of Pluronic® PE10400 and 0.7 g of a PMMA solution at 200 mg/mL in dichloromethane were weighted. Cyclohexane

and dichloromethane were mixed in a dark reaction flask, slowly to allow diffusion of the dichloromethane in the cyclohexane since the solvents are not very miscible. Afterwards, 75 mg of Pluronic® PE10400 were solubilized in 1 mL of dichloromethane and slowly added to the reaction flask. 500 µL of a levofloxacin solution at 39 mM in H_2O was added quickly to the reaction flask and 0.7 g of a PMMA solution at 200 mg/mL in dichloromethane was added drop by drop very slowly to the reaction flask to allow proper diffusion of the polymer. The mixture was sonicated for 2 min at 90% amplitude and 6 output. After the 2 min of sonication, the emulsion was put in a thermal bath at 50 ºC for 45 min under continuous mechanical stirring. The suspension formed was centrifuged for 5 min at 10000 rpm and dried in a vacuum overnight.

The synthesis of nanocapsules without levofloxacin where the volume of levofloxacin solution was replaced with water milli-q to serve as control for the release assays and the microbiological assays was also performed.

2.3. Characterization of the nanocapsules

2.3.1. UV-Visible spectroscopy

A spectrum of levofloxacin was obtained for a sample at 0.0416 mg/mL. The analysis was performed from 250 nm to 800 nm with 200 nm/min scan speed on a quartz cell with 10 mm optical path. It was not possible to perform the analysis in cyclohexane because levofloxacin isn't soluble in cyclohexane.

The spectrum for PMMA was also measured in a solution of 0.765 mg/mL in dichloromethane for the 350000 g/mol molecular weight PMMA and 0.539 mg/mL in dichloromethane for the 120000 g/mol molecular weight PMMA.

Samples of the synthesized nanocapsules for UV-Visible spectroscopy were prepared by dispersing 10 mg of nanocapsules in 3 mL of water and left stirring for 8 h. The obtained solution was filtered with a 0.2 µm Cellulose acetate filter to remove suspended empty capsules and minimize scattering caused by the empty capsules. 120 mg of the filtered sample were diluted with 2.88 mg of water milli-q. Full spectra from 250 nm to 800 nm of the samples was taken to evaluate possible impurities in the samples and quantification was performed with these spectra at 333 nm using the calibration curve prepared (levofloxacin standards used: 1.63 x 10^{-6} M; 2.00 x 10^{-6} M; 4.43 x 10^{-6} M; 6.21 x 10^{-6} M; 8.02 x 10^{-6} M; 1.01 x 10^{-5} M).

2.3.2. Fluorescence spectroscopy

Fluorescence spectroscopy was performed on the samples used for the levofloxacin calibration curve. Excitation spectra were obtained from 240 nm to 450 nm using a 2.00 nm increment. The entrance slit was 1.00 nm, the exit slit was 1.00 nm and first intermediate slit was 1.00 nm (right angle). Emission spectra were obtained from 350 nm to 650 nm with a 2.00 nm increment, 5.00 nm entrance slit and exit slit of 5.00 nm and an excitation wavelength of 330 nm.

For the PMMA nanocapsule samples, the parameters used were the same as for the levofloxacin samples. Fluorescence spectra were also performed on the supernatant of the reaction, first cleaning of the sample and on a second cleaning of the sample, to evaluate how many cycles were needed to clean the sample. The samples used were also the filtered ones previously used for the quantification.

2.3.3. ^{1}H NMR spectroscopy

Samples were prepared using 10 mg of nanocapsules, 1.5 mg of trioxane and 500 µL of $CDCl_3$ to completely dissolve the PMMA shell and release all the levofloxacin present in the capsules. 64 scans were performed on the sample to reveal the peaks of levofloxacin.

2.3.4. Fluorescence confocal microscopy

The sample was resuspended in cyclohexane and dropped in a coverslip. The nanocapsules were imaged using the laser scanning confocal microscope with excitation at 780 nm and the emission was collected from 400 to 700 nm. Images with 1024 by 1024 pixels were collected at a scan rate of 400 Hz per frame.

2.3.5. Dynamic Light Scattering

To evaluate the size of the nanocapsules, a sample right after each synthesis was analyzed since aggregation of the nanocapsules was a frequent problem after a short period of time.

2.4. Drug release studies

500 µL of PBS (Sigma Aldrich, St. Louis, Missouri, USA) + Tween®20 (0.05%) (PanReac AppliChem, Barcelona, Spain) were added to 10 mg of each sample of nanocapsules and left at a water bath for 1, 2, 4, 6 and 24h at 37 ℃. 200 µL of the samples at the end of their corresponding period of time were transferred to a 96-well microplate and absorbance was measured at 333 nm. At least 4 replicates of each sample were evaluated.

Calibration curves were obtained in PBS + Tween®20 (0.05%) to assess the release of levofloxacin from the nanocapsules. The analysis was conducted at 333 nm because PMMA does not absorb at that wavelength. For the tests in 96-well plates, calibration curves were prepared with 65.6, 32.8, 16.4, 8.2, 4.1, 2.05, and 1.02 µg/mL standards of levofloxacin in PBS + Tween®20 (0.05%). The calibration curves were repeated in each assay.

2.5. Agar disk-diffusion test

The Kirby-Bauer diffusion assay was performed according to Clinical and Laboratory Standards Institute (CLSI), (2015)[51]. *Staphylococcus aureus* (ATCC®25923 strain obtained from American Type Culture Collection) inoculum was prepared in culture media (Brain Heart Infusion media) to obtain, approximately $1x10^8$ Colony Forming Unit mL^{-1}. An aliquot was spread into solid Mueller-Hinton Agar (MHA). Furthermore, levofloxacin-loaded nanocapsules of PMMA with 2 different molecular weight (120000 and 350000 g/mol), blank PMMA nanocapsules (average mass = 20 mg) and one paper disk (containing 5 µg of levofloxacin) were positioned on the solid agar. The plate was incubated at 37 °C for 20h and the parameter used to study the antimicrobial activity was the mean diameter of the inhibition zone formed around the disk, after incubation. The diameters of the inhibition halos were measured with a caliper. Assays were performed in three independent experiments.

2.6. Biofilm inhibition assay

For the biofilm inhibition assays, *S. aureus* inocula were prepared from direct colony suspension of selected strains (24 h slants), adjusted to 1.0 McFarland units and further diluted in Brain Heart Infusion (BHI) medium with glucose at 1% (w/V). In each well of the 24-well plates, small PMMA cement (CMW, Johnson) plates were

previously glued and prepared according to Bettencourt *et al.,* (2004)[52]. The final inoculum concentration was 3 $\times 10^6$ CFU mL^{-1}. Suspensions of nanocapsules (20mg/mL, loaded with levofloxacin and blank) were added to the 24-well plates. After incubation, at 37 °C for 24 h under dynamic conditions (160 rpm). The PMMA plates were fixed with different ethanol solutions, 75, 90 and 100% (V/V) for 40 min [53]. Moreover, plain PMMA cement plates were tested with and without inoculated medium to access the assay response to biofilm formation in the absence of an inhibitor and the response to culture media, respectively. Assays were carried out in three independent experiments.

Images of the biofilms were obtained through Scanning Electron Microscopy. To increase the conductivity of the specimens, they were coated with a thin layer of conductive gold film under vacuum in an argon atmosphere.

3. Results and discussion

3.1. Synthesis of nanocapsules

The poly(methyl methacrylate) (PMMA) nanocapsules were successfully synthesised with both the 350 000 g/mol and 120 000 g/mol molecular weight polymers, following the scheme in Figure 3.1.

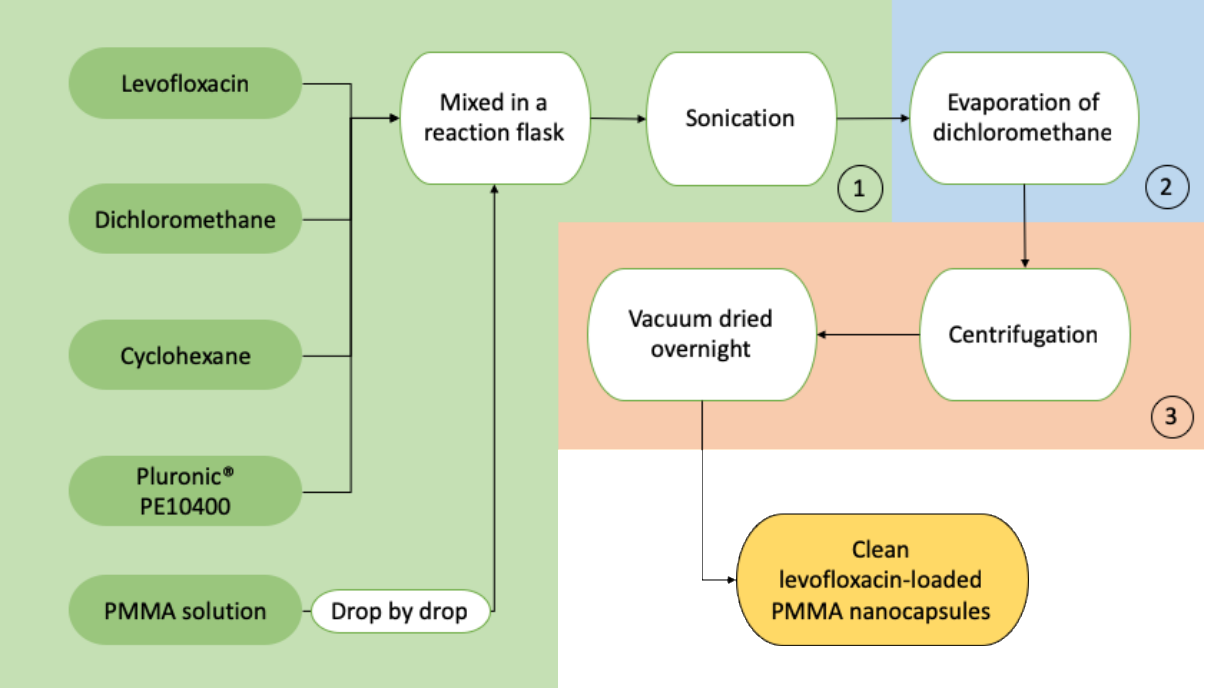

Figure 3.1 - Schematic of the synthesis of PMMA nanocapsules containing levofloxacin through nanoprecipitation by solvent evaporation (1: Preparation of the miniemulsion; 2: Nanoprecipitation of the PMMA; 3: Cleaning of the nanocapsules)

With the 120 000 g/mol PMMA, after sonication a white emulsion was obtained. During evaporation at 50 °C, the emulsion remained white for 40 min and afterwards it turned transparent with a white suspension of nanocapsules visible. Although a layer of polymer was formed in the reaction flask, no agglomerates of PMMA were formed. An average of 40 mg of capsules were obtained for each synthesis. For a total of 7 synthesis, 290 mg of nanocapsules were obtained.

