

UNIVERSITY OF LJUBLJANA
FACULTY OF PHARMACY

EVA ZUPANČIČ

**PREPARATION AND CHARACTERISATION OF
MINOCYCLINE-LOADED
POLYMETHYLMETHACRYLATE MICRO- AND
NANOPARTICLES FOR BONE CEMENT PREPARATION**

**IZDELAVA IN VREDNOTENJE
POLIMETILMETAKRILATNIH MIKRO- IN
NANODELCEV Z VGRAJENIM MINOCIKLINOM ZA
PRIPRAVO KOSTNEGA CEMENTA**

Ljubljana, 2010

I, Eva Zupančič, student of pharmacy at the University of Ljubljana, Faculty of Pharmacy performed the thesis research work within Erasmus mobility exchange program at the University of Lisbon, Faculty of Pharmacy, at the department of Pharmaceutical Technology. Host supervisor was Prof. Dr. Helena Florindo home supervisor was Prof. Dr. Julijana Kristl. Formulations of micro- and nanoparticles were made at the Faculty of Pharmacy, University of Lisbon. The size determination was performed at the Instituto Nacional de Engenharia, Tecnologia e Inovação (INETI), Lisbon, Portugal. SEM photographs were obtained at the Instituto Superior Técnico, Lisbon, Portugal.

Acknowledgments

I am grateful to Prof. Dr. António José Almeida from the University of Lisbon, Faculty of Pharmacy (FFUL), Portugal for the opportunity to grant the graduation thesis topic and for his helpful advices. Also, I thank my mentor from FFUL Prof. Dr. Helena Isabel Florindo, respectively for her encouragement, enthusiasm, trust, helpful advices, unconditional support during the research work and critical reading. I sincerely thank my Slovenian mentor Prof. Dr. Julijana Kristl for encouragement, trust, advices and critical reading. I am grateful to Prof. Dr. Ana Francisca Bettencourt from FFUL for her help in designing the PMMA plates and to Ana Matos from University Lusófona for cooperation during the preparation process of micro- and nanoparticles.

Special thanks go to my family for encouragement and moral support during my research time in Lisbon and the time of writing. I am really grateful to my friends for always being there and for supporting me by making me laugh.

Statement

I declare that I made this thesis research work under the supervision of Prof. Dr. Julijana Kristl and Prof. Dr. Helena Florindo.

Eva Zupančič

Ljubljana, September 2010

Graduation commission president: Prof. Dr. Janja Marc

Graduation commission member: Assist. Prof. Dr. Marko Anderluh

Table of contents

Acknowledgments	i
Abstract.....	iv
Povzetek	vi
List of abbreviations	vii
1 INTRODUCTION	1
1.1. Total hip arthroplasty and possible infections.....	1
1.2. Acrylic bone cement.....	2
1.2.1. Acrylic bone cement with antibiotic incorporated	5
1.3. Antibiotics	5
1.3.1. Tetracyclines.....	6
1.4. Micro- and nanoparticles as a control delivery systems of antibiotics.....	7
1.4.1. Micro- and nanoparticles	8
1.4.2. Preparation of micro- and nanoparticles.....	9
1.5. Antioxidants	9
1.5.1. Vitamin E.....	10
2 AIM	13
3 MATERIALS AND METHODS	14
3.1. Materials	14
3.2. Equipment.....	14
3.3. Preparation of PMMA particles.....	16
3.3.1. Microparticles.....	16
3.3.2. Nanoparticles.....	18
3.4. Preparation of acrylic bone cement plates with micro- and nanoparticles.....	20
3.5. Quantitative determination of minocycline	22
3.5.1. Calibration curve	22
3.5.2. Determination of minocycline concentration	23
3.6. Physicochemical characterisation of PMMA particles.....	25
3.6.1. Particle size.....	25
3.6.2. Particle morphology	25
3.7. Two different <i>in vitro</i> minocycline release studies	26
3.7.1. Minocycline release from lyophilised PMMA micro- and nanoparticles ...	26

3.7.2.	Minocycline release from acrylic bone cement plates.....	27
4	RESULTS AND DISCUSSION.....	28
4.1.	PMMA micro- and nanoparticles	28
4.1.1.	Characterisation of PMMA micro- and nanoparticles.....	28
4.1.2.	Particle morphology	29
4.1.3.	Plate morphology.....	32
4.1.4.	Particle size.....	33
4.2.	Quantitative determination of minocycline	41
4.2.1.	Minocycline encapsulation and loading efficiency	42
4.3.	<i>In vitro</i> minocycline release study.....	46
4.3.1.	Minocycline release from lyophilised PMMA micro- and nanoparticles ...	46
4.3.2.	Minocycline release from acrylic bone cement plates.....	47
5	CONCLUSIONS	50
6	REFERENCES	52

Abstract

Total hip arthroplasty is the replacement of a hip with a metal prosthesis. To make it more stable, orthopaedic surgeons anchor prosthesis with acrylic bone cement. During or after the operation process an infection can occur. A prolong antibiotic release would be desire to prevent eventual infection. Therefore, antibiotics-loaded acrylic bone cement micro- and nanoparticles can be an interesting system. The goal of the graduation thesis research work is to develop a formulation method and to prepare two drug delivery systems: polymethyl methacrylate (PMMA) micro- and nanoparticles containing the antibiotic minocycline.

There were some differences in the formulation process of micro- and nanoparticles. For microparticles, 250 mg of PMMA was dissolved in 5 ml of the organic solvent dichloromethane (DCM). On the other hand, the organic phase of nanoparticles was composed by 100 mg of PMMA dissolved in 6 ml of DCM. Moreover, a 2.5% (w/v) PVA solution was used as an external phase in the formulation of PMMA microparticles, while 1.25% (w/v) PVA solution was used for the preparation of nanoparticles. Minocycline-loaded microparticles had a volume mean diameter (VMD) of 787 nm and a polydispersity index (PI) of 0.223. The fundamental batch (mpMino1) had VMD 787 nm and PI 0.234. On the other hand, minocycline-loaded nanoparticles had VMD of 356 nm and their PI was 0.246. Fundamental nanoparticles (npMino1) had VMD of 353 nm and PI 0.217. The incorporation of sucrose did not significant influence on the VMD ($p > 0.05$). Incorporation of TPGS into the internal phase had significantly increased particle size ($p = 0.043$). From the obtained results, we could conclude that the size of microparticle was nearly two times larger compared to that of nanoparticles. The PI between particles is quite comparable, although higher deviations for nanoparticles would be expected. Surface morphology of plain, antibiotic-loaded and TPGS-loaded micro- and nanoparticles was analysed by SEM. Nanoparticles had a fine spherical shape with smooth surface. As concerns microparticles, a spherical shape with a rough surface was observed. Encapsulation (E.E) and loading efficiencies (L.E) were determined using an indirect method. Microparticles (mpMino1) had an E.E of 38.50% and L.E of 0.77%. Minocycline-loaded nanoparticles (npMino1) had an E.E of 30.45% and L.E of 0.60%. As concerns other batches, the salting-out method resulted in higher E.E and L.E. *In vitro* minocycline release from PMMA micro- and nanoparticles and from plates containing those vehicles was measured using two different methods. No drug release from PMMA particles was

observed. On the other hand, minocycline release from acrylic bone cement plates was successful. The release profile from the plates containing microparticles was lower compared to that from plates containing nanoparticles. The minocycline release 24 hours after the beginning of the assay was 0.13% (w/w) for PmM1 and 0.95% (w/w) for PnM1. On 5th day 1.8% (w/w) of minocycline was released from the PmM1 and 4.1% (w/w) from the PnM1. The difference increased in the following days, which can be due to particle size, since smaller particles have larger surface area.

For future work, it would be relevant to optimize the formulation of micro- and nanoparticles of PMMA in order to ensure a uniform particle size distribution. Moreover, it would be interesting to evaluate the influence of minocycline, sucrose and TPGS on the mechanical properties of the acrylic bone cement

Keywords: microparticles, nanoparticles, acrylic bone cement, PMMA, minocycline, total hip arthroplasty

Povzetek

Pri popolni artroplastiki kolka umetno protezo učvrstijo s kostnim cementom. Slabost posegov je pogost pojav infekcij. Odločili smo se za razvoj sistemov s podaljšanim sproščanjem za preprečevanje teh infekcij. Izdelali smo dva dostavna sistema, in sicer: polimetil metakrilat (PMMA) mikrodcelce (MD) in nanodelce (ND) z vgrajenim minociklinom. Za izdelavo MD smo uporabili 250 mg polimera PMMA, 5 ml diklorometana (DCM) in 2,5% (w/v) PVA. ND pa smo izdelali z 100 mg PMMA, 6 ml DCM in 1,25% (w/v) PVA. Povprečni volumski premer (VMD) MD je bil 787 nm, polidisperzni indeks (PI) pa 0,223. Osnovna serija MD (mpMino1) je imela VMD 786 nm in PI 0,234. VMD ND je bil 356 nm, PI pa 0,246. Osnovna serija ND (npMino1) je imela VMD 353 nm in PI 0,217. Dodatek saharoze pri formulacijah MD in ND ni signifikantno vplival na VMD ($p > 0,05$). Mikroskopska analiza MD in ND je pokazala, da so ND fine okrogle oblike z gladko površino, medtem ko so imeli MD grobo površino. Izkoristek enkapsulacije (E.E) ter celokupni izkoristek (L.E) vključitve minociklina v formulirane delce smo določili s posredno metodo. MD (mpMino1) so imeli E.E 38,5% in L.E 0,77%. Medtem ko so imeli ND (npMino1) E.E 30,45% in L.E 0,60%. V primeru serij kjer je bila saharoza uporabljena kot izsoljevalec, je le-ta povečala E.E in L.E teh delcev. V *in vitro* študiji sproščanja minociklina iz kostnega cementa, se je po petih dneh iz PmM1 sprostilo 1,8 % minociklina, medtem ko se je v istem času iz PnM1 sprostilo 4,1 % antibiotika. Sproščanje antibiotika je v veliki meri odvisno od velikosti delcev, saj manjši ko so delci, večja je njihova specifična površina.

V prihodnje, bi bilo smotrno optimizirati formulacijo MD in ND PMMA, da bi zagotovili enakomerno porazdelitev velikosti delcev (VMD). Ocenili bi lahko tudi vpliv antibiotika, saharoze in TPGS na mehanske lastnosti kostnega cementa. Saj so le-te bistvenega pomena za njeno uspešno klinično uporabo.

Ključne besede: mikrodcelci, nanodelci, kostni cement, PMMA, minociklin, popolna artroplastika kolka

List of abbreviations

PMMA	polymethyl methacrylate
MMA	methyl methacrylate
DCM	dichloromethane
PVA	polyvinyl alcohol
VMD	volume mean diameter
PI	polydispersity index
SEM	scanning electron microscopy
E.E	encapsulation efficiency
L.E	loading efficiency
pKa	dissociation constant
<i>logP</i>	logarithm of partition coefficient
w/o	water-in-oil
w/o/w	(water-in-oil)-in-water
ROS	reactive oxygen species
TPGS/vitamin E TPGS	D- α -tocopherol polyethylene glycol 1000 succinate
HLB	hydrophilic–lipophilic balance
UV	ultraviolet
Ph. Eur.	Pharmacopoeia Europea

1 INTRODUCTION

Biomaterials are defined as materials for designing, developing and producing the objects used for medical purposes. They are used especially in dentistry, cardiovascular, orthopaedic interventions and cosmetics. In the orthopaedic group, many different types of biomaterials have been used for prosthesis and to anchor prosthesis to the bone. Independently of the nature or application of the biomaterials, they should be biocompatible and should not injure the tissue where they are inserted in. A biomaterial widely use in orthopaedic surgery is the acrylic bone cement [1].

1.1. Total hip arthroplasty and possible infections

Total hip arthroplasty is the replacement of a hip with a metal prosthesis. To make it more stable, orthopaedic surgeons anchor prosthesis with acrylic bone cement. Arthroplasty is one of the most frequent and successful operations in orthopaedics. However, the list of potential complications is extensive. A side from the life threatening complications of total hip replacement, no post-operative complication can be more devastating than infection. The rate of infection for total hip arthroplasty ranges from 0.5 % to 3.0 %. The frequency is low, but when present is difficult to treat [2, 3].

Infection at the site of a total hip arthroplasty can be classified into three basic categories: acute postoperative infections, delayed infections and late haematogenous infections. The first ones are caused by contamination at the time of the operation. These infections are usually treated with intravenous antibiotics. Delayed infection usually appears at least eight weeks after the operation in the form of an indolent, chronic, low-grade infection. The worsening pain and loosening of the prosthesis are treated with a two-stage reconstruction. The final ones, late haematogenous infections can happen at any time with a presentation similar to that of the acute infection. *Staphylococcus aureus* is the most common cause of acute infection after surgery, whereas *Staphylococcus epidermidis* and *Staphylococcus albus* are common causes of late infection [2]. Because of the risk of infection, antibiotics are incorporate in the bone cement.

1.2. Acrylic bone cement

Acrylic bone cement, chemically polymethyl methacrylate (PMMA) is insoluble in water, freely soluble in organic solvents (DCM, anhydrous ethanol). This biomaterial is mostly used in orthopaedic surgery to anchor cemented prosthesis to the bone. Self-curing acrylic bone cement is extensively used in certain types of total hip and total knee replacements and is potential utility wherever mechanical attachment of metal to living bone is necessary. Acrylic bone cement increases the quality of patients' lives and life period of prosthesis. The mechanical performances needed for bone cement are a good capacity to distribute applied stress evenly, a good behaviour in fatigue and a good adherence with the metallic surface of prosthesis and the bone [4]. The main function of surgical bone cements is to transfer load from the prosthesis to the bone.

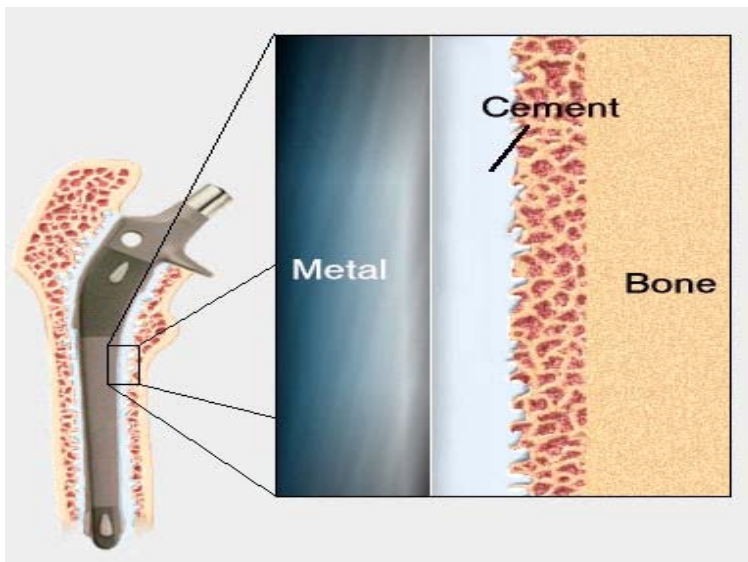
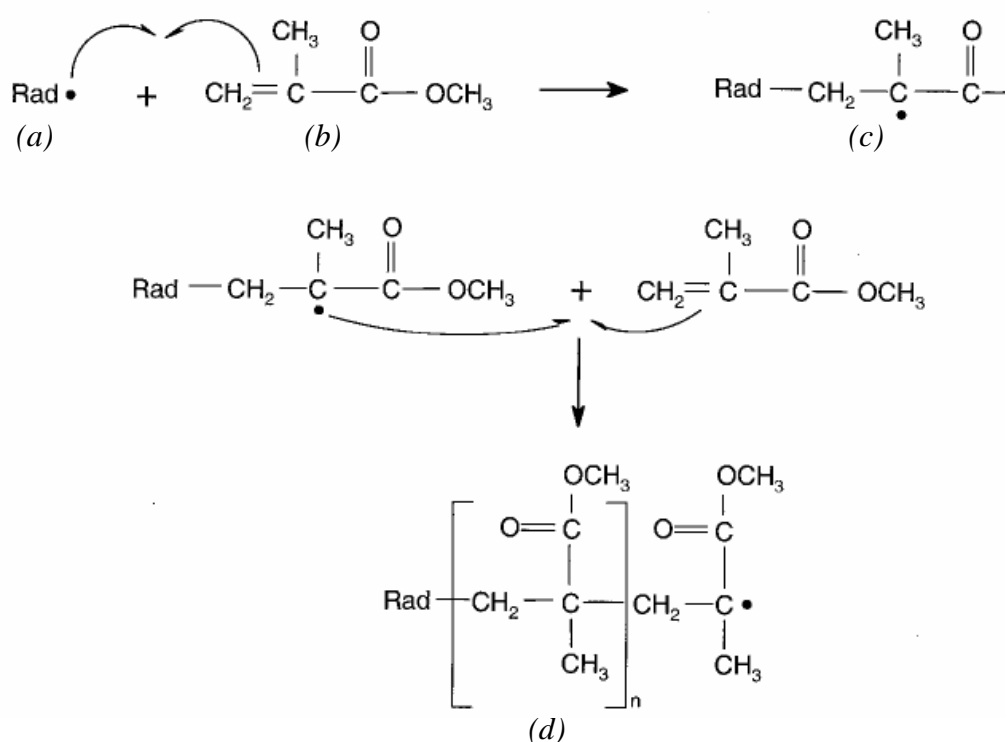


Figure 1. Schematic presentation of interface between bone-acrylic bone cement and acrylic bone cement-metal prosthesis (Adapted from [8]).

The advantage of PMMA when compared to other biomaterials is its polymerization *in situ* which makes it possible to mould it during the surgical process and therefore adapt it to the bone and to the metal prosthesis (Figure 1) [5, 6, 7].

Commercial acrylic bone cement consists of powder and liquid form. Powder form is composed of pre-polymerized PMMA, benzoyl peroxide and barium sulphate [4, 5, 8, 9]. Benzoyl peroxide is the initiator of the polymerizing reaction of acrylic bone cement, while the barium sulphate makes the acrylic bone cement visible during the X-rays diagnostic [4,

8, 10]. The liquid component contains methyl methacrylate (MMA) monomer, *N,N*-dimethyl-*p*-toluidine, hydroquinone, ethanol and ascorbic acid. *N,N*-dimethyl-*p*-toluidine acts as an activator of polymerisation, when the powder and liquid components are mixed [4, 9]. When the mixture contains high concentration of MMA, it is important that monomers do not react prematurely and, for that reason, ascorbic acid and hydroquinone are added. Ethanol is the solvent for ascorbic acid [9]. The polymerization reaction of acrylic bone cement proceeds as a radical chain reaction (Figure 2). Ideally polymerization is finished when all monomer has reacted.



I.

Figure 2. *In situ* polymerization of acrylic bone cement. **I.** Polymerization starts with formation of tertiary radical (c) activated initiator is needed to start the reaction (a), which binds to the double bond of methyl methacrylate (b). During the propagation chain is extended to form PMMA (d) (Adapted from [9]).

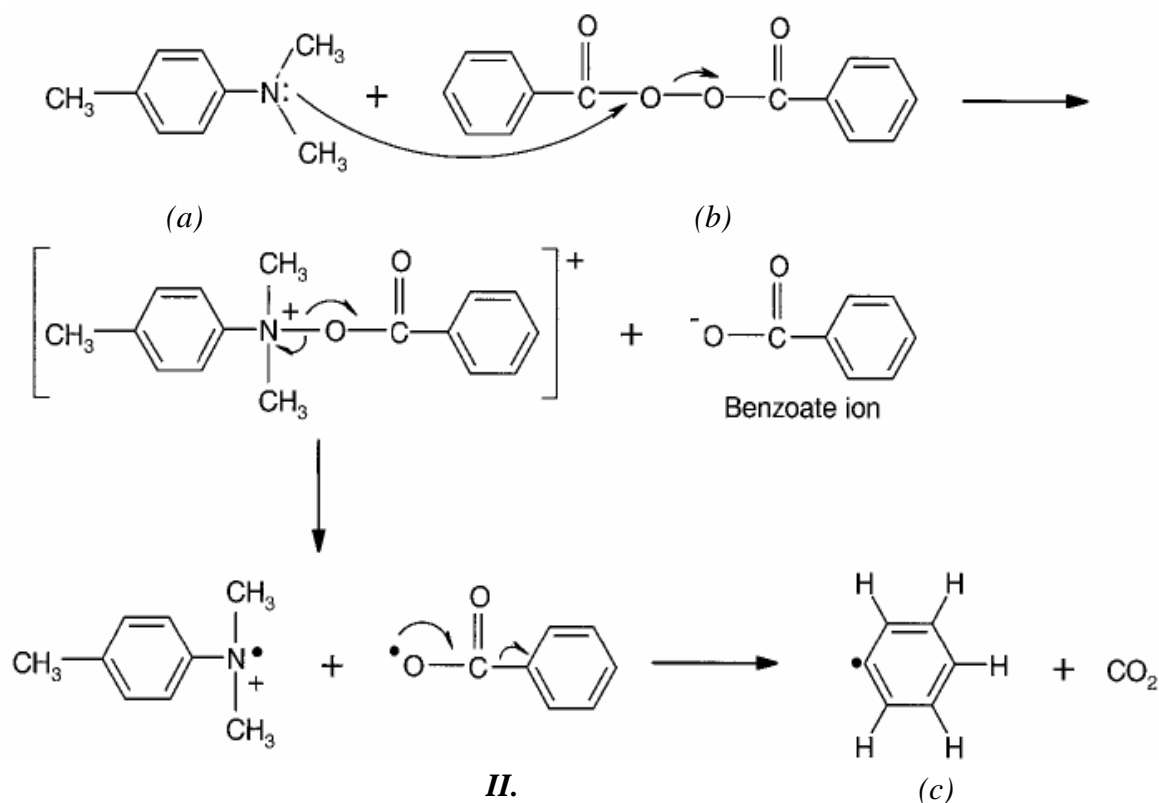


Figure 3. Initiation of polymerization. **II.** Reaction of initiator (benzoyl peroxide (a)) with activator (*N,N*-dimetil-*p*-toluidine (b)) yields phenyl radical (c). Activation of initiator occurs when powder and liquid component are mixed (Adapted from [9]).

This biomaterial has many advantages, like polymerization *in situ*, which gives an opportunity to adjust bone cement to bone and prosthesis, and has good mechanical characteristics. PMMA can be used as a vehicle for different active substances (antibiotics and anti-inflammatory drugs), antioxidants and contrast agents [5, 10]. Acrylic bone cement has as well some disadvantages on long term, occurring loss of prosthesis and requiring another surgical intervention [1, 5, 11, 12, 13].

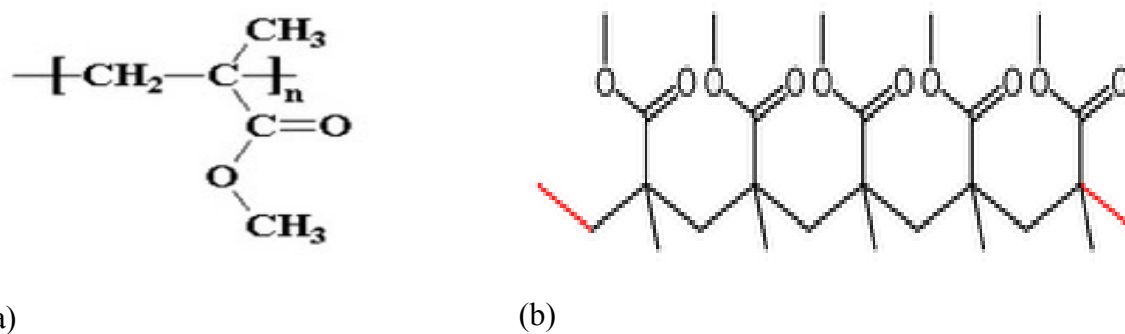


Figure 4. Chemical structure of non-polar PMMA polymer. (a) Monomer unit of PMMA (Adapted from (9)). (b) Linear chain of polymer PMMA (Adapted from [8]).

1.2.1. Acrylic bone cement with antibiotic incorporated

Main problems associated with cemented arthroplasty are necrosis, diminution of acrylic bone cement, fragile interface between acrylic cement-bone and acrylic cement-prosthesis and inflammation. The use of antibiotic-loaded PMMA has become a widespread technique. Antibiotic-impregnated cement has improved the outcomes of the postoperative infections after total hip arthroplasty [1, 11]. The advantages of antibiotic-impregnated cement are:

- local antibiotic therapy,
- maintenance of joint mobility,
- reduction of scar tissue,
- minimization of soft tissue contraction
- facilitate re-implantation

The impregnation of bone cement with antibiotic is a complex process. Not all antibiotic qualifies to be incorporated into bone cement. The desirable characteristics of antibiotics are: powder form, wide antibacterial spectrum, bactericidity at low concentrations, release from cement in minimal inhibitory concentrations for prolonged period, thermal stability, minimal or no risk of allergy, low influence on the mechanical properties of the bone cement and low serum protein binding. Some antibiotics are may undergo decomposition when mixed with the cement, or during its polymerization process. A low degree of antibiotic release from the cement can also limit its application [3].

1.3. Antibiotics

Antibiotics are compounds that kill bacteria or inhibit their growth. They are classified as either bactericidal or bacteriostatic antibiotics. Bactericidal antibiotics kill bacteria and are growth phase dependent, while bacteriostatic prevent cell division. In practice, both of these are capable of ending a bacterial infection caused by microorganisms. Antibiotics can be categorized on their target specificity into two groups: narrow-spectrum antibiotics and broad-spectrum antibiotics. Narrow-spectrum antibiotics target particular types of bacteria, while broad-spectrum antibiotics affect a wide range of bacteria. Antibiotics which target

the bacterial cell wall (penicillins, cephalosporins), or interfere with essential bacterial enzymes (quinolones, sulfonamides) usually are bactericidal in nature. Those which target protein synthesis (aminoglycosides, macrolides and tetracyclines) are usually bacteriostatic (Figure 4) [14, 15, 16, 17, 18].