With the 350 000 g/mol PMMA, a white emulsion was also obtained as in the previous polymer. However, during evaporation at 50 °C the emulsion turned transparent after just 2 min. After 45 min, a nanocapsule suspension was obtained and a small agglomerate was found in the bottom of the reaction flask. An average of 57 mg of nanocapsules was obtained. For a total of 6 synthesis, 343 mg of nanocapsules were obtained. For control, nanocapsules without levofloxacin were also synthesized and the results were all similar.

The solubility of the different molecular weight polymers might be the reason for the difference in the agglomerates observed at the end of the synthesis. According to Glöckner, (2000)[54], heavier polymers tend to have a lower solubility and form agglomerates easily. In fact, the 350 000 g/mol PMMA took longer to solubilize in dichloromethane than the 120 000 g/mol (while to solubilize the 120 000 g/mol PMMA around 30 min of stirring was enough, with the 350 000 g/mol PMMA it had to be left stirring overnight). It was also observed that

the amount of nanocapsules in average was lower for the 120000 g/mol polymer. This can be explained due to the higher solubility and slower precipitation of the 120000 g/mol polymer which allowed it to agglomerate in a film at the flask's walls instead of precipitating at the interface of the droplets, decreasing the amount of nanocapsules obtained. [34]

This drug delivery system has the advantage of being simple to synthesise. No degradation of the system is needed as opposing to *Peng et al., (2010)* [36] where the degradation of the microgel was necessary to achieve biocompatibility. This work also has the problem of possible excess monomer still present and free radicals derived from the polymerization process. Our work avoids this problem completely by not having a polymerization step. [24]

3.2. Characterization of the nanocapsules

3.2.1. Nanocapsule size distribution

Dynamic Light Scattering (DLS) measurements show an hydrodynamic diameter of (331 ± 50) nm and (545 ± 358) nm for the levofloxacin-loaded nanocapsules of 120 000 g/mol and 350 000 g/mol PMMA, respectively (Figure 3.2 and 3.3). All measurements were done immediately after synthesis.

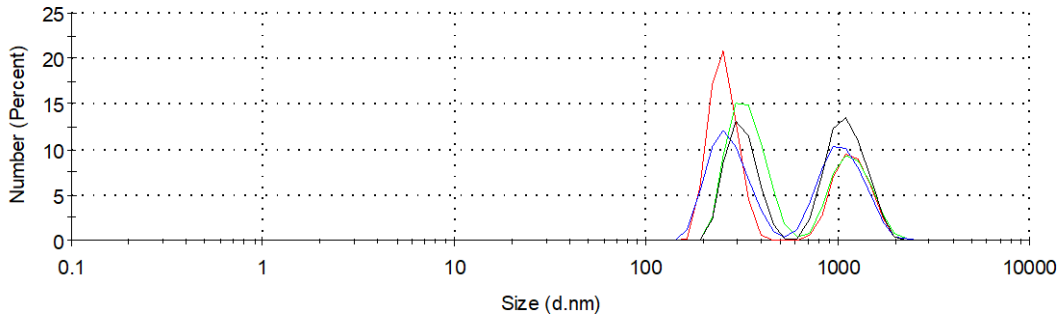

Figure 3.2 - Size distribution by number obtained by Dynamic Light Scattering for the levofloxacin-loaded nanocapsules of 120000 g/mol PMMA (The peak to the right correspond to agglomeration of the nanocapsules in solution. The correct measurement is assumed to be the peak to the left).

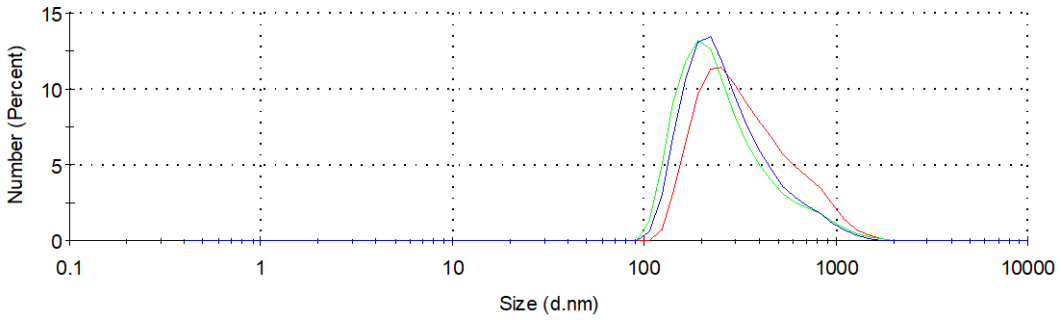

Figure 3.3 - Size distribution by number obtained by Dynamic Light Scattering for the levofloxacin-loaded nanocapsules of 350000 g/mol PMMA

Agglomeration of the nanocapsules in cyclohexane solution was a problem for DLS measurements (Figure 3.2) because the nanocapsules tended to precipitate and agglomerate in solution. It causes the hydrodynamic

radius measured to be larger than the real one since we are measuring two nanocapsules agglomerated instead of a singular one.

The distribution size of the nanocapsules is in the same range as for other nanoparticulate systems described in the literature. For example, the work of Gupta *et al.,* (2011)[27] obtained nanoparticles of approximately 200 nm in diameter. With the work of Bettencourt *et al.,* (2010)[55], the average size of the nanoparticles was approximately 400 nm. By comparing with our PMMA nanocapsules with the same polymer, 350000 g/mol, we verify that our nanocapsules are larger.

3.2.2. Presence of levofloxacin in the nanocapsules

To confirm the presence of levofloxacin in the nanocapsules, fluorescence spectroscopy analysis was also conducted (Figures 3.4 and 3.5), since this compound is fluorescent.

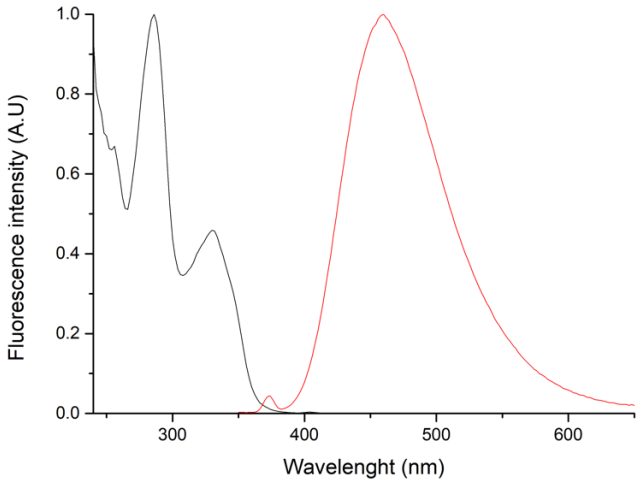

Figure 3.4 – Excitation (black line) and emission (Red line) fluorescence spectra of Levofloxacin in water

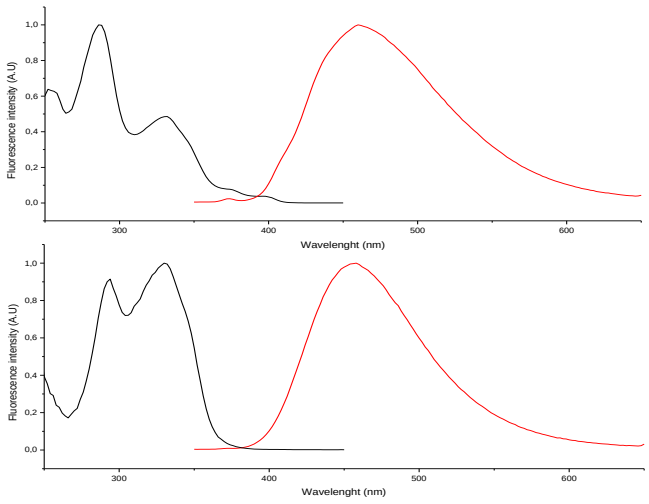

Figure 3.5 - Excitation (Black line) and emission (Red line) of a sample of nanocapsules (Top: 120 000 g/mol PMMA nanocapsules; Bottom graph: 350 000 g/mol PMMA nanocapsules) in water

The fluorescence emission spectra of the capsules in dispersion (Figure 3.5) confirm the presence of levofloxacin in both of them, with the emission band maximum at 460 nm. It corresponds to the values of the pure levofloxacin in solution (Figure 3.4). The increase in intensity of the excitation band at the 330 nm can be due to the formation of aggregates of levofloxacin, possibly due to interaction with the 350 000 g/mol PMMA.

Fluorescence spectrum was measured for the supernatant after each centrifugation to verify how many times the samples needed to be washed with cyclohexane (Figure 3.6). It was shown that only one wash was necessary to remove completely the excess levofloxacin that was not encapsulated.