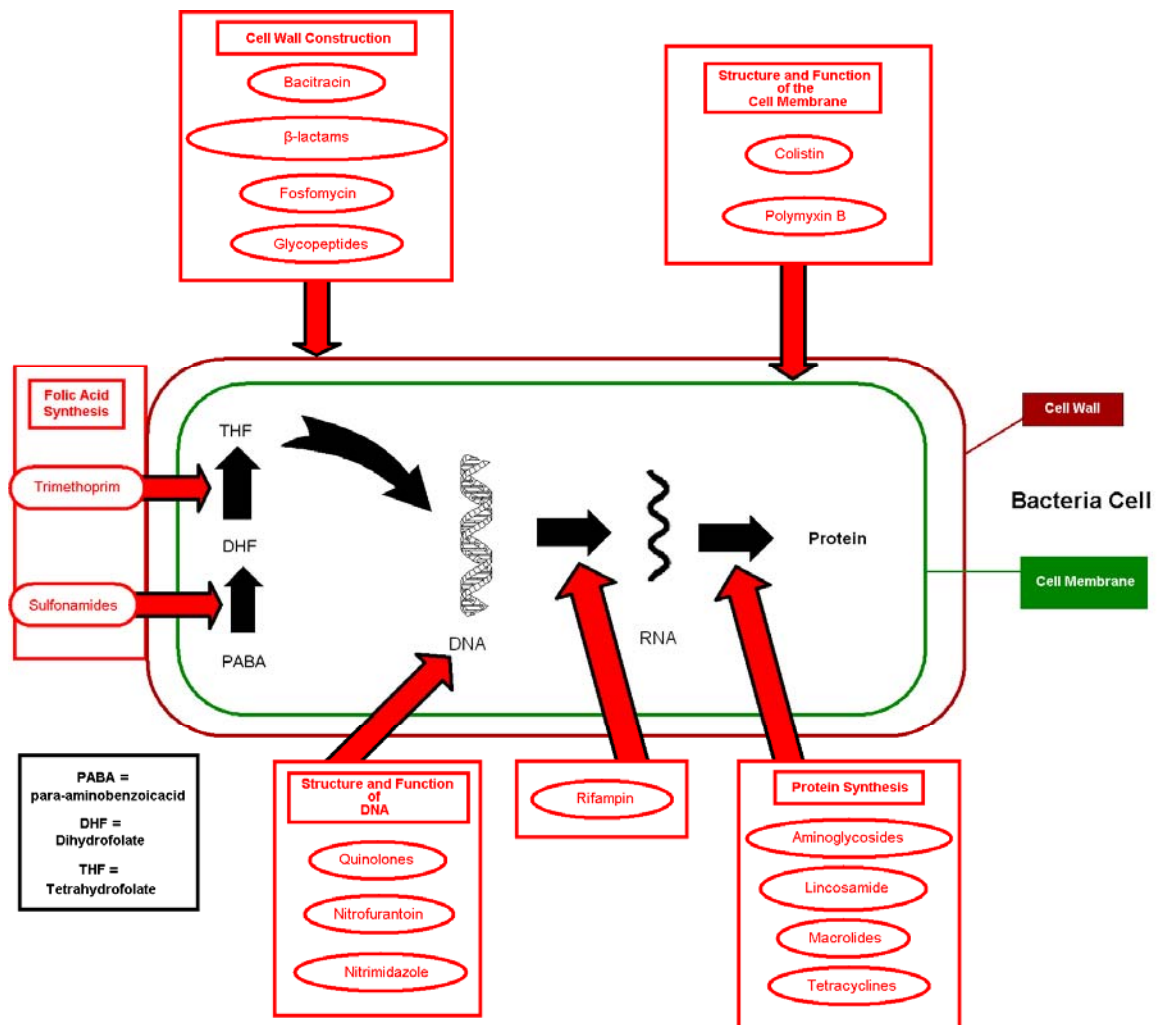


Figure 5. Target site of antibiotics (Adapted from [19]).

1.3.1. Tetracyclines

Tetracyclines are broad spectrum bacteriostatic antibiotics, with selective activity against the procariont cells which reuptake the antibiotic by an active transport process. They bind to the ribosome 30S subunit, preventing the binding of aminoacyl transfer RNA and inhibiting protein synthesis and cell growth [17, 18, 19]. They are active against Gram-

positive and Gram-negative bacteria, *Mycoplasma spp.*, *Rickettsia spp.*, *Chlamydiaceae*, spirochaetes and some protozoa [16, 17, 20].

1.3.1.1. Minocycline

Minocycline is a broad spectrum bacteriostatic antibiotic and has better cell-wall penetration than other tetracyclines [17, 18, 19]. Minocycline is more lipophilic than the other members of the group and is therefore capable of disrupting cytoplasmic membrane function and for this reason has as well bactericidal properties. Dissociation constants of minocycline are $pK_{a1} = 3.3$, $pK_{a2} = 7.2$ and $pK_{a3} = 9.3$. The logarithm of partition coefficient ($\log P$) for minocycline is $\log P = + 0.3$ and it was determined with *HyperChem* program.

Minocycline is active against *Staphylococcus aureus*, *Neisseria meningitidis*, various enterobacteria, *Acinetobacter*, *Bacteroides*, *Haemophilus*, *Nocardia*, and some mycobacteria, including *M. leprae*. Minocycline decreases the production of substances causing inflammation, such as prostaglandins and leukotrienes, while increases the production of interleukin-10, a substance that reduces inflammation [17, 18, 19, 20].

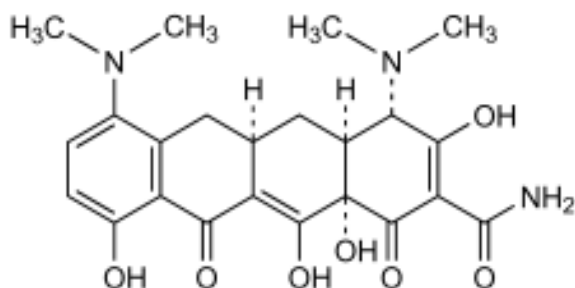


Figure 6. Chemical structure of minocycline (Adapted from [20]).

1.4. Micro- and nanoparticles as a control delivery systems of antibiotics

Micro- and nanoparticles have an important role among the drug delivery technology. Controlled drug delivery technology involves a multidisciplinary scientific approach. It has numerous advantages compared to conventional dosage forms, which include improved efficacy, reduced toxicity, and improved patient compliance. Drug delivery systems have

been developed and diversified in recent years and some of the reasons why micro- and nanoparticles are classified as drug delivery systems are:

- Size and size distribution of particles have an important impact on drug release. It is known that smaller particles have a larger surface area. Therefore, usually nanoparticles have faster drug release compared to microparticles. Microparticles are larger and allow higher amount of drug to be encapsulated in the matrix, but in the case of nanoparticles the drug is distributed at or close to their surface.
- The drug release profile mainly depends on the incorporation method. Diffusion or erosion of the matrix occurs in micro- and nanospheres in which the drug is physically and uniformly dispersed [21, 22].

1.4.1. Micro- and nanoparticles

Particulate dispersions or solid particles are two of the most common forms in which micro- and nanoparticles are defined. The drug is dissolved, entrapped, encapsulated in the matrix or attached to the particle surface, depending on the preparation techniques. Micro- and nanocapsules are systems where the drug is surrounded by a polymeric membrane. Micro- and nanospheres are matrix systems in which the drug and the polymer are evenly dispersed. Polymeric compound as the selected matrix material has some advantages: they increase the stability of active agent (drugs or proteins), affect the particle size and morphology and improve their release properties. Particle size, surface characteristics and controlled drug release are the main characteristics of micro- and nanoparticles as drug delivery systems. A controlled release increases the therapeutic efficiency of the drug and minimizes its side effects. Micro- and nanoparticles as controlled drug delivery system can be administered by several routes, such as oral, parenteral and nasal [21, 22]. The main difference between microparticles and nanoparticles is their size. The differences in the emulsifying agent concentration, the velocity of homogenization and the polymer concentration used in the formulation of different particles have a profound impact on their size. Nanoparticles have diameters lower than 500 nm. On the other hand, the size of microparticles is around 1000 nm [21].

1.4.2. Preparation of micro- and nanoparticles

There are three methods most commonly used in the preparation of micro- and nanoparticles: solvent evaporation technique, polymerization method and coacervation.

Solvent evaporation method is the most frequent, being based on the dissolution of the polymer in an organic solvent, such as DCM, acetone, ethylacetate. DCM is also a solvent for the hydrophobic drugs. The polymer solution is emulsified with a surfactant to form an oil-in-water (o/w) emulsion. The organic solvent is evaporated either by continuous stirring or by reducing the pressure. A double w/o/w emulsion needs to be formed in case of hydrophilic drug, as in this case, the drug has to be dissolved in the internal aqueous phase.

In the polymerization method the polymerization medium is an aqueous solution with various stabilizers and surfactants, in which monomers are polymerized to form nanoparticles. Drug incorporation can be achieved by dissolving the drug in the polymerization medium or by adsorption onto nanoparticles surface after polymerization.

The coacervation method is mostly used for chitosan, gelatine and sodium alginate. This method is based on the mixture of two aqueous phases, chitosan and the other containing a polyanion sodium tripolyphosphate. The amino group of chitosan has a positive charge which interacts with the negatively charged tripolyphosphate and forms coacervate [21].

1.5. Antioxidants

Inflammation is a common reaction that occurs after the arthroplasty surgery. Reactive species from acrylic resin polymerization can cause prosthesis loosening by inducing oxidative stress and producing reactive oxygen species (ROS). Therefore antioxidants have been used in the formulation of micro- and nanoparticles to suppress free radical injury which may contribute to prosthesis loosening [12, 13, 23]. Antioxidants are able to prevent or minimize the oxidation of other molecules. These molecules terminate chain reactions by removing free radical intermediates, and inhibit other oxidation reactions by oxidizing themselves [24]. Free radicals are of endogen and external origin. In normal circumstances the respiratory chain is one of the main sources of endogen free radicals. In the human body there is a balance between free radicals and antioxidants. Imbalance, when there is

surplus of free radicals, leads to the oxidative stress. That means that free radicals react with all components of the cell, including proteins, lipids, and DNA and can provoke inflammation [24, 25].

1.5.1. Vitamin E

Some vitamins are antioxidants. Those are vitamin C, A and E (Figure 6 (a)) (25, 26, 27).

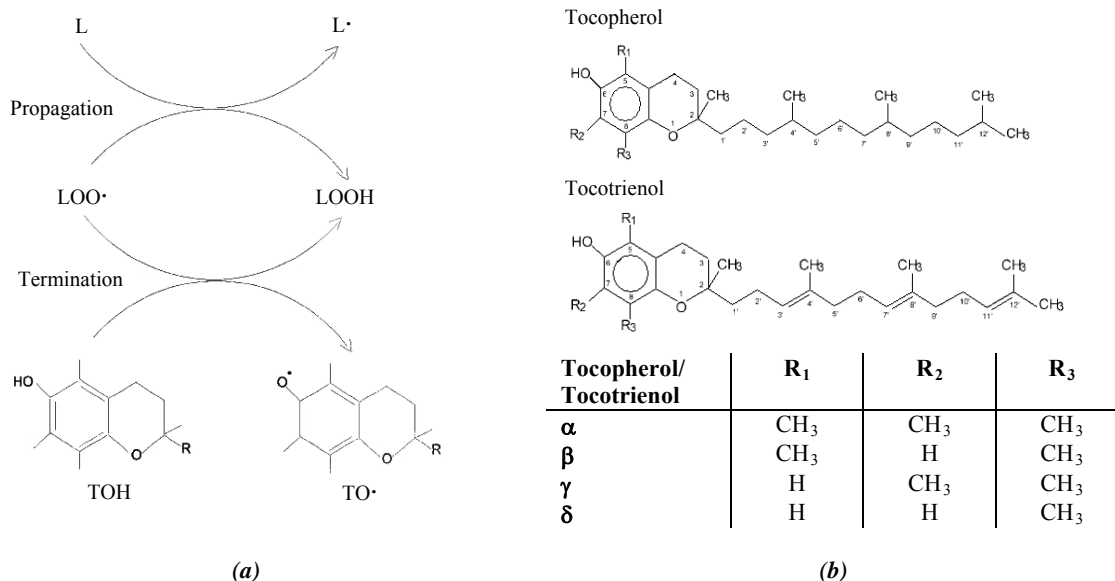


Figure 7. (a) Antioxidative mechanism of vitamin E (α -tocopherol). (b) Chemical structures of tocopherols (α , β , γ , δ) and tocotrienols (α , β , γ , δ) (Adapted from [28, 29]).

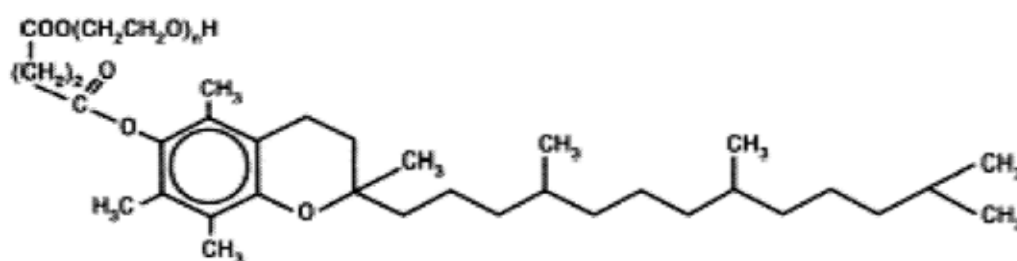
Vitamin E is the name for a group of potent, lipid-soluble, chain-breaking antioxidants. Structural analyses have revealed that molecules having vitamin E antioxidant activity include four tocopherols (α , β , γ , δ) and four tocotrienols (α , β , γ , δ) (Figure 7(b)).

At the time of discovery, vitamin E was known as an essential nutrient for reproduction. In later studies it was defined as cellular antioxidant which prevents lipid peroxidation and other radical-driven oxidative events in lipid environment, especially for preventing diseases with inflammations like cardiovascular diseases, atherosclerosis, cancer and neurodegenerative diseases [26, 30, 31, 32].

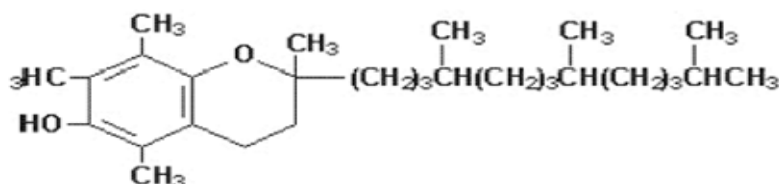
One form of vitamin E, α -tocopherol, is the most abundant form in nature and has the highest biological activity [26, 30, 31, 32, 33, 34]. α -Tocopherol has several mechanisms of action, including inhibition of protein kinase C, which decreases the activity of cyclooxygenase (COX). It also inhibits a production of interleukin 1 (IL1) and superoxide anion ($O_2^{\cdot -}$). The other mechanism leads to the decrease of the genetic expression of NF-

κ B and the reduction of cytokines, prostaglandins and leukotrienes [34]. α -Tocopherol is a substance with high antioxidative potential so it reacts with air oxygen making it difficult to manipulate.