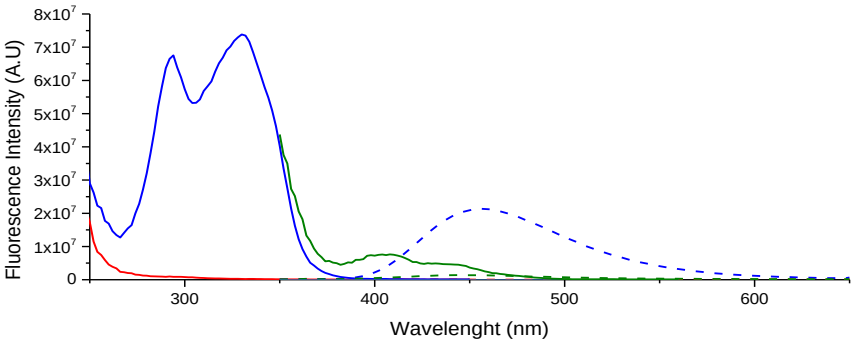

Figure 3.6 - Excitation (continuous line) and Emission (dashed line) fluorescence spectra of a nanocapsule sample (350 000 g/mol PMMA) (Blue), reaction supernatant after centrifugation (green) and after the first wash (red). All samples were measured in cyclohexane.

3.2.3. Quantification of levofloxacin encapsulation

To characterize the synthesized antibiotic loaded nanocapsules, a calibration curve was performed with levofloxacin in water for quantification of the released drug (Figure 3.7).

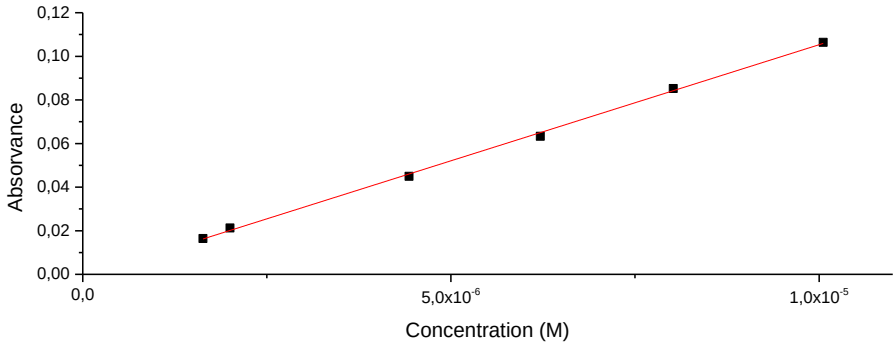

Figure 3.7 - Calibration curve for levofloxacin in water (equation: Absorbance = (10.7 ± 0.1) x Concentration + (1 ± 1) x 10^{-3}; R^2 = 0,9996).

The points for the calibration curve were measured using different well-known concentrations of levofloxacin (Figure 3.8).

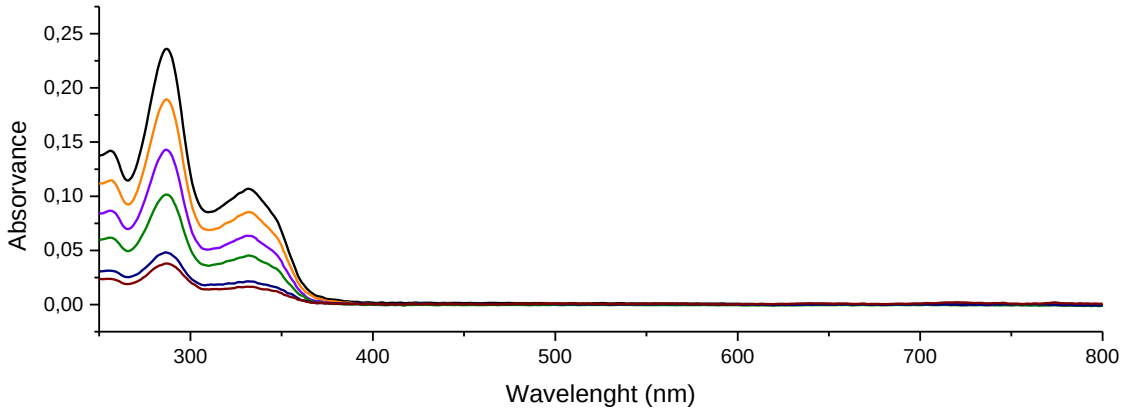

Figure 3.8 - UV-Visible spectra for each sample of levofloxacin in water used to build the calibration line (From top to bottom: 1.63 x 10^{-6} M; 2.00 x 10^{-6} M; 4.43 x 10^{-6} M; 6.21 x 10^{-6} M; 8.02 x 10^{-6} M; 1.01 x 10^{-5} M). Peaks in higher wavelengths can indicate some degradation. No peaks were found meaning degradation is absent (Exposure to light can easily cause the levofloxacin to be degraded).

Using the calibration curve (Figure 3.7) it was later possible to quantify the amount of levofloxacin released by the nanocapsules. By measuring a UV-Visible spectrum of a sample (as explained in section 2.3.1) the amount of levofloxacin was calculated (Figure 3.9).

The sample analysed was the result of multiple syntheses, because the amount of nanocapsules obtained in each synthesis was not enough for the needed tests. Therefore, the obtained results were representative of the sample used for the microbiological and drug release assays.

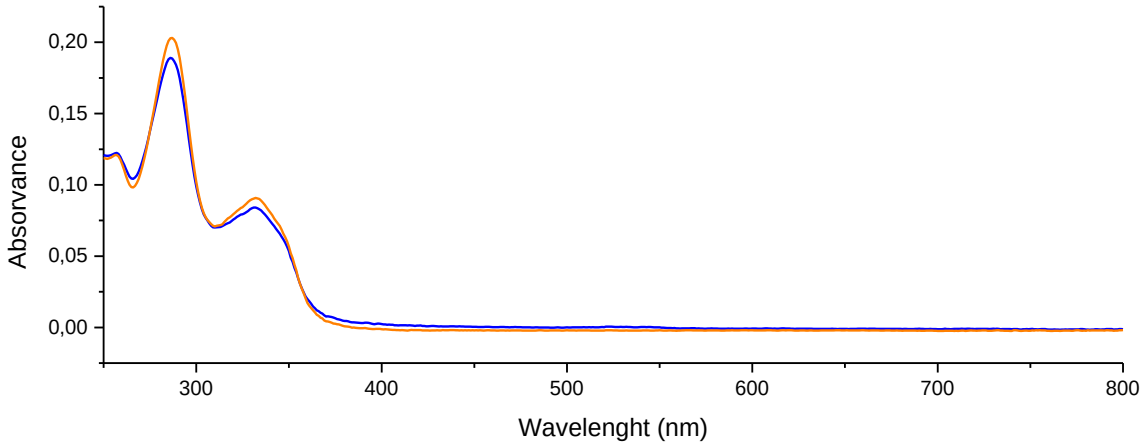

Figure 3.9 - UV-Visible Spectra obtained for the samples (as mentioned in section 2.3.1) for quantification (Orange: 350000 g/mol PMMA nanocapsules; Blue: 120000 g/mol PMMA nanocapsules).

For the nanocapsules synthesized with the 120 000 g/mol PMMA, the amount of levofloxacin calculated was 0.08136 mg/10 mg of nanocapsules (encapsulation efficiency (EE) = 5%). For the nanocapsules synthesized with the 350000 g/mol PMMA, the amount of levofloxacin calculated was 0.08182 mg/10 mg of nanocapsules (encapsulation efficiency (EE) = 7%).

The results show that the encapsulation efficiency is low. This can be explained due to the destabilization of the emulsion during the evaporation process. Pluronic® PE 10400 is more soluble in cold water than in hot

water. [56] The evaporation of dichloromethane destabilizes the droplets agglomerated because the surfactant is not soluble in cyclohexane, further destabilizing the miniemulsion.

Although the encapsulation efficiency was low, the amount of encapsulated drug was enough to be quantified by UV-Visible spectroscopy (Figure 3.9). The levofloxacin that was not encapsulated can be easily recovered for further use.

3.2.4. Purity of nanocapsule content

To further evaluate the nanocapsules, NMR spectroscopy was performed to verify if the organic solvents were present in the samples. The presence of organic solvents can be toxic for living organisms because they disrupt the function of the lipid membrane of the cells as a barrier. [57] Cyclohexane can cause several health problems for living organisms, for example, in humans can cause central nervous system depression, drowsiness, irritability, dizziness, gastrointestinal disturbance, lung irritation, chest pain and pulmonary edema [58].

NMR was also performed to check if degradation of levofloxacin did not occur during synthesis, because this leads to loss of antimicrobial activity and can be an health hazard. [59] It was also performed for levofloxacin dissolved in $CDCl_3$ (Figure 3.10) to compare with the nanocapsules' spectra.

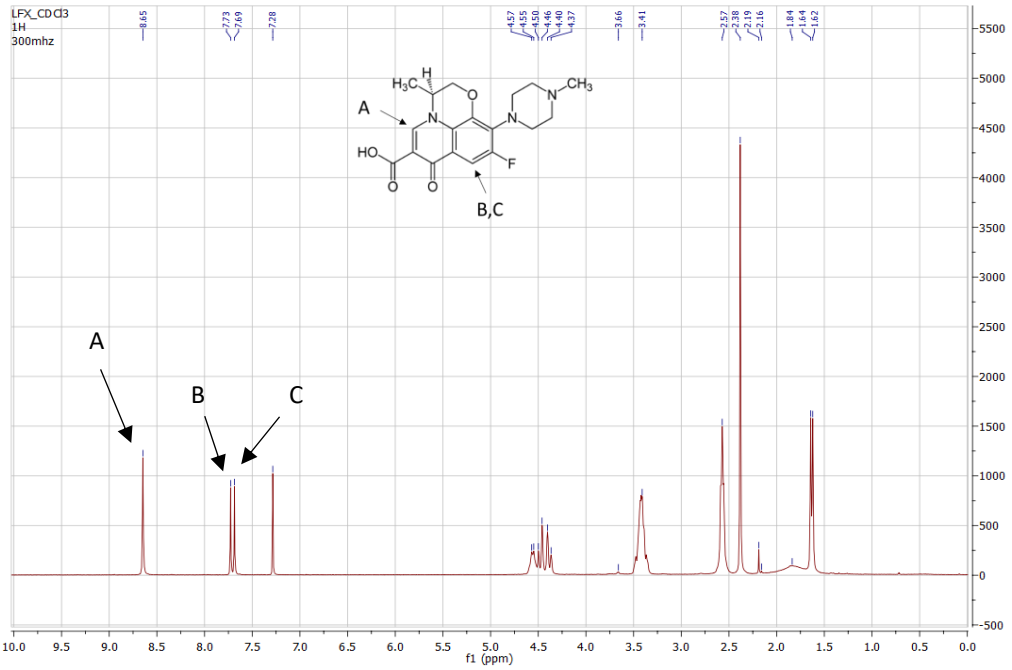

Figure 3.10 - ^1H NMR spectrum of levofloxacin in $CDCl_3$. A, B and C mark levofloxacin peaks.