Many different derivatives of vitamin E have been developed and are used in the pharmaceutical industry. A totally new type of emulsifier is D- α -tocopherol polyethylene glycol 1000 succinate (vitamin E TPGS or TPGS). Vitamin E TPGS is a water-soluble derivative of natural vitamin E, produced by the esterification of vitamin E succinate with polyethylene glycol 1000. TPGS is a safe and effective form of vitamin E for reversing or preventing vitamin E deficiency. Vitamin E TPGS has high stability and can be used as a solubiliser, absorption enhancer and as a vehicle for lipid-based drug delivery formulations [35, 36, 37]. The chemical structure of vitamin E TPGS comprises both lipophilic and hydrophilic parts, resulting in amphiphilic properties. Moreover, its lipophilic alkyl tail (α -tocopherol) and hydrophilic polar head portion (polyethylene glycol) are bulky and have large surface areas (Figure 8). Such characteristic makes it a good emulsifier, which can emulsify a wide range of water-oil immiscible systems. The hydrophile-lipophile balance (HLB) of TPGS is about 13. TPGS has the same mechanism of action like vitamin E (Figure 7) [35].



Vitamin E TPGS



Vitamin E

Figure 8. Chemical structure of TPGS and vitamin E (Adapted from [35]).

Direct incorporation of vitamin E into acrylic bone cement is not possible, because it interferes with radical polymerization. Results of this interference are prolongation of

polymerization, modifications in mechanical characteristics and increased flexibility of acrylic bone cement. Incorporation of antioxidant into the micro- or nanoparticles does not interfere with *in situ* polymerization of acrylic bone cement [38, 39].

2 AIM

The aim of the graduation thesis is to develop a formulation method and to prepare and characterise two drug delivery systems: polymethyl methacrylate (PMMA) micro- and nanoparticles containing antibiotic minocycline, which will be then incorporated into bone cement. To modify particle size into the micro and nano scale some excipients will be tested in various proportions. Variations will be made in the amount of incorporated PMMA, minocycline, sucrose and TPGS. For each minocycline particles, plain particles will be produced as controls.

The PMMA particle size, surface morphology, minocycline encapsulation, loading efficiency and drug release profile will be evaluated. The drug release from particles incorporated in the plates will also be studied.

3 MATERIALS AND METHODS

3.1. Materials

1. Minocycline
2. Polyvinyl alcohol (PVA)
3. Dichloromethane (DCM)
4. Polymethyl methacrylate (PMMA)
5. D- α -Tocopherol polyethylene glycol 1000 succinate (TPGS)
6. Sucrose
7. Purified water

3.2. Equipment

1. Balance Kern EG 220-3 NM , serial number: 087690049, Kern&Sohn, Germany;
2. Homogenisator Ultraturrax T10, IKA Laboratories, UK;
3. Homogenisator Silverson® SL-2, Silverson Machines, UK;
4. Centrifuge Allegra 64R, serial number: A2VO8A20, Beckman Coulter Inc., USA;
5. Freeze drying chamber Christ Alpha 1-4, serial number: 6364B (1995), B.Braun Biotech International, Germany;
6. Centrifuge Heraeus, Megafuge 1.0R, serial number: 75003060/02, BD/BE-ADEIFAN, Cacem, Portugal.;
7. Shaking water bath, The Mickle Laboratory Engineering CO.LTD, serial number: 966 038 754, Gomshall, Surrey, England;
8. Laboratorial material: beakers; glass vials; spatules; measuring cilinder; micropipettes 5000 μ l, 1000 μ l, 200 μ l, 20 μ l; magnetic stirrers; vials for freeze drying; eppendorfs; Al foil; heaters; thermometers; graduated conical polypropylene centrifuge tubes, 15 ml capacity.

Table I: Description of materials that had been used in the formulation of micro and nanoparticles

MATERIAL	REFERENCE	CHARACTERISTICS	PRECAUTION	NOTES
Polymethyl methacrylate (PMMA)	Aldrich (Batch 07506TH, MW 350000)	Light white spherical particles (50 μm). Insoluble in water, soluble in organic solvents. Tg = 122 °C	Mucous irritating (skin, eyes and respiratory tract). It is recommended to use gloves.	Contain barium sulphate (9.1% w/w) and benzoyl peroxide (2.05% w/w)
Minocycline	Sigma M9511	A yellow, hygroscopic, crystalline powder. Sparingly soluble in water; slightly soluble in alcohol. It dissolves in solutions of alkali hydroxides and carbonates. Practically insoluble in chloroform and in ether.	Store in airtight containers. Protect from light.	
Dichloromethane (DCM)	Merck 6050	A clear, colourless, volatile liquid. Sparingly soluble in water; miscible with alcohol.	Store in airtight containers. Protect from light.	
Polyvinyl alcohol, 87-89% hydrolyzed (PVA)	Aldrich, 363170-500G, Batch: 10918AE	White, odourless powder. MW 13-23 kDa. Freely soluble in water; slightly soluble in dehydrated alcohol; practically insoluble in acetone. It is a non-ionic surfactant that is used in pharmaceutical manufacturing as a stabilizing agent and as a viscosity-increasing agent and lubricant.	Store in airtight containers. Inflammable at high temperatures. Melting point 200 °C. Light sensitive. Mucous irritating (skin, eyes and respiratory tract).	
D- α -Tocopherol polyethylene glycol 1000 succinate (TPGS)	Sigma (WA23136, Batch: 1413144)	A clear, colourless, or yellowish viscous oily liquid. Practically insoluble in water; freely soluble in dehydrated alcohol, in acetone, in dichloromethane, and in fatty oils. Melting point 36 °C. Solubility 1 g/10 ml H ₂ O.	Hygroscopic. Store at 4 °C. Protect from light.	
Sucrose	HiMedia Laboratories, RM 134	A white or almost white, crystalline powder. Very soluble in water; slightly soluble in alcohol; practically insoluble in ether.	Hygroscopic. Store in a cool, dry place below 25 °C. Away from bright light.	Use for a water solution 10% (w/v) and 10% (w/w PMMA)
Purified water (Ph.Eur.6.3)		Uncoloured, tasteless liquid. Produced with double distillation.		

3.3. Preparation of PMMA particles

3.3.1. Microparticles

PMMA microparticles were prepared by a modified double emulsion w/o/w solvent evaporation method, using PVA as a stabiliser at room temperature [39, 40]. 250 mg of polymer was dissolved in 5 ml DCM and emulsified by homogenisation using an Ultra-Turrax T10 (IKA-Laboratories, UK) for 2 min at 24,000 rpm, with 5.0 mg of antibiotic in 10 % (w/v) PVA solution (internal aqueous phase). The w/o emulsion was added dropwise into 30 ml of a 2.5 % (w/v) PVA solution, and homogenised for 6 min at 10,000 rpm with the Silverson homogeniser (SL-2, Silverson Machines, UK). The double emulsion w/o/w was magnetically stirred for 3 h at room temperature for organic solvent evaporation and to allow microparticle formation. The PMMA microparticles were harvested two times by centrifugation (10,000 rpm, 20 min, 10 °C; Centrifugen Allegra 64R, Beckman Coulter Inc., USA), and were washed with 10% (w/v) sucrose solution before each centrifugation and, at the end, dispersed in 10% (w/w_{PMMA}) sucrose solution. Supernatants recovered after each centrifugation were kept frozen at -20° C until future analysis. All samples of microparticles were subsequently freeze-dried in Christ Alpha 1-4. Glass vials of frozen samples were covered with parafilm with holes, so that water could sublime during the lyophilisation process. These vials were put in beakers (300 ml) and screwed to the plastic stopper of the freeze-drier. The freeze-drier chamber and the frozen samples were connected to a source of vacuum; the pressure was reduced to 0.03 mbar. After 48 hours of lyophilisation a fine, free-flowing dry powder was obtained. Samples were stored in a desiccator [40, 41, 42]. This is the basic preparation process of particles (mpBlank1, mpMino1, mpBlank0.75 and mpMino0.75).

To improve technological and pharmacokinetic properties of microparticles, various excipients were added in the formulation process. Sucrose (2.5% (w/v) or 5%(w/v)) in the external phase was used as a salting-out agent (mpBlank1_S2.5%, mpMino1_S2.5%, mpBlank0.75_S2.5%, mpMino0.75_S2.5%, mpBlank1_S5%, mpMino1_S5%, mpBlank0.75_S5%, mpMino0.75_S5%). For batches mpBlank_TPGS(i.p) and mpMino_TPGS(i.p), TGPS was added to the internal phase. For batches

mpBlank_TPGS(o.p) and mpMino_TPGS(o.p), TPGS was incorporated into the organic phase.

The formulation details are shown in Tables II and III.

Table II: In formulation of mpMino1, the theoretical loading was 2.0 % $w_{\text{MINOCYCLINE}}/w_{\text{PMMA}}$

FORMULATIONS	ORGANIC PHASE	INTERNAL PHASE	EXTERNAL PHASE
mpBlank1	250 mg PMMA + 5 ml DCM	1000 μ l of PVA 10% (w/v)	30 ml of PVA 2.5% (w/v)
mpMino1	250 mg PMMA + 5 ml DCM	1000 μ l solution of antibiotic in PVA 10% (w/v)	30 ml of PVA 2.5% (w/v)

Table III: Variations in the formulation of microparticles

FORMULATIONS	ORGANIC PHASE	INTERNAL PHASE	EXTERNAL PHASE
mpBlank1_S2.5%	250 mg PMMA + 5 ml DCM	1000 μ l of PVA 10% (w/v)	30 ml solution of PVA 2.5% (w/v)+ sucrose 2.5% (w/v)
mpMino1_S2.5%	250 mg PMMA + 5 ml DCM	1000 μ l solution of antibiotic in PVA 10% (w/v)	30 ml solution of PVA 2.5% (w/v)+ sucrose 2.5% (w/v)
mpBlank1_S5%	250 mg PMMA + 5 ml DCM	1000 μ l of PVA 10% (w/v)	30 ml solution of PVA 2.5% (w/v) + sucrose 5% (w/v)
mpMino1_S5%	250 mg PMMA + 5 ml DCM	1000 μ l solution of antibiotic in PVA 10% (w/v)	30 ml solution of PVA 2.5% (w/v) + sucrose 5% (w/v)
mpBlank0.75	250 mg PMMA + 5 ml DCM	750 μ l of PVA 10% (w/v)	30 ml of PVA 2.5% (w/v)
mpMino0.75	250 mg PMMA + 5 ml DCM	750 μ l solution of antibiotic in PVA 10% (w/v)	30 ml of PVA 2.5% (w/v)
mpBlank0.75_S2.5%	250 mg PMMA + 5 ml DCM	750 μ l of PVA 10% (w/v)	30 ml solution of PVA 2.5 % (w/v)+ sucrose 2.5 % (w/v)
mpMino0.75_S2.5%	250 mg PMMA + 5 ml DCM	750 μ l solution of antibiotic in PVA 10% (w/v)	30 ml solution of PVA 2.5% (w/v)+ sucrose 2.5% (w/v)
mpBlank0.75_S5%	250 mg PMMA + 5 ml DCM	750 μ l of PVA 10% (w/v)	30 ml solution of PVA 2.5% (w/v) + sucrose 5% (w/v)
mpMino0.75_S5%	250 mg PMMA + 5 ml DCM	750 μ l solution of antibiotic in PVA 10% (w/v)	30 ml solution of PVA 2.5% (w/v)+ sucrose 5% (w/v)
mpBlank_TPGS(i.p)	250 mg PMMA + 5 ml DCM	1000 μ l of PVA 10% (w/v)+ TPGS 10 mg	30 ml of PVA 2.5% (w/v)
mpMino_TPGS(i.p)	250 mg PMMA + 5 ml DCM	1000 μ l solution of antibiotic in PVA 10% (w/v) + TPGS 10 mg	30 ml of PVA 2.5% (w/v)
mpBlank_TPGS(o.p)	250 mg PMMA + 5 ml DCM + TPGS 10 mg	1000 μ l of PVA 10% (w/v)	30 ml of PVA 2.5% (w/v)
mpMino_TPGS(o.p)	250 mg PMMA + 5 ml DCM + TPGS 10 mg	1000 μ l solution of antibiotic in PVA 10% (w/v)	30ml of PVA 2.5% (w/v)

Batches mpMino1_S2.5%, mpMino1_S5%, mpMino_TPGS(i.p) and mpMino_TPGS(o.p) contained 2.0% $w_{\text{MINOCYCLINE}}/w_{\text{PMMA}}$ in the internal phase. On the other hand, batches mpMino0.75, mpMino0.75_S2.5% and mpMino0.75_S5% contained 1.5% $w_{\text{MINOCYCLINE}}/w_{\text{PMMA}}$. For each minocycline-loaded microparticles, plain particles were produced as controls.

3.3.2. Nanoparticles

Formulation process of nanoparticles is comparable to the one of microparticles. The main difference is the concentration of polymer. The quantity of reagents that were used in the preparation of nanoparticles was different compared to the PMMA microparticles: 100 mg of polymer was dissolved in 6 ml DCM and emulsified. An internal aqueous phase consisted of 10%(w/v) PVA solution, which contained the 2.0 mg of antibiotic. The w/o emulsion was added dropwise into the 30 ml of a 1.25% (w/v) PVA solution and homogenised with the Silverson homogeniser. The resultant w/o/w emulsion was magnetically stirred at room temperature for 3 h to evaporate the organic solvent and allow nanoparticle formation. The PMMA nanoparticles were harvested by centrifugation (15,000 rpm, 30 min at 10 °C), washed two times with 10% (w/v) sucrose solution before each centrifugation and dispersed in 10% (w/ w_{PMMA}) sucrose solution. All samples were subsequently freeze-dried as describe earlier. After the lyophilisation, samples were stored in a desiccators [40, 41, 42]. Variations in the formulation of nanoparticles are comparable to those of microparticles.

The formulations details are shown in Tables IV and V.

Table IV: In formulation of nanoparticles npMino1, the theoretical loading was 2.0% $w_{\text{MINOCYCLINE}}/w_{\text{PMMA}}$

FORMULATIONS	ORGANIC PHASE	INTERNAL PHASE	EXTERNAL PHASE
npBlank1	100 mg PMMA + 6 ml DCM	1000 µl of PVA 10% (w/v) solution	30 ml of PVA 1.25% (w/v)
npMino1	100 mg PMMA + 6 ml DCM	1000 µl solution of antibiotic in PVA 10% (w/v)	30 ml of PVA 1.25% (w/v)

Table V: Variations in the formulation of nanoparticles

FORMULATIONS	ORGANIC PHASE	INTERNAL PHASE	EXTERNAL PHASE
npBlank1_S1.25%	100 mg PMMA + 6 ml DCM	1000 µl of PVA 10% (w/v)	30 ml solution of PVA 1.25% (w/v) + sucrose 1.25% (w/v)
npMino1_S1.25%	100 mg PMMA + 6 ml DCM	1000 µl solution of antibiotic in PVA 10% (w/v)	30 ml solution of PVA 1.25% (w/v) + sucrose 1.25% (w/v)
npBlank1_S2.5%	100 mg PMMA + 6 ml DCM	1000 µl of PVA 10% (w/v)	30 ml solution of PVA 1.25% (w/v)+ sucrose 2.5% (w/v)
npMino1_S2.5%	100 mg PMMA + 6 ml DCM	1000 µl solution of antibiotic in PVA 10% (w/v)	30 ml solution of PVA 1.25% (w/v) + sucrose 2.5% (w/v)
npBlank0.75	100 mg PMMA + 6 ml DCM	750 µl of PVA 10% (w/v)	30 ml of PVA 1.25% (w/v)
npMino0.75	100 mg PMMA + 6 ml DCM	750 µl solution of antibiotic in PVA 10% (w/v)	30 ml of PVA 1.25% (w/v)
npBlank0.75_S1.25%	100 mg PMMA + 6 ml DCM	750 µl of PVA 10% (w/v)	30 ml solution of PVA 1.25% (w/v) + sucrose 1.25% (w/v)
npMino0.75_S1.25%	100 mg PMMA + 6 ml DCM	750 µl solution of antibiotic in PVA 10% (w/v)	30 ml solution of PVA 1.25% (w/v) + sucrose 1.25% (w/v)
npBlank0.75_S2.5%	100 mg PMMA + 6 ml DCM	750 µl of PVA 10% (w/v)	30 ml solution of PVA 1.25% (w/v) + sucrose 2.5% (w/v)
npMino0.75_S2.5%	100 mg PMMA + 6 ml DCM	750 µl solution of antibiotic in PVA 10% (w/v)	30 ml solution of PVA 1.25% (w/v) + sucrose 2.5% (w/v)
npBlank_TPGS(i.p)	100 mg PMMA + 6 ml DCM	1000 µl of PVA 10% (w/v)+ TPGS 10 mg	30 ml of PVA 1.25% (w/v)
npMino_TPGS(i.p)	100 mg PMMA + 6 ml DCM	1000 µl solution of antibiotic in PVA 10% (w/v)+ TPGS 10 mg	30 ml of PVA 1.25% (w/v)
npBlank_TPGS(o.p)	100 mg PMMA + 6 ml DCM + TPGS 10 mg	1000 µl of PVA 10% (w/v)	30 ml of PVA 1.25% (w/v)
npMino_TPGS(o.p)	100 mg PMMA + 6 ml DCM + TPGS 10 mg	1000 µl solution of antibiotic in PVA 10% (w/v)	30 ml of PVA 1.25% (w/v)

Batches npMino1_S2.5%, npMino1_S1.25%, npMino_TPGS(i.p) and npMino_TPGS(o.p) contained 2.0% $w_{\text{MINOCYCLINE}}/w_{\text{PMMA}}$ in the internal phase. Batches npMino0.75, npMino0.75_S2.5% and npMino0.75_S1.25% contained 1.5% $w_{\text{MINOCYCLINE}}/w_{\text{PMMA}}$. For each minocycline-loaded nanoparticle plain particles were made as controls.

3.4. Preparation of acrylic bone cement plates with micro- and nanoparticles

- Reagents:

1. Acrylic bone cement DePuy® CMW1 :

Table VI: Comercial acrylic bone cement DePuy® CMW1 (Johnson&Johnson)

COMPONENTS	% (w/w)
<u>Powder</u>	
Polymethyl methacrylate	88.85
Benzoyl peroxide	2.05
Barium sulphate	9.10
<u>Liquid</u>	
Methyl methacrylate	98.50
Hydroquinone (ppm)	75
N,N-dimethyl- <i>p</i> -toluidine	≤1.50
Ethanol	There is no reference of the quantity
Ascorbic acid	There is no reference of the quantity

2. Lyophilised micro- and nanoparticles (Tables II, III, IV and V)

- Equipment and materials:

1. Balance Kern EG 220-3 NM, serial number: 087690049, Kern&Sohn, Germany;
2. Mould;
3. Laboratorial material: spatula; Petri dish; micropipette 1000 µl.

- Experiment:

In this assay, four different acrylic bone cement plates were prepared with micro- (PmB1, PmM1) or nanoparticles (PnB1, PnM1). Each plate was prepared by polymerization *in situ* of PMMA (1.0 g) and particles (10.0 mg) with MMA (500 µl). PMMA (50.0 mg) was mixed with 10.0 mg of particles (micro- and nanoparticles, blank or loaded with minocycline), until the mixture was homogenised. The remaining 950.0 mg of PMMA was added by the principle of increasing weight, so that the powders were homogeneously mixed. MMA (500 µl) was added to the powder mixture and homogenised with spatula in a Petri dish. When the mixture gained consistence, it was transferred to the mould, where the polymerization process has finished.

All four plates were prepared as previously described.



Figure 9. Material used in the preparation of acrylic bone cement. In the flask it is methyl methacrylate (MMA) and in the Petri dish it is polymethyl methacrylate (PMMA) with 10.0 mg of lyophilised particles. On the right side of the picture is the mould.



Figure 10. Homogenization of powder mixture (PMMA and lyophilised particles) with MMA with spatula in a Petri dish.



Figure 11. Acrylic bone cement mixture in the mould.



Figure 12. Plate of acrylic bone cement after completed polymerization.

Table VII: Description of four different plates formulated by polymerization *in situ*

FORMULATION	MASS (g)	LENGTH (mm)	WIDTH (mm)	THICKNESS (mm)
		(a)	(b)	(c)
PmB1	0.754	24.1	24.6	1.18
PmM1	0.720	23.2	25.0	1.08
PnB1	0.809	25.0	24.8	1.18
PnM1	0.737	23.0	25.1	1.08

Plate PmB1 had mpBlank1 lyophilised microparticles incorporated. PmM1 contained minocycline-loaded microparticles (mpMino1). Plates PnB1 and PnM1 contained respectively the lyophilised nanoparticles npBlank1 and npMino1.

3.5. Quantitative determination of minocycline

- Reagents:

1. Purified water (Table I);
2. Polyvinyl alcohol (PVA) (Table I);
3. Dichloromethane (DCM) (Table I);
4. Acetone
5. Minocycline (Table I);
6. Lyophilised micro- and nanoparticles (Tables II, III, IV and V)

- Equipment and materials:

1. Balance Kern EG 220-3 NM (max: 220 g, min: 0,02 g), serial number: 087690049, Kern&Sohn, Germany;
2. Spectrophotometer U-2001, serial number: 0051-020, Hitachi Instruments Inc, USA;
3. Laboratorial material: micropipettes 20 µl, 200 µl, 1000 µl and 5000 µl; Eppendorf® tubs 2 ml; spatulas; beakers; quartz cuvettes.

- Experiment:

The method of selection to quantify minocycline is the UV spectrophotometry [43]. The absorbance was determined at the wavelength of maximum absorption of the minocycline ($\lambda=343.0$ nm). The linearity of this method was verified and elaborated by 2 calibration curves. The “mother solution” was prepared immediately before each assay. All samples that were kept frozen had to be defrosted before the experiment.

3.5.1. Calibration curve

Eight minocycline solutions with different concentrations were prepared during the development of this quantification method. PVA_{2.5%} was the media for microparticles, whether PVA_{1.25%} was used in the quantification of minocycline-nanoparticle loading. In both cases the “mother solution” had a concentration of 500 ppm (microparticles: 10 mg minocycline/ 20 ml PVA_{2.5%} = 500 µg/ml = 500 ppm; nanoparticles: 10 mg minocycline/ 20 ml PVA_{1.25%} = 500 µg/ml = 500 ppm).

From each “mother solution”, eight different solutions of minocycline have been prepared with a final volume of 2 ml and presenting the final concentrations: 30 µg/ml, 25 µg/ml, 20 µg/ml, 15 µg/ml, 10 µg/ml, 5 µg/ml, 2.5 µg/ml and 1 µg/ml (Table VIII). All solutions were prepared in duplicates in 2 ml Eppendorf® tubes.

Table VIII: Composition of the minocycline solutions, which were used to demonstrate the linearity of the method

SOLUTIONS	FINAL CONCENTRATION (µg/ml)	VOLUME OF “MOTHER SOLUTION” (ml)	VOLUME OF PVA _{2.5%} ⁽¹⁾ (ml)
S1	1	4	1996
S2	2.5	10	1990
S3	5	20	1980
S4	10	40	1960
S5	15	60	1940
S6	20	80	1920
S7	25	100	1900
S8	30	120	1880

⁽¹⁾ Note: PVA_{2.5%} is referring to the microparticles. As regards nanoparticles, PVA_{1.25%} has been used with the same volume.

The linearity was demonstrated by measuring the absorbance of each solution that had been prepared from the main one (“mother solution”). The absorbance was measured with a UV spectrophotometer at the wavelength 343.0 nm (this was the λ_{maximum} determined for minocycline), using quartz cuvettes. To determine the baseline, both cuvettes were filled with PVA_{2.5%} solution (in the case of nanoparticles, PVA_{1.25%} was use as a baseline). Thus, the absorbance of minocycline solutions was determined from the most diluted to the most concentrated solutions (from S1 to S8, Table VIII). The blank was a PVA_{2.5%} solution (nanoparticles- PVA_{1.25%}). The quartz cuvette was washed with purified water and ethanol before each reading.

As a result, four calibration curves were elaborated two for each type of particles (micro- and nano-).

3.5.2. Determination of minocycline concentration

3.5.2.1. Indirect method

Supernatants, that were kept frozen in screw-capped tubes after each centrifugation during the formulation process of micro- and nanoparticles, were used in this indirect method.

Before each determination, supernatants were defrosted at the room temperature and 2 ml of each sample (Tables II, III, IV and V) were put into an Eppendorf® tube. Marked Eppendorf® tubes were put into the micro-centrifuge (Centrifuge Allegra 64R, Beckman Coulter Inc., USA) with rotor F2402H for 20 minutes at 10°C and 15,000 rpm. After centrifugation, the absorbance of minocycline in supernatants was measured at the wavelength 343.0 nm. With this absorbance value, it was possible to calculate the amount of minocycline that was not incorporated in particles. In the case of microparticles, the PVA_{2.5%} was used as a baseline. As regards nanoparticles, the baseline was made with PVA_{1.25%}.

3.5.2.2. Direct method

Direct determination was performed with several organic solvents, because of particular PMMA solubility.

In the first experiment DCM was used as a solvent. An accurate amount of plain particles (10.0 mg) was weighed and dispersed in 2.5 ml of DCM. This dispersion was divided into five Eppendorfs® tubes and 1000 µl of purified water was added for extraction of the antibiotic. This resultant dispersion was carefully shaken on a Vortex and the aqueous phase from each Eppendorf® was collected into a vial. Again, 1000 µl of purified water was added to the organic phase in all five Eppendorf® tubes. After a new re-extraction, the aqueous phase was collected into other vial. The absorbance of each collected aqueous phase was measured at wavelength $\lambda=343.0$ nm using quartz cuvette and purified water as a baseline and control.

The second experiment was carried out with DCM and ethanol. Particles (10.0 mg) were dispersed in 1 ml of DCM and 1.5 ml of ethanol. The suspension was centrifuged and the absorbance of minocycline in the supernatant was read against ethanol.

Plain particles (10.0 mg) were also dispersed in 2.5 ml of acetone. The suspension was put on stirring, but the PMMA was not completely dissolved. This dispersion was then centrifuged and the supernatant absorbance was measured at 343.0 nm against acetone.

In the last experiment, PMMA particles were dissolved with a mixture of DCM and acetone. Particles (10.0 mg) were dispersed in 1.5 ml of DCM and 1.5 ml of acetone was added to this suspension. After stirring, particles were still not dissolved. Therefore, this

suspension was centrifuged and the absorbance of minocycline in the supernatant was measured at $\lambda=343.0$ nm against acetone.

3.6. Physicochemical characterisation of PMMA particles

3.6.1. Particle size

- Reagents:

1. Purified water (Table I);
2. Lyophilised micro- and nanoparticles (Tables II, III, IV and V)

- Equipment and materials:

1. Zetasizer Nano Series, Malvern Instruments Ltd., UK;
2. Balance Kern EG 220-3 NM, serial number: 087690049, Kern&Sohn, Germany;
3. Laboratorial material: micropipettes 20 μ l, 100 μ l, 200 μ l, Eppendorf® tubs, plastic cuvettes 1 ml

- Experiment:

Each sample of particles (5 mg; micro- and nanoparticles), plain or loaded with the antibiotic minocycline, was suspended in 200 μ l of purified water in Eppendorf® tubes. All samples were analysed in duplicates. These samples were further diluted by adding 20 μ l of each suspension to 100 μ l of purified water. This dilution was performed in order to avoid instrumental errors.