In the spectrum of Figure 3.11, we can verify that levofloxacin is present in the capsules by the 3 peaks between 9 and 7 ppm which correspond to the peaks of levofloxacin (Marked in Figure 3.10, 3.11 and 3.12 as A, B and C). The peak at 8.1 ppm is not found in the free form of levofloxacin due to its low intensity. The same was

verified for the 350 000 g/mol PMMA capsules (Figure 3.12), although here there is some overlapping with the PMMA peaks.

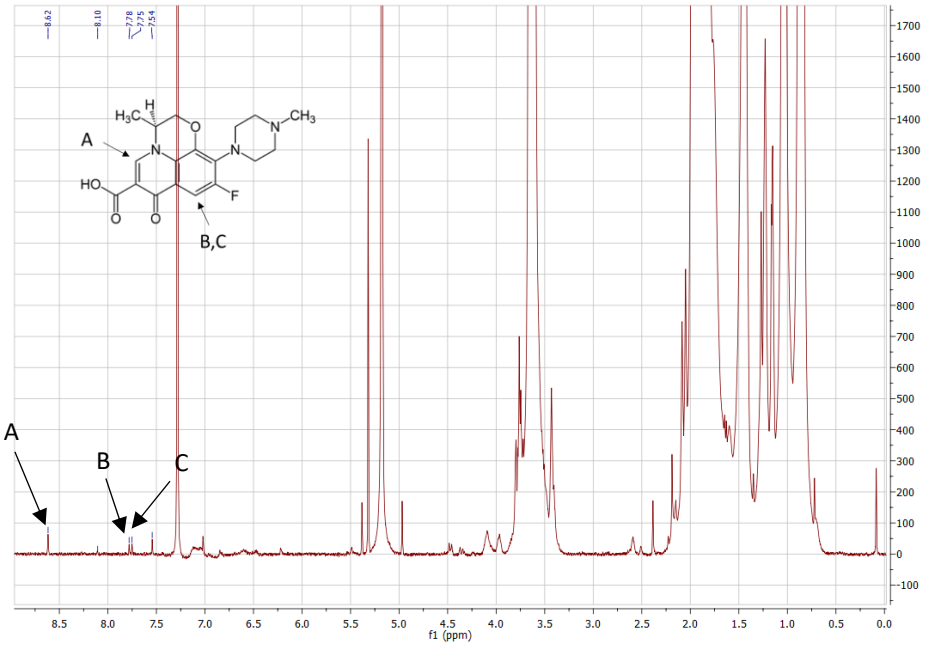

Figure 3.11 - ^{1}H NMR Spectrum of 120000 g/mol PMMA nanocapsules in $CDCl_3$. A, B and C mark levofloxacin peaks.

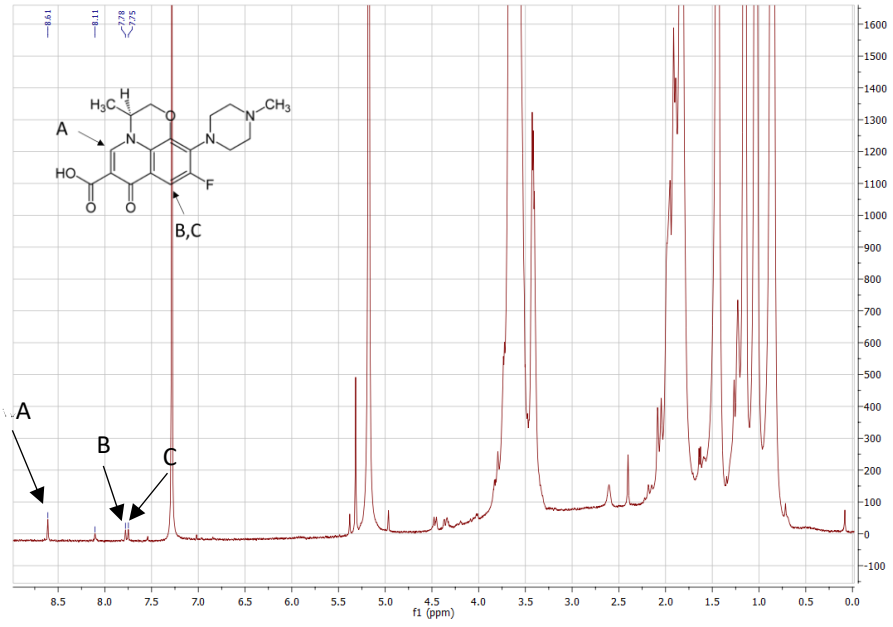

Figure 3.12 - ^{1}H NMR spectrum of 350000 g/mol PMMA nanocapsules in $CDCl_3$, A, B and C mark levofloxacin peaks.

The peak at 5.3 ppm corresponds to the peak of dichloromethane in $CDCl_3$ showcasing that a small portion of organic solvent is still present in the sample. [60] The presence of other possible trace impurities such as cyclohexane cannot be evaluated due to the peaks being overlapped with the PMMA peaks (Cyclohexane NMR peak in $CDCl_3$ corresponds to 1.4 ppm). [60] However, due to the results of the microbiological studies performed (further information in section 3.4) we can establish two possibilities: the dichloromethane present in the

samples evaporated by the time the microbiological studies were performed, or the quantity of dichloromethane present was not significant to cause an effect on those studies. We assume these hypotheses because growth of *S. aureus* was verified with the blank nanocapsules which underwent the same process of synthesis as the levofloxacin loaded PMMA nanocapsules.

Gupta *et al.,* (2011)[27] only needs acetone and water for the synthesis while our levofloxacin-loaded PMMA nanocapsules rely on the use of two organic solvents. This translate on the presence of dichloromethane on the NMR spectrum previously explained. Their synthesis process resulted in smaller nanoparticles which was relevant to their work. Larger nanoparticles induced higher tear formation and faster removal of the dose administered. Gupta *et al.,* (2011)[27] nanoparticles were designed to treat ocular infections. Due to the ocular area being an extremely sensible zone to irritation, biocompatibility and the absence of organic solvents were important factors.

3.2.5. Fluorescence Confocal Microscopy

The images of fluorescence microscopy reveal the encapsulation of levofloxacin inside the nanocapsules (Figure 3.13). Encapsulated levofloxacin was however difficult to observe due to photobleaching during image acquisition.

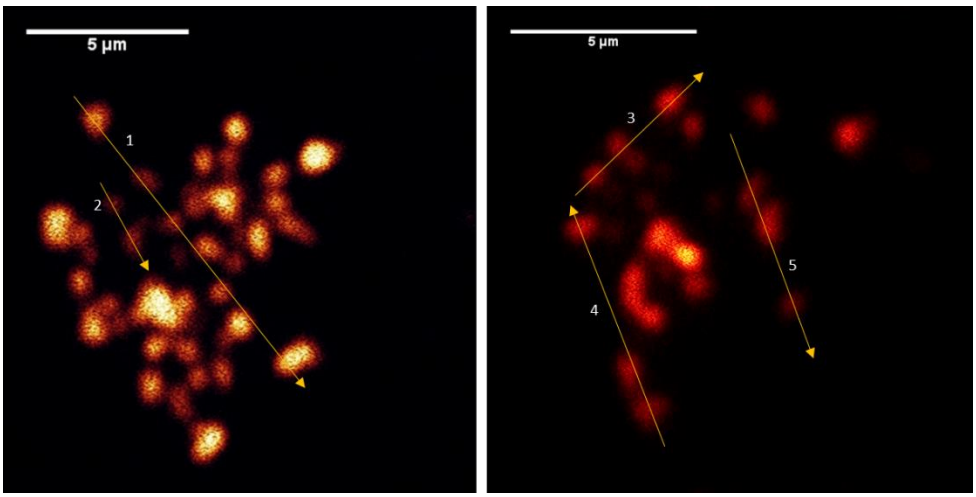

Figure 3.13 – Fluorescence microscopy images of 120000 g/mol PMMA nanocapsules (yellow lines corresponds to where an intensity profile was traced).

We can estimate average sizes through the fluorescence intensity profile and apply a Gaussian fit using multiple peaks to obtain the full width at half maximum. This gives the size of the nanocapsule represented by each peak. Average value of the sizes measured is 658 ± 34 nm (Full list of values obtained, and graphics are in Appendix 1). This diameter is higher than the hydrodynamic diameter measured by DLS due to the limit of detection of the fluorescence microscope. [61] From DLS measurements we verify that the nanocapsules size is below the detection limit of fluorescence confocal microscopy. [61]

3.3. Drug release studies

An overview of the method used to synthesise the nanocapsules is shown in Figure 3.14. The dry nanocapsules were dispersed in a medium of Phosphate-buffered saline containing Tween® 20 and left incubating for up to 24 hours to allow the levofloxacin to be released into the medium. After a determined period of time, a portion of the medium was recovered to a 96-well microplate and absorbance was read at 333 nm. Using a calibration curve prepared in the same 96-well microplate, quantification of the levofloxacin released was performed.

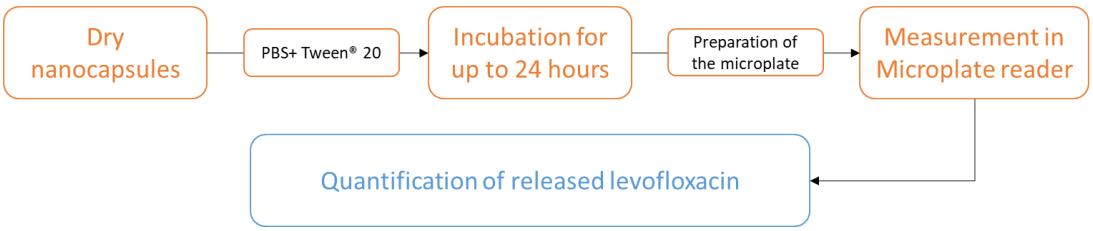

Figure 3.14 - Scheme of the release assays' procedure. Were conducted in a biomimetic fluid (PBS) and a surfactant (Tween® 20), at physiological temperature (37 ℃).

The drug's release profile from the 120 000 g/mol PMMA nanocapsules is shown in Figure 3.15. Up to 6 hours, most of the load was released to the medium. Afterwards, the drug release rate decreased until the 24-hour mark. This delay in release is due to a diffusion process through the polymeric shell causing the levofloxacin to slowly be solubilized and spread to the involving medium, according to Lee and Yeo, (2015)[62]. When comparing the concentration achieved at 24 hours and the minimum inhibitory concentration of levofloxacin (0.25 mg/mL)[63], we can relate that 10 mg of nanocapsules in 0.5 mL is not enough to inhibit the activity of *S. aureus.*

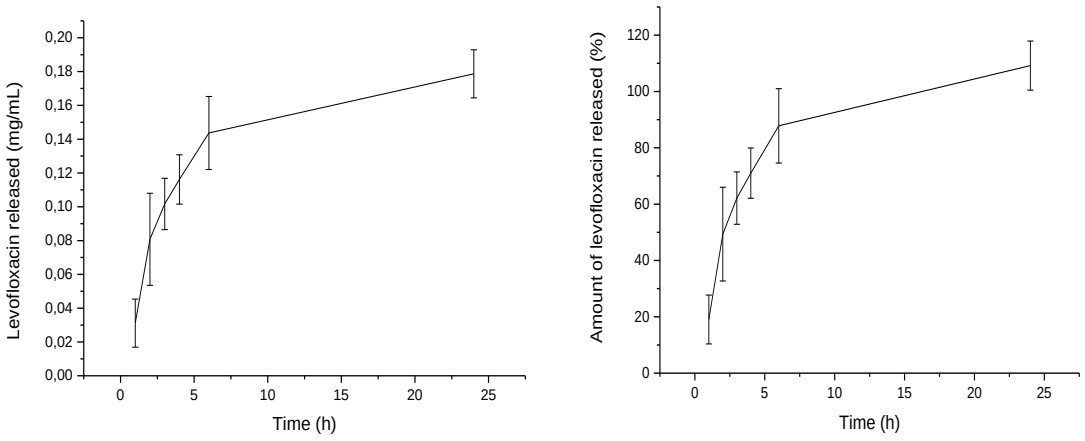

Figure 3.15 – Release profile of the Levofloxacin-loaded 120000 g/mol PMMA nanocapsules performed for 1, 2, 3, 4, 6 and 24 hours. Results are presented as mean ±SD (left: release profile in mg/ml per unit of time; right: release profile in percentage per unit of time).

Despite all the microbiological tests against *S. aureus* (further information in section 3.4) for the 350 000 g/mol PMMA nanocapsules being successful, for the drug release studies we decided to abandon these nanocapsules. *In vitro* release studies were not feasible due to the difficulties in resuspending the capsules in

the medium, which resulted in inconsistent data. Difficulties in obtaining a homogeneous powder, which was easily obtained with the 120 000 g/mol PMMA nanocapsules, was also one of the reasons not to perform those tests with the nanocapsules obtained with the 350 000 g/mol PMMA.

By comparing the spectrum of levofloxacin after 24 hours of release (Figure 3.16) and of the pure levofloxacin previously traced (Figure 3.7) we can verify that no drug degradation occurred.

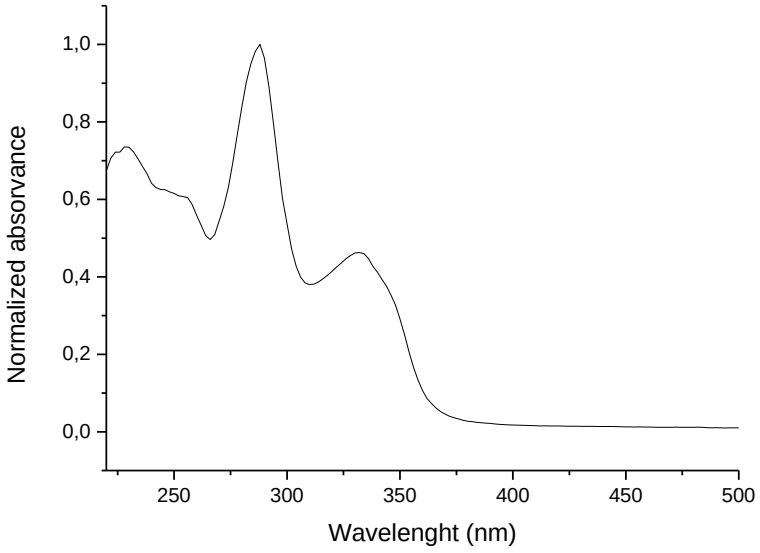

Figure 3.16 - UV-Visible spectrum of the released levofloxacin after 24 hours.

In terms of drug release, our system behaves as fast in releasing the drug to the medium as other previous works such as Pillai *et al.,* (2008)[13]. In both studies, the drug release profiles are similar, showing a high release in the first hours and stabilizing towards the 24-hour mark. The same can be verified for the work of Gupta *et al.,* (2011)[27]. In comparison with the work of Bettencourt, *et al.,* (2010)[55], although it was a different PMMA drug delivery system with a different compound (tocopherol acetate), the *in vitro* release assay methodology was the same. In that work, we can verify a much slower release profile which stabilized at 80 hours. When those PMMA particles were mixed with bone cement, only 37% of the load was released instead of the full load. Our system is much faster releasing the encapsulated levofloxacin than the one in Bettencourt *et al.,* (2010)[55], it only needs 6 hours to release the major part of the encapsulated antibiotic. This can prove advantageous to acute osteomyelitis since a faster release of the antibiotic is needed because it is a fast spreading infection. Instead, for the work of Bettencourt *et al.,* (2010)[55], which is about the delivery of a vitamin (tocopherol acetate), the slow release is appropriate to avoid excretion from the body and be maintained in adequate concentration for a long period of time. In the work of Iannitelli *et al.,* (2011)[29] that consisted on carvacrol-loaded poly (DL-lactide-*co*-glycolide) (PLGA) nanoparticles we verified a similar release profile with ours. A large amount of the antibiotic (in their case, carvacrol) was released after 6 hours and all the load was released at the 24-hour mark. This was important for this application because carvacrol only has an antimicrobial and biofilm-destabilization effect within the first 3 hours of exposure. For our work this is not as important but levofloxacin still needs a concentration of 0.25 mg/mL to achieve the minimum inhibitory concentration (MIC) against *S. aureus*. [63]

Because of this, it is required to obtain that minimum concentration as fast as possible and maintain it. Levofloxacin-loaded chitosan nanoparticles from Gevariya *et al.,* (2011)[28] display a linear drug release profile opposed to our drug release profile. This allows the supply of a consistent amount of levofloxacin, as opposed to our system in which a majority of the drug is released in the first 6 hours. Our system maybe more adequate to cases in which a rapid release of drug is necessary opposing a steady inflow of antibiotics *in situ.*

3.4. Activity against *Staphylococcus aureus*

3.4.1. Agar disk-diffusion test

The agar or Kirby-Bauer diffusion test (Figure 3.17) was successful for a 24-hour period showing inhibition halos for both types of levofloxacin-loaded PMMA nanocapsules. The inhibition halos displayed dimensions of (35 ± 1) mm and (37 ± 2) mm for the 120 000 g/mol and 350 000 g/mol molecular weight PMMA nanocapsules respectively.

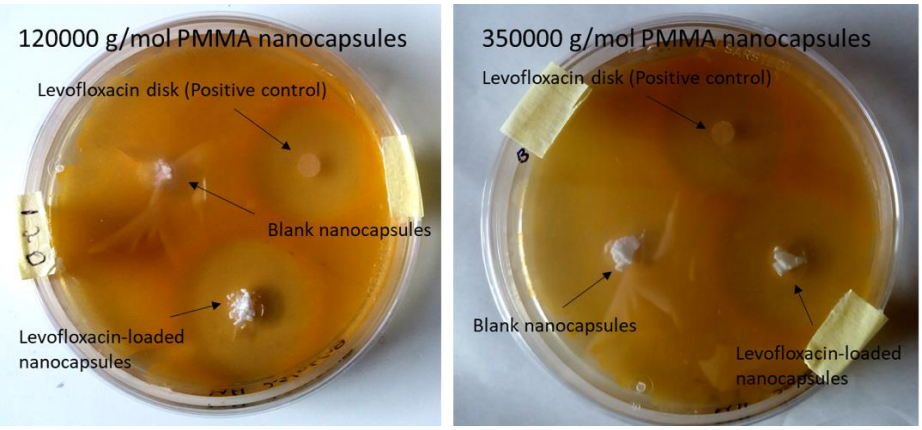

Figure 3.17 – Kirby-Bauer diffusion test results showing inhibition of the growth of S. aureus in the vicinity of the nanocapsules and positive control. No inhibition was verified in the vicinity of the blank nanocapsules.

The Kirby-Bauer diffusion test confirmed the efficiency of the nanocapsules in inhibiting the growth of *S. aureus* in an agar media. This result shows that our nanocapsules allow the diffusion of the antibiotic on a semi-solid medium. Similar inhibition results were verified by Ferreira *et al.*, (2017)[41] with the same antibiotic released from a PMMA cement against the same bacterial strain.