3.6.2. Particle morphology

Scanning electron microscopy (SEM, Phillips/FEI XL30 SEM) was used to corroborate the size and to analyse particle surface morphology. Before operation, a thin layer of sample powder was stuck on a double-sided tape attached onto aluminium stubs. The particle surface was then coated with a gold film. Coating was made under vacuum in an argon atmosphere with thickness of approximately 20 nm, prior to SEM analysis [38, 39, 40].

3.7. Two different *in vitro* minocycline release studies

3.7.1. Minocycline release from lyophilised PMMA micro- and nanoparticles

- Reagents:

1. PBS pH 7,4
2. Lyophilised micro- and nanoparticles (Tables II, III, IV and V)

- Equipment and materials:

1. Balance Kern EG 220-3 NM, serial number: 087690049, Kern&Sohn, Germany;
2. Shaking water bath, The Mickle Laboratory Engineering Co. Ltd, serial number: 966 038 754, Gomshall, Surrey, England;
3. Spectrophotometer U-2001, serial number: 0051-020, Hitachi Instruments Inc, USA;
4. Laboratorial material: micropipettes 20 µl, 100 µl, 200 µl, Eppendorf® tubs, quartz cuvette 2 ml

- Phosphate buffer saline with pH 7.4 (PBS 7.4):

The buffer helps to maintain a constant pH. Osmolarity and ion concentrations of the solution usually match those of the human body (isotonic). Preparation (Farmacopeia Portuguesa vol.4): Dissolve 2.38 g of dibasic sodium phosphate R, 0.19 g of monobasic potassium phosphate R and 8.0 g of sodium chloride R in water R and fill to 1000.00 ml with the same solvent. Adjust pH.

- Experiment:

The release properties of minocycline from the lyophilised particles were measured in duplicate in PBS (Phosphate buffer saline) at pH 7.4, under *in vitro* conditions. 20.0 mg of minocycline-loaded nanoparticle powder (npBlank1, npMino1 and npBlank_TPGS(i.p.), npMino_TPGS(i.p.)) was suspended in 2 ml of PBS in screw-capped tube. As regard microparticles, 30.0 mg of antibiotic-loaded microparticles powder (mpBlank1, mpMino1 and mpBlank_TPGS(i.p.), mpMino_TPGS(i.p.)) was suspended in 2 ml of PBS in screw-

capped tube. The tubes were placed in an orbital shaking water bath, which was maintained at 37 °C and shaken horizontally at 110 min⁻¹.

At pre-defined time points (2 h, 4 h, 6 h, 24 h, 48 h, 72 h, 7th day, 9th day, 14th day and 16th day), the tubes were collected and centrifuged at 6,000 rpm for 30 min. The supernatant solution (1.8 ml) was collected from each tube for UV analysis and the pellet was re-suspended in 1.8 ml of fresh PBS and then put back into the shaking water bath to continuous release.

3.7.2. Minocycline release from acrylic bone cement plates

- Reagents:

1. PBS pH 7.4
2. Plates (Table VII)

- Equipment and materials:

1. Shaking water bath, The Mickle Laboratory Engineering Co. Ltd, serial number: 966 038 754, Gomshall, Surrey, England;
2. Spectrophotometer U-2001, serial number: 0051-020, Hitachi Instruments Inc, USA;
3. Laboratorial material: micropipettes 5000 µl; screw-capped tubes; quartz cuvette 2 ml

- Experiment:

The release characteristics of minocycline from plates were measured in PBS pH 7.4 under *in vitro* conditions. Each plate (PmB1, PmM1, PnB1 and PnM1) was suspended in 2 ml of PBS in screw-capped tube. The tubes were placed in an orbital shaking water bath, under the same conditions as describe earlier.

At pre-defined time points (24 h, 48 h and 5th day), the tubes were collected from the orbital shaker water bath and 1.8 ml of supernatant solution from each tube was analysed using an UV spectrophotometer. After the absorbance determination, the supernatants were put back into the screw-capped tubes, which were maintained in the shaking water bath to proceed the release. At each time point the samples were treated as mentioned above.

4 RESULTS AND DISCUSSION

4.1. PMMA micro- and nanoparticles

4.1.1. Characterisation of PMMA micro- and nanoparticles

In microparticle formulation process, the organic phase was composed of 250 mg of PMMA powder dissolved in 5 ml of DCM. On the other hand, the organic phase of nanoparticles was composed of 100 mg of PMMA powder dissolved in 6 ml of DCM. Differences in external phase between micro- and nanoparticles were related to PVA concentration: 2.5% (w/v) PVA solution was used in the formulation of microparticles, while nanoparticles were formulated with a 1.25% (w/v) PVA solution. There was no difference in the formulation process between the internal phases of micro- and nanoparticles (Table IX).

In order to protect a hydrophilic drug and to prolong its further delivery, the multiple emulsions w/o/w were formulated. Freeze-drying technology was used to produce dry powder, as the powder form is much more stable. Some molecules, known as lyoprotectants, protect freeze-dried material. In the formulation process 10% (w/w_{PMMA}) sucrose solution was used as a lyoprotectant.

Table IX: Differences in formulation of micro and nanoparticles

	MICROPARTICLES	NANOPARTICLES
ORGANIC PHASE	250 mg PMMA + 5 ml DCM	100 mg PMMA + 6 ml DCM
INTERNAL PHASE	PVA _{10%} or solution of minocycline in PVA _{10%}	PVA _{10%} or solution of minocycline in PVA _{10%}
EXTERNAL PHASE	30 ml PVA _{2.5%}	30 ml PVA _{1.25%}

Variations in the formulation were made in order to improve technological and pharmacokinetic properties of micro- and nanoparticles. Different concentrations of sucrose solution (1.25% (w/v), 2.5% (w/v) or 5% (w/v)) were added to the external phase. Sucrose was used as a salting-out agent. Many others compounds, especially salts as MgCl₂ or NaCl could be use for salting-out. The sucrose was chosen, according to the positive results of the study that was conducted previously in the same laboratory. In this study of tigecycline-loaded micro- and nanoparticles higher E.E and L.E were obtained when sucrose was incorporated in the vehicles. Microparticles mpBlank1_S2.5%,

mpMino1_S2.5%, mpBlank0.75_S2.5%, mpMino0.75_S2.5% and nanoparticles npBlank1_S2.5%, npMino1_S2.5%, npBlank0.75_S2.5%, npMino0.75_S2.5% were prepared using a 2.5% (w/v) sucrose solution (S is a abbreviation for sucrose). A 5% (w/v) sucrose solution was used in the formulation of mpBlank1_S5%, mpMino1_S5%, mpBlank0.75_S5%, mpMino0.75_S5%. Nanoparticles npBlank1_S1.25%, npMino1_S1.25%, npBlank0.75_S1.25%, npMino0.75_S1.25% had incorporated 1.25% (w/v) sucrose solution. TPGS is a drug solubiliser and bio-enhancer, as previously referred in the Introduction (Section 1.5.1 Vitamin E). In a previous study performed in our lab, higher loadings were obtained when TPGS was used in PMMA particle formulation [38, 39]. In addition, it is an antioxidant agent and for these reasons we decided to incorporate this compound in the minocycline-loaded PMMA particles.

4.1.2. Particle morphology

Main characteristics of prepared micro- and nanoparticles were determined by a scanning electronic microscope (SEM). The following photographs were obtained for mpBlank1, mpMino1, mpBlank_TPGS(i.p.) and mpMino_TPGS(i.p.) microparticle batches and npBlank1, npMino1, npBlank_TPGS(i.p.) and npMino_TPGS(i.p.) nanoparticle batches (Figures 13 and 14). The SEM photos show some differences between micro- and nanoparticles. All nanoparticles had a fine spherical shape with various degree of smooth surface. From the zoom-in picture we noticed pores on the particle surfaces and single particles showed certain roughness on their surface although multi-particle images gave a relatively smoother surface morphology. Microparticles also had a spherical shape and showed a rough surface even on multi-particle images. Although the topography of particles may be complex, the roughness and pores observed on the surface could provide diffusion release mechanism. Smooth surface of nanospheres supports the assumption that the drug release from nanoparticles might be caused by both diffusion and matrix erosion. The amount of PMMA powder (Table IX) not only had impact on particles size, but also had a great influence on their surface characteristics. In Figures 13 and 14 it is possible to observe two different size populations of particles within the same type of particles. This may be a consequence of the homogenisation technique during a formulation process. No considerable difference was observed between microparticles that contain vitamin E TPGS and those without this excipient (Figure 13 (c), (d)). Rough surface characteristics

are comparable among blank microparticles mpBlank1, mpBlank_TPGS(i.p.) and minocycline-loaded microparticles mpMino1, mpMino_TPGS(i.p.). The resemblance is noticeable as well, as concerns two size populations of particles. Nanoparticles were equally similar regardless their composition, except in case of batch npBlank_TPGS(i.p.), as it is obvious that these particles are bigger than npBlank1. This outcome can be due to the incorporated vitamin E TPGS, which has a high molecular weight.

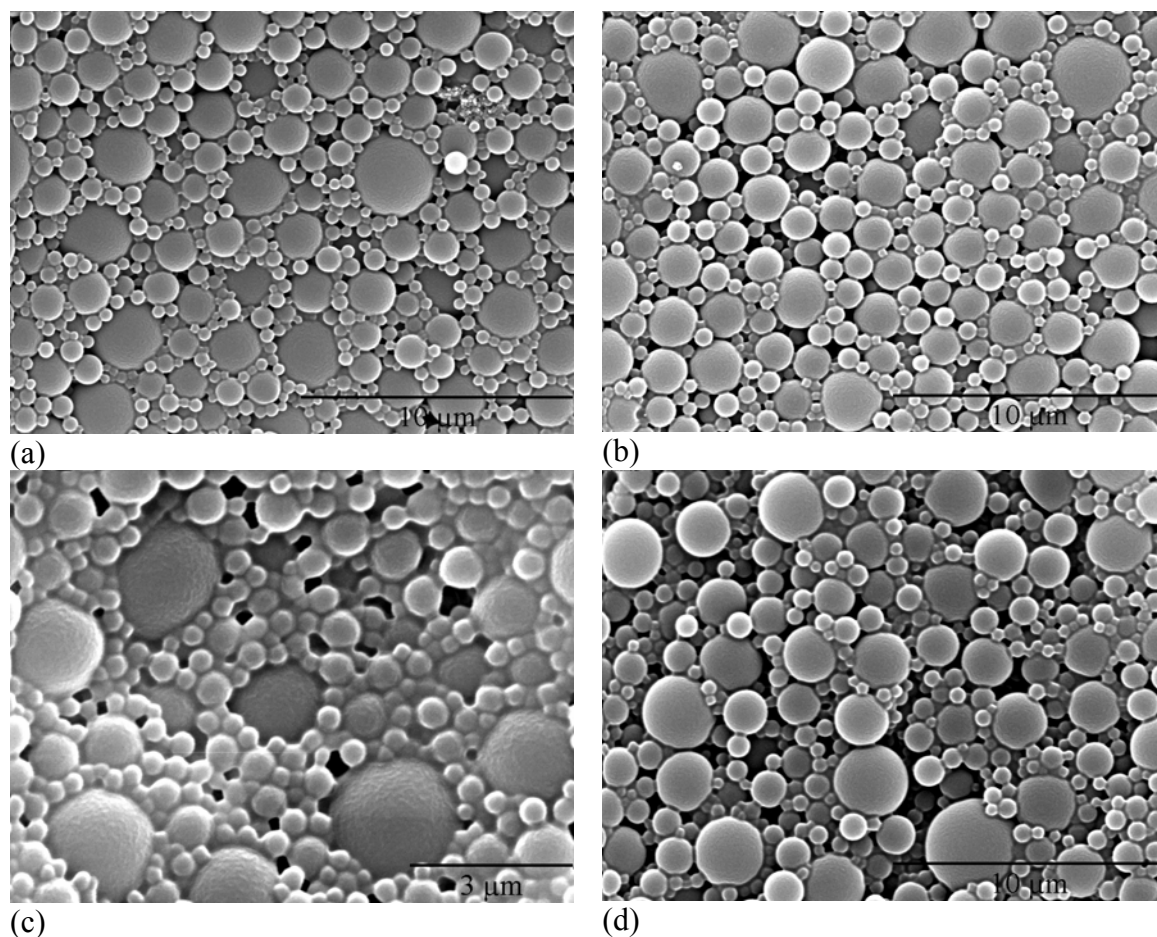


Figure 13. SEM photographs of PMMA microparticles: (a) mpBlank1 lyophilized particles; (b) minocycline-loaded microparticles mpMino1; (c) and (d) microparticles that contain TPGS in the internal phase; (c) mpBlank_TPGS(i.p.) and (d) mpMino_TPGS(i.p.) microparticles.