The Kirby-Bauer diffusion test gives an insight on how effective our drug delivery system and has the advantages of being fast (only takes 24 hours), easy to perform (non-expensive or specialized equipment needed) and gives a direct correlation between the activity of the aqueous solution of the tested antibiotic and the one released from the drug delivery.

By correlating the quantity of free antibiotic and the quantity of antibiotic encapsulated in the 20 mg of PMMA nanocapsules, we can verify that the amount encapsulated was much higher (approximately 162 μg/20 mg of nanocapsules) than the amount of levofloxacin in the paper disk (5 μg). The fact that the inhibition halos are similar in size can be explained by the difficulty in the diffusion of the encapsulated levofloxacin in the semi-solid medium of the test. Nevertheless, the main purpose of this test was to show that the encapsulated

antibiotic was not affected by the synthesis procedure, maintaining its antimicrobiological activity once encapsulated.

3.4.2. Biofilm inhibition assay

The microbiological activity of the nanocapsules was also assessed by an inhibition biofilm assay test (more information in section 2.6).

SEM images showed the inhibition of the formation of *S. aureus* biofilms on bone cement when exposed to the Levofloxacin-loaded PMMA nanocapsules (Figure 3.18). We can verify the formation of biofilm with the PMMA nanocapsules without levofloxacin (Figure 3.18 – B and D) and in the positive control (bone cement without any of our levofloxacin-loaded PMMA nanocapsules, Figure 3.18 - A). In the images corresponding to the levofloxacin-loaded PMMA nanocapsules (Figure 3.18 – C and E) it is visible that no biofilm was formed. We conclude that the antimicrobiological effect came from the action of levofloxacin since there was biofilm formed in the presence of blank nanocapsules and no biofilm formation was verified in the presence of the loaded nanocapsules.

On the image regarding the bone cement with the 120000 g/mol PMMA nanocapsules (Figure 3.18 - C) some bacteria are visible, but it is assumed as it is only vestigial bacteria and will not develop a biofilm.

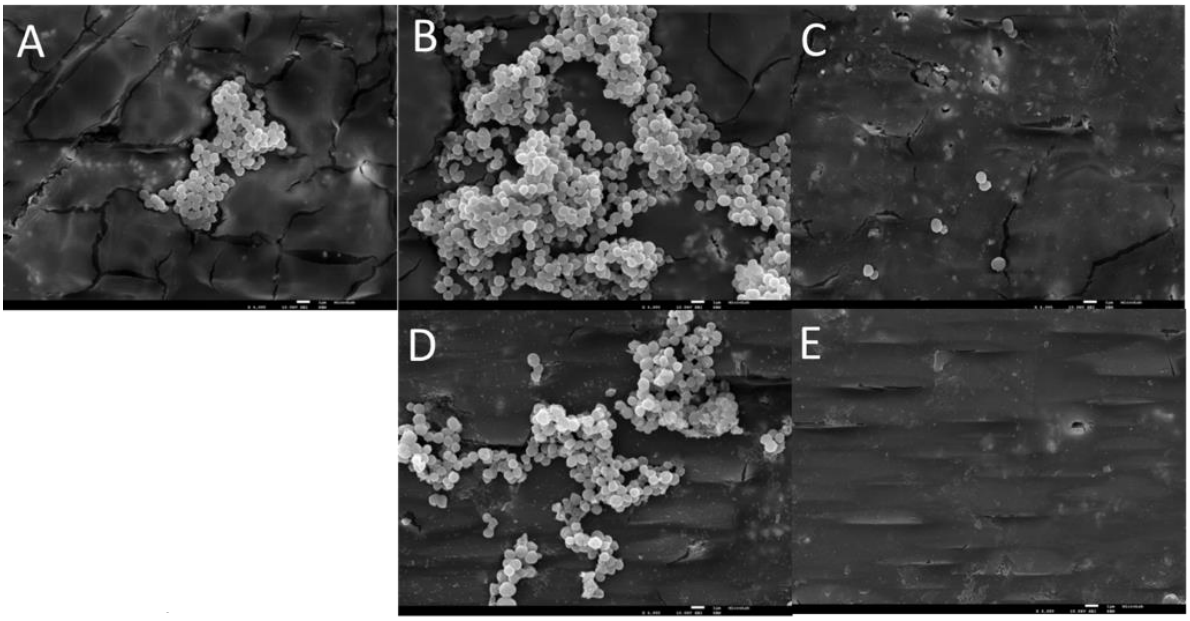

Figure 3.18 - Scanning Electron Microscopy images of the bone cements with the various samples of PMMA nanocapsules (Scale: 1 μm). (A: Bone cement without any nanocapsules and antimicrobial agent; B: Bone cement with blank 120000 g/mol PMMA nanocapsules; C: Bone cement with levofloxacin-loaded 120000 g/mol PMMA nanocapsules; D: Bone cement with blank 350000 g/mol PMMA nanocapsules; E: Bone cement with levofloxacin-loaded 350000 g/mol PMMA nanocapsules) The white spheres are Staphylococcus aureus *bacteria.*

Inhibition of *S. aureus* biofilm was also verified in Ferreira *et al.*, (2017) after exposure to levofloxacin loaded PMMA bone cement. However, in the mentioned study the drug was not encapsulated. Instead it was loaded directly in the bone cement. Our work is designed to site-targeting the release of levofloxacin and inhibit the formation of biofilms on the surface of a commercial bone cement. Moreover, our work can be applied

directly to bone instead of having the need to apply a bone cement. This is useful to prevent infections in situations that the application of a bone cement is not needed contrary to the work of Ferreira *et al.,* (2017) in which it is obliged to apply a bone cement to obtain the desired inhibition effect.

The applications of the novel levofloxacin-loaded PMMA nanocapsules can be various as suggested in Figure 3.19. Nanocapsules can be applied directly on a bone to treat an existing infection or on a prosthesis/bone cement to also prevent and/or treat infections. This novel drug delivery system allows the treatment and prevention of bone infections despite the previous procedures if performed to treat a previous bone related problem (fracture, bone removal and application of a prosthesis are some examples).

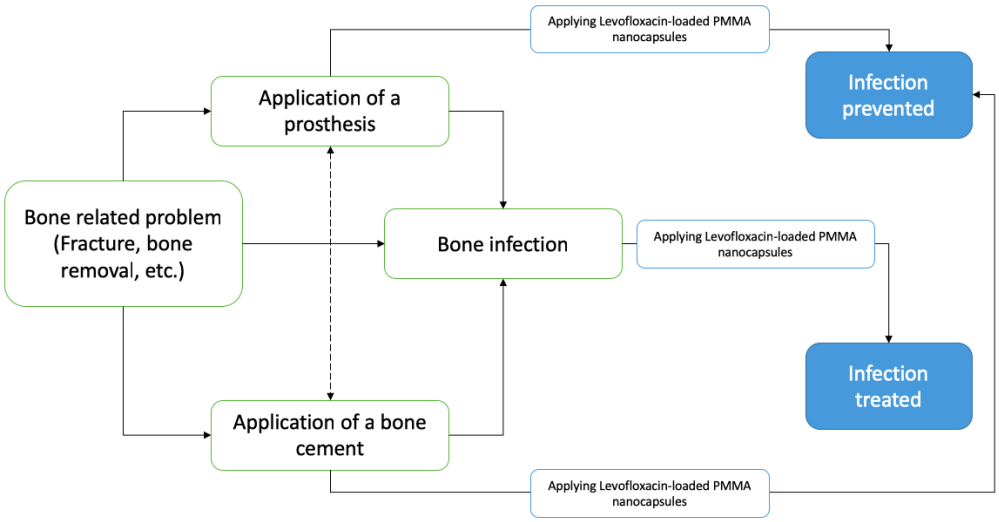

Figure 3.19 – Scheme of the potential applications of the novel levofloxacin-loaded PMMA nanocapsules. Levofloxacin-loaded nanocapsules can be applied directly in the bone or applied on a bone cement/prosthesis to prevent or treat infections.

Overall, the microbiological tests showed that the novel levofloxacin loaded PMMA nanocapsules were highly effective against *S. aureus* as assessed by the agar-diffusion (Figure 3.17) and biofilm inhibition assays (Figure 3.18). Also, *S. aureus* growth in both tests was verified with the blank nanocapsules proving that only the antimicrobial agent was in play on inhibiting the growth of *S. aureus*.

Through the drug release assays, we acknowledged that 10 mg of nanocapsules were not enough to achieve the minimum inhibitory concentration. However, the 20 mg of nanocapsules used for the antimicrobial tests were enough to achieve inhibition of *S. aureus* as verified through the disk diffusion and biofilm inhibition assays (Figure 3.17 and 3.18).

The efficiency of the local drug delivery system against *S. aureus* was proven and both microbiological tests allow us to perceive that the levofloxacin was not affected by the process of nanoencapsulation. It retained its main function as an antimicrobial agent and that it was indeed encapsulated with success.

4. Conclusions

The synthesis of nanoparticulate drug delivery system with activity against *S. aureus*, a main pathogenic associated with bone infections was successfully achieved, comprehending the main goal of this work.

Two types of PMMA nanocapsules containing levofloxacin were prepared, with polymer of 120000 g/mol and 350000 g/mol molecular weight. Both exhibited similar characteristics in terms of the quantity of levofloxacin encapsulated and hydrodynamic radius. We also concluded that the process of synthesis was easily reproducible and fairly optimized. Through UV-Visible spectroscopy it was possible to confirm the presence of levofloxacin in our nanocapsules and quantify that amount, resulting in approximately 81 μg per 10 mg of nanocapsules with both types of PMMA (350000 g/mol and 120000 g/mol molecular weight). We also confirmed that cyclohexane did not remove any encapsulated levofloxacin and as such, the nanocapsules could be washed with cyclohexane which is removed before usage.

Fluorescence microscopy confirmed that the design firstly planned which was to encapsulate the levofloxacin in a PMMA nanocapsule, was achieved with success. We verified the presence of levofloxacin inside the nanocapsules. NMR spectroscopy also confirmed the presence of levofloxacin in the PMMA nanocapsules and the absence of residual organic solvents from the synthesis process. This is important due to the toxicity of these solvents to living organisms since they destabilize the lipid membrane, affecting its function.

Drug release studies conducted with nanocapsules prepared with the 120 000 g/mol polymer gave us an insight on how the nanocapsules behaved in an aqueous environment. The released load was assessed over a 24-hour timespan, rapidly releasing before the 6-hour mark and starting to stabilize afterwards. Degradation of the levofloxacin was not observed. This type of release can prove advantageous to stop the rapid formation of biofilms as in the case of acute osteomyelitis.

Antimicrobial susceptibility assays showed that our levofloxacin-loaded PMMA nanocapsules had antimicrobial effects as intended for inhibiting the growth of *S. aureus* even in its biofilm form. Despite the amount of levofloxacin encapsulated not being high, these tests proved to be successful. Also, it was proved that the blank PMMA nanocapsules had no effect on the growth of *S. aureus* showing that the carrier had no pharmacological effect. This observation leads us to conclude that the microbiological effect was originated by the action of levofloxacin released by the nanocapsules. The 350000 g/mol PMMA nanocapsules still work as intended and both microbiological tests were successful. This proves the efficiency of this type of PMMA nanocapsules as a drug delivery system even though the release assays were not feasible for this PMMA nanocapsules.

In conclusion, the main goal of this work was achieved with success and the newly developed levofloxacin-loaded PMMA nanocapsules showed great promise as a nanoparticulate drug delivery system against *S. aureus.*

5. Future perspectives

Although the results obtained were very promising, some work must be performed in order to improve the nanocapsule drug delivery system.

The synthesised amount of the levofloxacin-loaded PMMA nanocapsules was low compared with the amount of PMMA and levofloxacin used for each synthesis. Further improvements on the amount of nanocapsules obtained are necessary. Controlling the evaporation of dichloromethane to have a more precise management of the nanoprecipitation of PMMA can help to increase the amount of nanocapsules obtained. This might be achievable through lower temperatures, blow down evaporation or vortex evaporation.

The amount of levofloxacin encapsulated was low in relation to the amount first introduced in the synthesis (the levofloxacin that was not encapsulated can however still be recovered). Although the amount of levofloxacin encapsulated was enough to inhibit the growth of *S. aureus*, a higher amount of levofloxacin might improve the efficiency of this drug delivery system. This fact however might also start to cause toxicity problems to the user if the encapsulated amount is too large, resulting in the problems previously mentioned with the traditional treatment with levofloxacin. Stabilization of the miniemulsion during the evaporation phase is critical to increase the amount of levofloxacin encapsulated. Possible ways to stabilize it can be achieved through a different evaporation process (as mentioned above) or a different surfactant more water-soluble at high temperatures.

Other molecular weights of PMMA can be used to prepare the nanoparticulate drug delivery system which can offer different properties to the ones showcased in this work. Higher or slower drug release rate or higher amount of levofloxacin encapsulated can be achieved this way. In this work we also noted that with the 350000 g/mol PMMA the nanocapsules were more resistant to stress resulting in poor homogenization of the samples when trying to create a powder. This can be used as an advantage to stop the antibiotic from being rapidly released due to breakdown of the polymer shell under mechanical stress.

The potential clinical application of the proposed novel drug delivery system is high. Its inclusion in bone cements or teeth implants to prevent the formation of biofilms, growth of pathogens and infections, which are prevalent in this type of materials, should be explored.

6. References

1. Adams L, Gilsbach JM, Krybus W, Meyer-Ebrecht D, Mösges R, Schlöndorff G. CAS - a Navigation Support for Surgery. In: Höhne KH, Fuchs H, Pizer SM, eds. *3D Imaging in Medicine*. Berlin, Heidelberg: Springer Berlin Heidelberg; 1990:411-423.

2. Registo Português de Artroplastias. *2013 Annual Report*.; 2013. http://www.rpa.spot.pt/getdoc/656b137c-4a0c-4bfc-a34d-8dafedaa0c62/Relatorio_Anual_2013.aspx.

3. Gbejuade HO, Lovering AM, Webb JC. The role of microbial biofilms in prosthetic joint infections: A review. *Acta Orthop*. 2015;86(2):147-158.

4. John L. Zeller, MD, PhD; Alison E. Burke, MA; Richard M. Glass M. Jama Patient Page. *Jama*. 2008;305(4):858.

5. Dym H, Zeidan J. Microbiology of Acute and Chronic Osteomyelitis and Antibiotic Treatment. *Dent Clin North Am*. 2017;61(2):271-282.

6. Lew DP, Waldvogel FA. Osteomyelitis. *Lancet*. 2004;364(1474-547X (Electronic)):369-379.

7. Santos-Ferreira I, Bettencourt A, Almeida AJ. Nanoparticulate platforms for targeting bone infections: meeting a major therapeutic challenge. *Nanomedicine (Lond)*. 2015;10(20):3131-3145.

8. Høiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O. Antibiotic resistance of bacterial biofilms. *Int J Antimicrob Agents*. 2010;35(4):322-332.

9. Mah TF, O'Toole GA. Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol*. 2001;9(1):34-39.

10. Peng KT, Chen CF, Chu IM, et al. Treatment of osteomyelitis with teicoplanin-encapsulated biodegradable thermosensitive hydrogel nanoparticles. *Biomaterials*. 2010;31(19):5227-5236.

11. Stapleton M, Sawamoto K, Alméciga-Díaz CJ, et al. Development of Bone Targeting Drugs. *Int J Mol Sci*. 2017;18(7):1-15.

12. Baleizão C, Farinha JPS. Hybrid smart mesoporous silica nanoparticles for theranostics. *Nanomedicine*. 2015;10(15):2311-2314.

13. Pillai RR, Somayaji SN, Rabinovich M, Hudson MC, Gonsalves KE. Nafcillin-loaded PLGA nanoparticles for treatment of osteomyelitis. *Biomed Mater*. 2008;3(3):1-7.

14. Kostova B, Kamenska E, Georgieva D, Balashev K, Rachev D, Georgiev G. Design and Concept of Polyzwitterionic Copolymer Microgel Drug Delivery Systems In Situ Loaded with Non-steroidal Anti-inflammatory Ibuprofen. *AAPS PharmSciTech*. 2017;18(1):166-174.

15. Miyazaki M, Yuba E, Hayashi H, Harada A, Kono K. Hyaluronic Acid-Based pH-Sensitive Polymer-Modified

Liposomes for Cell-Specific Intracellular Drug Delivery Systems. *Bioconjug Chem*. 2018;29(1):44-55.

16. Jyoti K, Pandey RS, Kush P, Kaushik D, Jain UK, Madan J. Inhalable bioresponsive chitosan microspheres of doxorubicin and soluble curcumin augmented drug delivery in lung cancer cells. *Int J Biol Macromol*. 2017;98:50-58.

17. Tiwari G, Tiwari R, Sriwastawa B, et al. Drug delivery systems: An updated review. *Int J Pharm Investig*. 2012;2(1):2-11.

18. Poletto FS, Beck RCR, Guterres SS, Pohlmann AR. Polymeric Nanocapsules: Concepts and Applications. In: Beck R, Guterres S, Pohlmann A, eds. *Nanocosmetics and Nanomedicines: New Approaches for Skin Care*. Berlin, Heidelberg: Springer Berlin Heidelberg; 2011:49-68.

19. Liechty WB, Kryscio DR, Slaughter B V, Peppas NA. Polymers for Drug Delivery Systems. *Annu Rev Chem Biomol Eng*. 2010;1:149-173.

20. Masood F. Polymeric nanoparticles for targeted drug delivery system for cancer therapy. *Mater Sci Eng C*. 2016;60:569-578.

21. Mohanraj VJ, Chen Y. Nanoparticles – A Review. *Trop J Pharm Res*. 2006;5(1):561-573.

22. Klee D, Höcker H. Polymers for Biomedical Applications: Improvement of the Interface Compatibility. In: Eastmond GC, Höcker H, Klee D, eds. *Biomedical Applications Polymer Blends*. Berlin, Heidelberg: Springer Berlin Heidelberg; 1999:1-57.

23. Vert M. Polymeric biomaterials: Strategies of the past vs. strategies of the future. *Prog Polym Sci*. 2007;32(8-9):755-761.

24. Bettencourt A, Almeida A. Poly (methyl methacrylate) particulate carriers in drug delivery. *J Microencapsul*. 2012;29(4):353-367.

25. Elvira C, Fanovich A, Fernández M, Fraile J, San Román J, Domingo C. Evaluation of drug delivery characteristics of microspheres of PMMA-PCL-cholesterol obtained by supercritical-CO2impregnation and by dissolution-evaporation techniques. *J Control Release*. 2004;99(2):231-240.

26. Vaishya R, Chauhan M, Vaish A. Bone cement. *J Clin Orthop Trauma*. 2013;4(4):157-163.

27. Gupta H, Aqil M, Khar RK, Ali A, Bhatnagar A, Mittal G. Biodegradable levofloxacin nanoparticles for sustained ocular drug delivery. *J Drug Target*. 2011;19(6):409-417.

28. Gevariya H, Gami S, Patel N. Formulation and characterization of levofloxacin-loaded biodegradable nanoparticles. *Asian J Pharm*. 2011:114-119.

29. Iannitelli A, Grande R, di Stefano A, et al. Potential antibacterial activity of carvacrol-loaded poly(DL-lactide-co-glycolide) (PLGA) nanoparticles against microbial biofilm. *Int J Mol Sci*. 2011;12(8):5039-5051.

30. Gupta H, Aqil M, Khar R, Ali A, Bhatnagar A, Mittal G. Nanoparticles laden in situ gel for sustained ocular drug delivery YR - 2013/4/1. *J Pharm Bioallied Sci*. 2013;5(2):162-165.