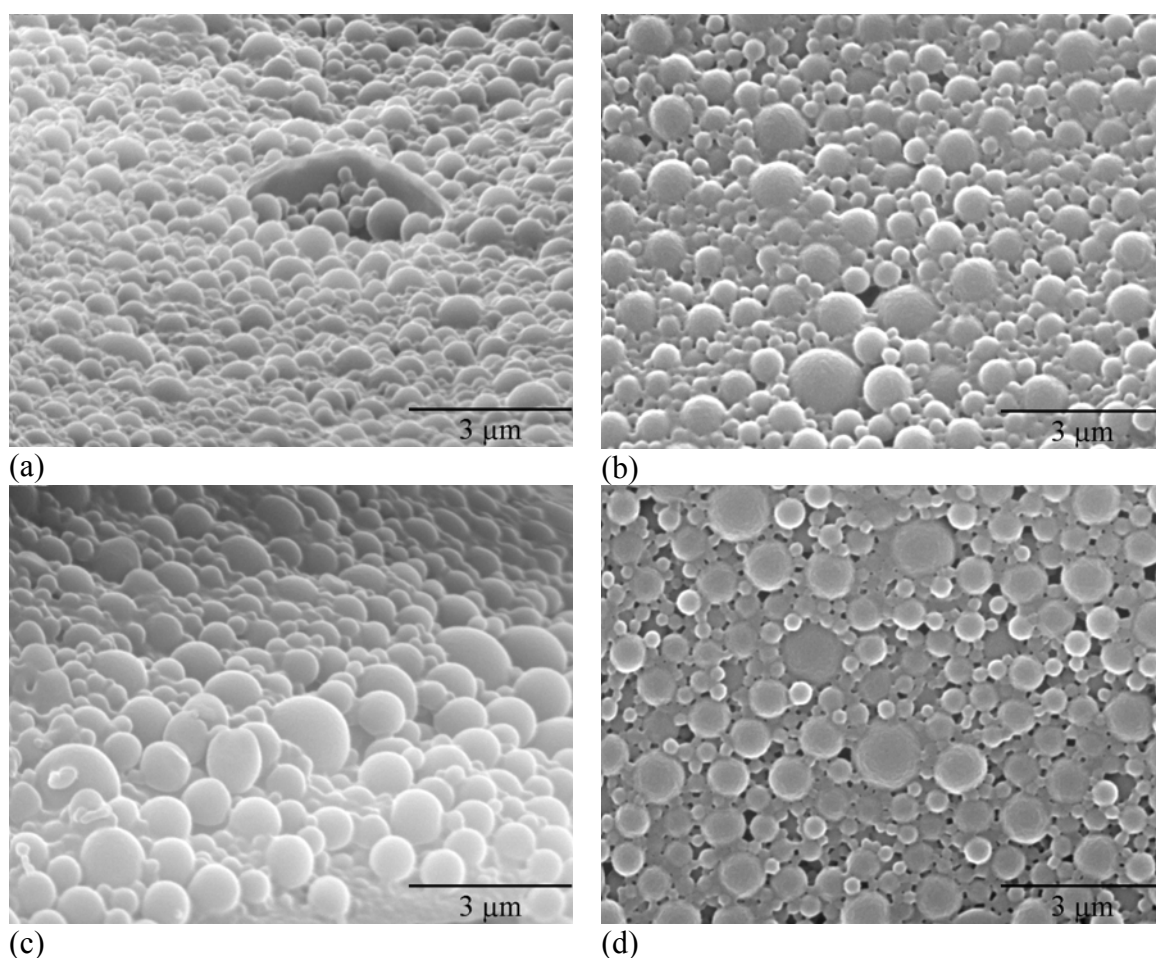


Figure 14. SEM photographs of PMMA nanoparticles: (a) npBlank1; (b) minocycline-loaded nanoparticles npMino1; (c) and (d) npBlank_TPGS(i.p.) and npMino_TPGS(i.p.), respectively.

As for the macroscopic aspects of lyophilised particles, they appear as a light powder. All minocycline-loaded micro- and nanoparticles had a slightly yellow coloration, compared to white plain particles. The coloration of loaded particles is related to the fact that the antibiotic minocycline is a yellow powder (Table I). Loaded microparticles were more yellow compared to nanoparticles, which are loaded with the same antibiotic quantity. From the macroscopic properties of antibiotic-loaded particles it was possible to assume that microparticles incorporate higher amount of minocycline than nanoparticles. These expectations were confirmed or rejected, by results of drug L.E and E.E.

4.1.3. Plate morphology

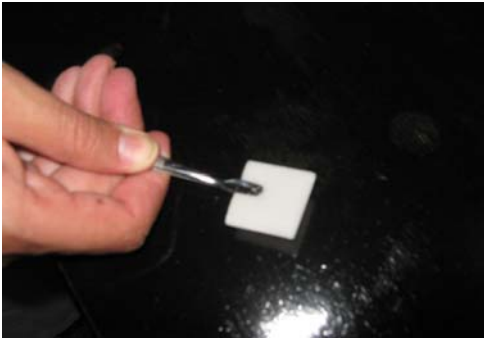
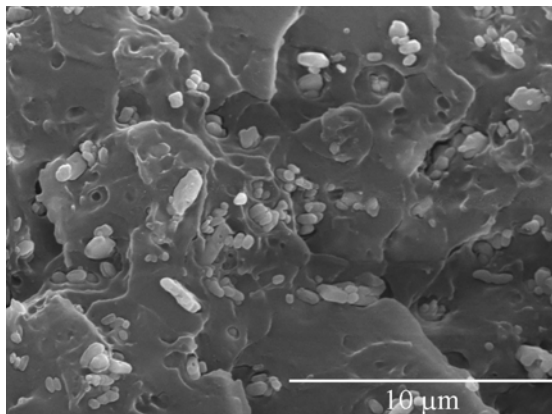
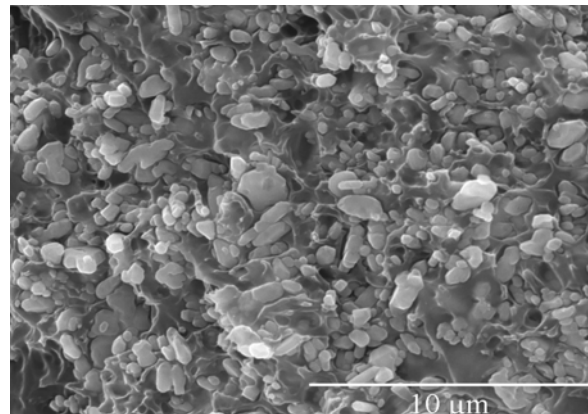


Figure 15. Macroscopic aspect of acrylic bone cement plate that contains 10 mg of nanoparticles.

From visual observation of the acrylic bone cement plate, we can not assume if the incorporated material were particles or just powder (Figure 15). That is why the observation with a scanning electronic microscope (SEM) was performed. However, even from the SEM Figure 16 it was not possible to distinguish unequivocally nanoparticles, as it is difficult to distinguish between the spherical shape of nanoparticles and the random morphology of the PMMA powder (Figure 16 (a)). The Figure 16 (c) was taken at zoom 30 μ m and it shows non-homogeneous distribution of empty areas and areas with incorporated nanoparticles. Distribution is difficult to improve, because each plate was homogenised with spatula for about 3 min, until the mixture gained consistence and was impossible to mould it.



(a)



(b)

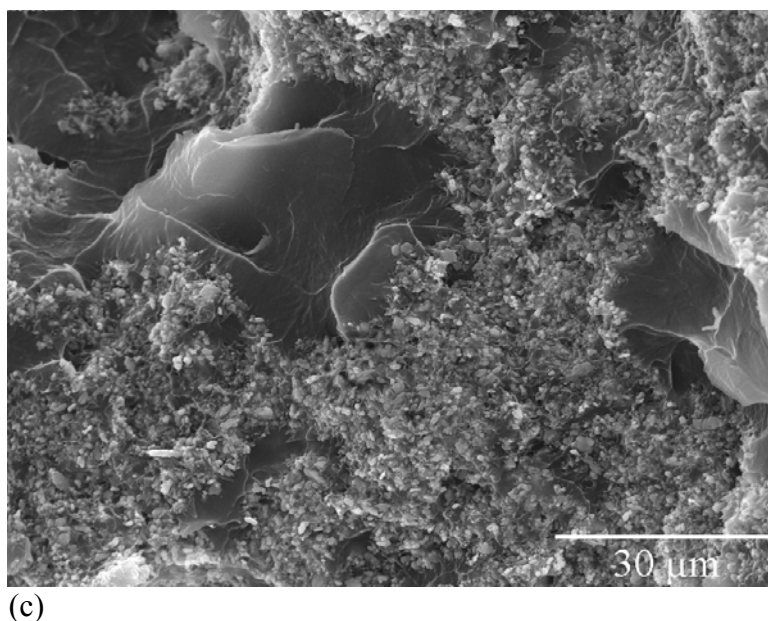


Figure 16. Bone cement plates, empty or loaded with nanoparticles. (a) Empty plates, composed by PMMA and MMA, without any nanoparticles incorporated. (b) Plates with 10 mg of incorporated nanoparticles (npMino1) in the mixture of PMMA and MMA. (c) Plate with empty areas and areas with incorporated npMino1 nanoparticles.

4.1.4. Particle size

In our study the VMD and polydispersial index (PI) were measured in duplicates, using a photonic correlation spectroscopy (Tables X, XI). Size and size distribution are the most important characteristics of micro- and nanoparticulate systems. They have an important impact on physicochemical properties, *in vitro* bio-distribution, optimum efficacy and toxicity. Drug loading, drug release and stability of particles also depend on particle size. An advantage, nanoparticles over microparticles, is a higher intracellular uptake and therefore can be available to a wider range of biological targets. Therefore, it is a challenge to formulate as small particles as possible, with a maximum stability. In this thesis work we intended to formulate microparticles smaller than 1000 nm and nanoparticles under 500 nm. In practice, these particles will be incorporated into the acrylic bone cement to anchor prosthesis to the bone. After a certain time when particles degrade, they will leave holes behind. Because of the risk of reduced bone density, these holes should be minimal.

The t-test was used to examine differences in VMD between the batches of minocycline-loaded particles and their plain control. The level of statistical difference was defined as $p < 0.05$.

4.1.4.1. Size and size distribution of microparticles

Table X: Average size and polydispersity index (PI) of formulated microparticles (mean \pm SD; n = 3);

FORMULATIONS	VMD (nm)	POLYDISPERSITY INDEX
mpBlank1	762 \pm 4.24	0.226
mpMino1	786 \pm 11.3	0.234
mpBlank1_S2.5%	807 \pm 6.36	0.170
mpMino1_S2.5%	834 \pm 15.6	0.238
mpBlank1_S5%	901 \pm 14.1	0.215
mpMino1_S5%	863 \pm 13.4	0.197
mpBlank0.75	701 \pm 5.66	0.217
mpMino0.75	712 \pm 15.6	0.228
mpBlank0.75_S2.5%	712 \pm 5.66	0.219
mpMino0.75_S2.5%	730 \pm 8.48	0.236
mpBlank0.75_S5%	749 \pm 21.9	0.220
mpMino0.75_S5%	793 \pm 26.2	0.265
mpBlank_TPGS(i.p.)	801 \pm 4.95	0.238
mpMino_TPGS(i.p.)	867 \pm 16.3	0.226
mpBlank_TPGS(o.p.)	789 \pm 3.21	0.225
mpMino_TPGS(o.p.)	781 \pm 6.44	0.219

Batch mpMino1, incorporated with 2.0% (w/w_{PMMA}) of minocycline, had VMD of 786 nm, with a PI of 0.234 (Zetasizer Nano Sizer, Malvern Instruments Ltd., UK; Figure 17). The VMD of blank particles mpBlank1 was 762 nm and PI was 0.226. These results were comparable (p = 0.105). Similarly, microparticles mpMino0.75, which had a theoretical loading of 1.5% (w/w_{PMMA}) of minocycline, had a VMD of 712 nm and PI 0.228 (Figure 18). Their blank control had a VMD of 701 nm and PI 0.217. In fact, the difference observed between the size of plain (mpBlank 0.75) and minocycline-loaded particles (mpMino0.75) was not significant (mpMino0.75 vs mpBlank 0.75 p = 0.180).

Also in the case of mpMino0.75 the quantity of internal phase that was used in the formulation process was lower than that used for mpMino1 production, the difference observed between these values were not significant (p = 0.09). From Figures 17 and 18, we could notice that in both cases samples had two populations of particles. It is probable that the population with lower intensity was due to the presence of particles aggregated in the sample prepared for this determination.

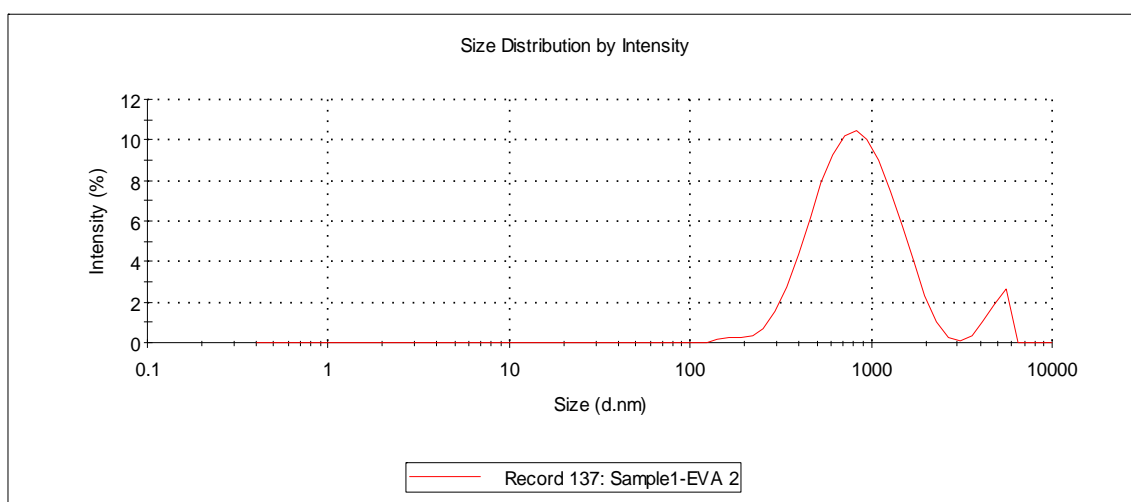


Figure 17. Size distribution of mpMino1 microparticles.