31. Flores FC, Rosso RS, Cruz L, Beck RCR, Silva CB. An innovative polysaccharide nanobased nail formulation for improvement of onychomycosis treatment. *Eur J Pharm Sci*. 2017;100:56-63.

32. Ferreira IS, Bettencourt A, Betrisey B, Goncalves LMD, Trampuz A, Almeida AJ. Improvement of the antibacterial activity of daptomycin-loaded polymeric microparticles by Eudragit RL 100: an assessment by isothermal microcalorimetry. *Int J Pharm*. 2015;485(1-2):171-182.

33. Prokopovich P, Köbrick M, Brousseau E, Perni S. Potent antimicrobial activity of bone cement encapsulating silver nanoparticles capped with oleic acid. *J Biomed Mater Res B Appl Biomater*. 2015;103(2):273-281.

34. Martínez Rivas CJ, Tarhini M, Badri W, et al. Nanoprecipitation process: From encapsulation to drug delivery. *Int J Pharm*. 2017;532(1):66-81.

35. Landfester K. Synthesis of Colloidal Particles in Miniemulsions. *Annu Rev Mater Res*. 2006;36(1):231-279.

36. Peng K-T, Chen C-F, Chu I-M, et al. Treatment of osteomyelitis with teicoplanin-encapsulated biodegradable thermosensitive hydrogel nanoparticles. *Biomaterials*. 2010;31(19):5227-5236.

37. Uhrich KE, Cannizzaro SM, Langer RS, Shakesheff KM. Polymeric systems for controlled drug release. *Chem Rev*. 1999;99(11):3181-3198.

38. Jiang Z, Wang P, Pan B, et al. Evaluation of Levofloxacin Release Characteristics from a Human Foldable Capsular Vitreous Body In Vitro. *J Ocul Pharmacol Ther*. 2012;28(1):33-40.

39. Greenberg RN, Newman MT, Shariaty S, Pectol RW. Ciprofloxacin, lomefloxacin, or levofloxacin as treatment for chronic osteomyelitis. *Antimicrob Agents Chemother*. 2000;44(1):164-166.

40. Matos AC, Ribeiro IAC, Guedes RC, et al. Key-properties outlook of a levofloxacin-loaded acrylic bone cement with improved antibiotic delivery. *Int J Pharm*. 2015;485(1):317-328.

41. Ferreira M, Rzhepishevska O, Grenho L, et al. Levofloxacin-loaded bone cement delivery system: Highly effective against intracellular bacteria and Staphylococcus aureus biofilms. *Int J Pharm*. 2017;532(1):241-248.

42. Kim B-N, Kim ES, Oh M-D. Oral antibiotic treatment of staphylococcal bone and joint infections in adults. *J Antimicrob Chemother*. 2014;69(2):309-322.

43. Ahmad I, Bano R, Sheraz MA, Ahmed S, Mirza T, Ansari SA. Photodegradation of levofloxacin in aqueous and organic solvents: a kinetic study. *Acta Pharm*. 2013;63(2):223-229.

44. Dudhani AR, Kosaraju SL. Bioadhesive chitosan nanoparticles: Preparation and characterization. *Carbohydr Polym*. 2010;81(2):243-251.

45. Farrant RD, Hollerton JC, Lynn SM, Provera S, Sidebottom PJ, Upton RJ. NMR quantification using an artificial signal. *Magn Reson Chem*. 48(10):753-762.

46. Anitha A, Deepagan VG, Divya Rani V V., Menon D, Nair S V., Jayakumar R. Preparation, characterization, in vitro drug release and biological studies of curcumin loaded dextran sulphate-chitosan nanoparticles. *Carbohydr Polym*. 2011;84(3):1158-1164.

47. Nakama KA, dos Santos RB, da Rosa Silva CE, et al. Establishment of analytical method for quantification of anti-inflammatory agents co-nanoencapsulated and its application to physicochemical development and characterization of lipid-core nanocapsules. *Arab J Chem*. 2018:1-14.

48. Nimesh S, Manchanda R, Kumar R, et al. Preparation, characterization and in vitro drug release studies of novel polymeric nanoparticles. *Int J Pharm*. 2006;323(1-2):146-152.

49. Keawchaoon L, Yoksan R. Preparation, characterization and in vitro release study of carvacrol-loaded chitosan nanoparticles. *Colloids Surfaces B Biointerfaces*. 2011;84(1):163-171.

50. Donlan RM. Biofilms: Microbial life on surfaces. *Emerg Infect Dis*. 2002;8(9):881-890.

51. Clinical and Laboratorial Standards Institute. M02 - A12 - Performance Standards for Antimicrobial Disk Susceptibility Tests. 2015. https://clsi.org/media/1631/m02a12_sample.pdf.

52. Bettencourt A, Calado A, Amaral J, et al. Surface studies on acrylic bone cement. *Int J Pharm*. 2004;278(1):181-186.

53. Pontes C, Alves M, Santos C, et al. Can Sophorolipids prevent biofilm formation on silicone catheter tubes? *Int J Pharm*. 2016;513(1):697-708.

54. Glöckner G. GRADIENT POLYMER CHROMATOGRAPHY: LIQUID CHROMATOGRAPHY. In: *Encyclopedia of Separation Science*. ; 2000:2975-2984.

55. Bettencourt A, Florindo HF, Ferreira IFS, et al. Incorporation of tocopherol acetate-containing particles in acrylic bone cement. *J Microencapsul*. 2010;27(6):533-541.

56. BASF. *Pluronic® PE Types*.; 2005. http://www.timing-ouhan.com/images/pluronicpetypes.pdf.

57. Weber FJ, de Bont JAM. Adaptation mechanisms of microorganisms to the toxic effects of organic solvents on membranes. *Biochim Biophys Acta - Rev Biomembr*. 1996;1286(3):225-245.

58. Sigma-Aldrich. *Cyclohexane Safety Data Sheet*.; 2018. https://www.sigmaaldrich.com/Graphics/COfAInfo/SigmaSAPQM/SPEC/22/227048/227048-BULK_____SIAL_____.pdf.

59. Gul W, Hamid F, Ayub S, Bhatti S. Degradation of selected Fluoroquinolones. *Eur J Pharm Med Res*. 2015;2(7):6-10.

60. Fulmer GR, Miller AJM, Sherden NH, et al. NMR Chemical Shifts of Trace Impurities: Common Laboratory Solvents, Organics, and Gases in Deuterated Solvents Relevant to the Organometallic Chemist. *Organometallics*. 2010;29(9):2176-2179.

61. Abbe E. Ueber einen neuen Beleuchtungsapparat am Mikroskop. *Arch für mikroskopische Anat*. 1873;9(1):469-480.

62. Lee JH, Yeo Y. Controlled Drug Release from Pharmaceutical Nanocarriers. *Chem Eng Sci*. 2015;125:75-84.

63. Orhan DD, Özçelik B, Özgen S, Ergun F. Antibacterial, antifungal, and antiviral activities of some flavonoids. *Microbiol Res*. 2010;165(6):496-504.

Appendices

Appendix 1

Table 0.1 – Table with full width at half height values for the fluorescence intensity profiles obtained through the fluorescence microscopy images obtained.

Peaks		Value (nm)	Standard error (nm)
Fluorescence Image 1			
1	1	766	21
	2	785	51
	3	592	35
	4	642	36
	5	567	44
	6	723	62
	7	575	13
2	1	448	37
	2	615	36
Fluorescence Image 2			
3	1	604	33
	2	582	37
	3	719	27
4	1	686	36
	2	835	41
	3	600	18
5	1	612	31
	2	596	28
	3	900	27
Average		658	34

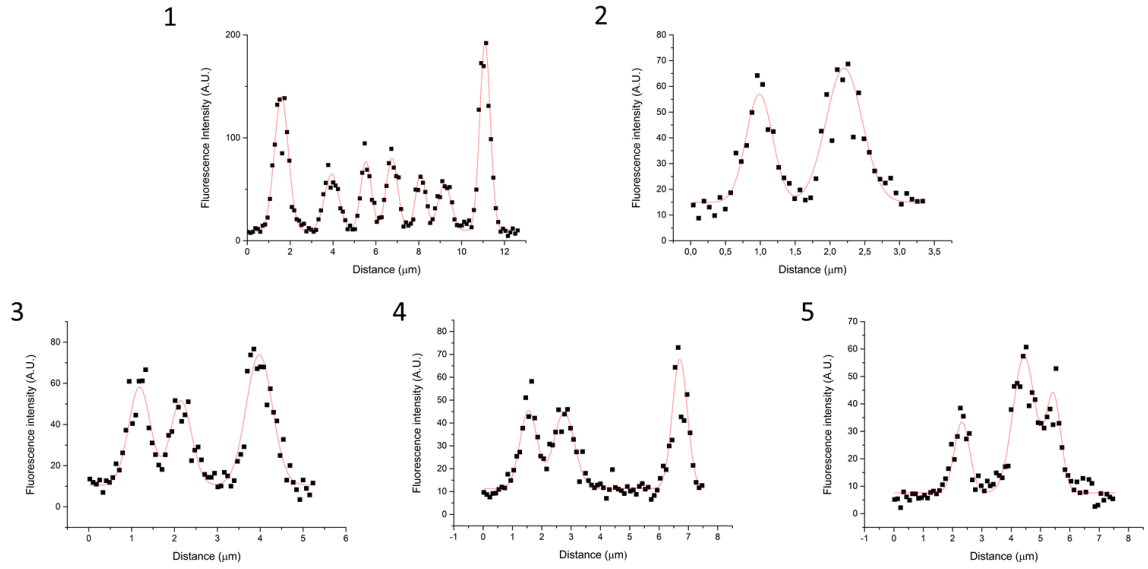

Figure 0.1 - Graphics of the fluorescence intensity profile obtained through fluorescence confocal microscopy (black dots) and the respective Gaussian fit (red line)