To increase both E.E and L.E some changes were performed in the formulation process using a salting-out agent. Sucrose was used in two different concentrations, 2.5% (w/v) and 5% (w/v) (Table III, Materials and methods). From the results presented in Table X, it was visible that the salting-out agent (sucrose) seems to augment the VMD. The size of plain particles with or without sucrose was significantly different for those prepared with 1 ml of 10% (w/v) PVA solution in the internal phase (mpBlank1 vs mpBlank1_S2.5% $p = 0.023$; mpBlank1 vs mpBlank1_S5% $p = 0.006$). On the other hand, the incorporation of 2.5% (w/v) of sucrose in particles prepared with 750 μ l of 10% (w/v) PVA solution did not significantly change the size of the correspondent plain ones (mpBlank0.75 vs mpBlank0.75_S2.5% $p = 0.106$). However, those prepared with higher amount of sucrose were of higher size than the plain ones (mpBlank0.75 vs mpBlank0.75_S5% $p = 0.04$), which was expected.

The incorporation of minocycline in those sucrose-loaded microparticles (mpBlank1_S2.5%, mpBlank0.75_S2.5%, mpBlank1_S5% and mpBlank0.75_S5%) did not change particle size (mpBlank1_S2.5% vs mpMino1_S2.5% $p = 0.076$; mpBlank1_S5% vs mpMino1_S5% $p = 0.152$; mpBlank0.75_S2.5% vs mpMino0.75_S2.5% $p = 0.05$; mpBlank0.75_S5% vs mpMino0.75_S5% $p = 0.21$). We were expect, that microparticles mpMino1_S5% loaded with minocycline would be larger when compared to the blank control. The size difference was less than 40 nm. Most likely the suspension of particles mpMino1_S5% was not properly dispersed and bigger particles sediment because of their physical instability. Lyophilised particles should be dispersed in

aqueous media just before they were put into the Zetasizer Nano Sizer and so we would not have any problems with sedimentation. Moreover, the particles could have been sonicated before their reading or Tween 80 could have been used to help to their dispersion. In order to confirm microparticle size, the size measurement with Malvern Mastersizer should have been performed, as the Zetasizer Nano Sizer is more adequate to measure the size of particles within the nanometric range. Unfortunately the mentioned equipment was not available and the measurement was not performed.

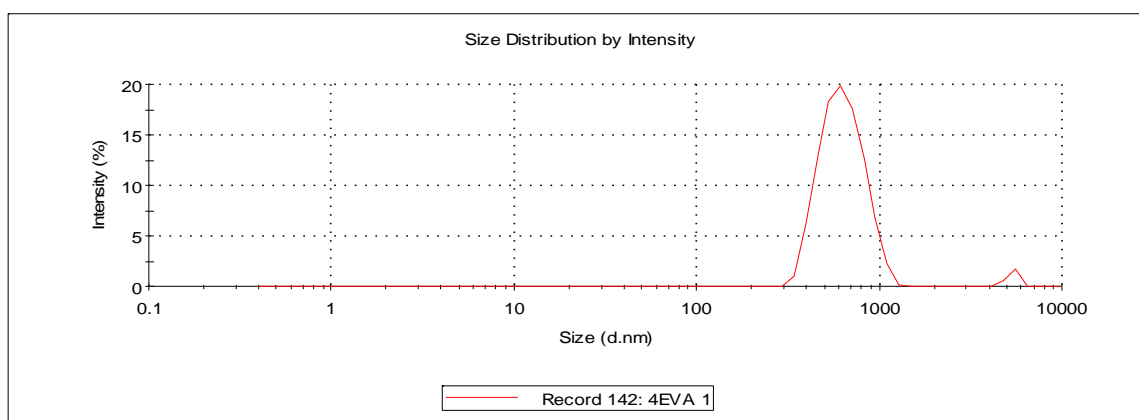


Figure 18. Size distribution of mpMino0.75 microparticles.

Size average of plain microparticles where TPGS was incorporated into internal (mpBlank_TPGS(i.p.)) or organic phase (mpBlank_TPGS(o.p.)), were comparable to formulation mpBlank1 ($p > 0.05$). The size of mpMino_TPGS(i.p.) particles were significantly higher than presented by mpBlank_TPGS(i.p.) ($p = 0.038$). VMD of mpMino_TPGS(i.p.) was 867 nm and was about 80 nm higher than size average of mpMino_TPGS(o.p.), which was 781 nm. The incorporation of TPGS in the internal phase of the double emulsion (mpMino_TPGS(i.p.)), significantly increased particle size, VMD of 867 ± 16.3 ($p = 0.043$). From Figure 19, it was possible to observe that the peak of the curve in the graph was not as clearly defined as on Figure 17. Also the intensity of the peak (Figure 19) was visibly lower and PI was wider, compared to microparticles mpMino1 (Figure 17).

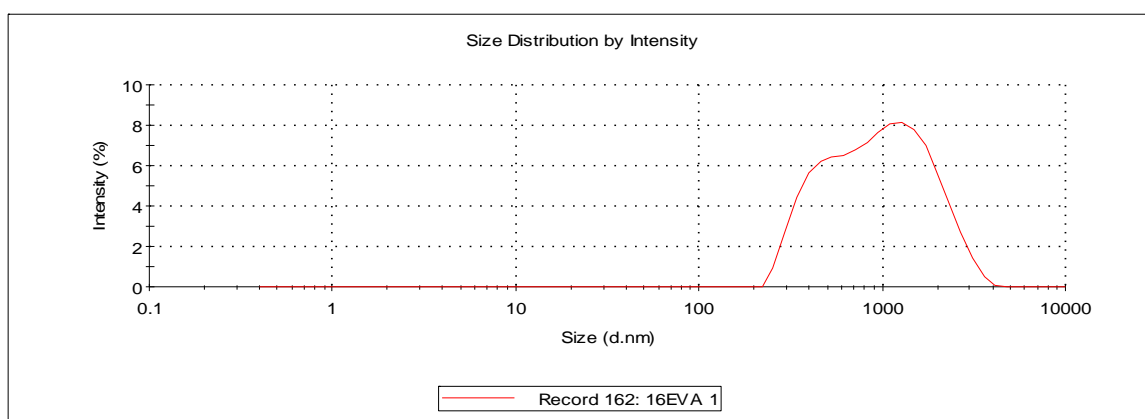


Figure 19. Size distribution of microparticles mpMino_TPGS(o.p.).

To conclude, average particle size of microparticles that was formulated in this study was 787 nm with PI 0.223. VMD of plain microparticles (mpMino1), which had incorporated 2% (w/w_{PMMA}) of theoretical antibiotic, was 786 nm. VMD of microparticles (mpMino0.75) that had incorporated 1.5% (w/w_{PMMA}) of theoretical minocycline was 712 nm. This could be attributed to the lower volume of minocycline that was incorporated. There is no major difference between blank and loaded microparticles in each batch.

4.1.4.2. Size and size distribution of nanoparticles

Table XI: VMD and polydispersity index (PI) of formulated nanoparticles (mean \pm SD; n= 3);

FORMULATIONS	VMD (nm)	POLYDISPERSITY INDEX
npBlank1	342 \pm 16.3	0.221
npMino1	353 \pm 16.3	0.217
npBlank1_S1.25%	340 \pm 11.3	0.256
npMino1_S1.25%	351 \pm 6.36	0.247
npBlank1_S2.5%	350 \pm 7.78	0.220
npMino1_S2.5%	359 \pm 2.12	0.205
npBlank0.75	329 \pm 17.7	0.191
npMino0.75	344 \pm 14.8	0.226
npBlank0.75_S1.25%	322 \pm 4.95	0.250
npMino0.75_S1.25%	335 \pm 16.3	0.329
npBlank0.75_S2.5%	347 \pm 5.66	0.247
npMino0.75_S2.5%	353 \pm 2.12	0.231
npBlank_TPGS(i.p.)	393 \pm 3.65	0.274
npMino_TPGS(i.p.)	399 \pm 6.13	0.249
npBlank_TPGS(o.p.)	375 \pm 4.95	0.246
npMino_TPGS(o.p.)	374 \pm 12.7	0.327

Photonic correlation spectroscopy (Zetasizer Nano Sizer) was used to determinate the VMD and PI of nanoparticles. Nanoparticles npMino1, containing 2.0% (w/w_{PMMA})

minocycline, had a VMD of 353 nm and their PI was 0.217 (Figure 20). Blank control particles npBlank1 had VMD of 342 nm. If we compare the npMino1 to plain nanoparticles npBlank1 VMD (Table XI), it is possible to state that the difference observed was not significant ($p = 0.28$). However, it would be expected that the incorporation of the antibiotic would increase particle size average, due to the size of the antibiotic molecule (Figure 5, Introduction).

Nanoparticles npMino0.75, which had incorporated 1.5% (w/w_{PMMA}) of theoretical minocycline, had a VMD of 344 nm and PI 0.226 (Figure 21). In the case of npMino0.75 parameters were smaller than npMino1, because of the lower volume of the incorporated internal phase. Even though, that difference was not significant ($p = 0.15$). NpMino0.75 sample presented two size populations (Figure 21), compared to npMino1 (Figure 20) that did not have any effect on PI, which is a mathematic parameter that help us to determinate the differences between batches of particles. If we compare the npMino0.75 to plain nanoparticles npBlank0.75 (Table XII), we can assume that the minocycline incorporation did not significantly increased size average ($p = 0.055$), and a similar situation was observed for npBlank1 and npMino1 ($p = 0.28$).

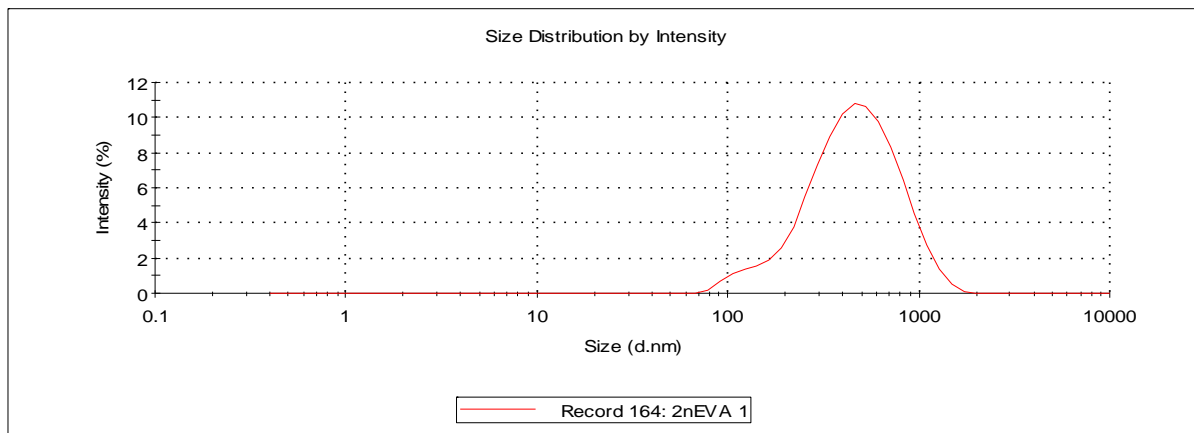


Figure 20. Size distribution of npMino1 nanoparticles.

Salting-out method with two different concentrations of sucrose 1.25 % (w/v) and 2.5 % (w/v) (Table V, Materials and methods), was used to increase both E.E and L.E of nanoparticles.

VMD of all batches with sucrose in the external phase (npBlank0.75_S1.25%, npMino0.75_S1.25%, npBlank0.75_S2.5% and npMino0.75_S2.5%) did not deviate for more than 20 nm from the average particle size. The increase of size average of batches that contains sucrose was not as obvious as it would be expected (Table XI). Batches with

sucrose had similar size results compared to the plain particles ($p > 0.05$). We could conclude that all the batches in this group had small deviations from the value of average particle size. The incorporation of minocycline in plain and sucrose-loaded particles did not significantly change particles size ($p > 0.05$).

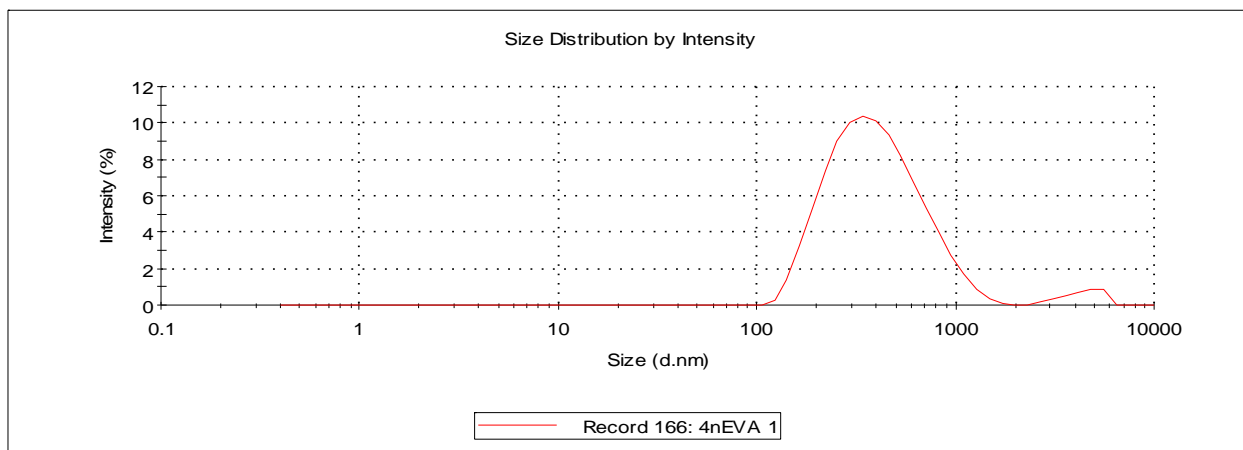


Figure 21. Size distribution of npMino0.75 nanoparticles.

From data in Table XI, we could conclude how the TPGS influenced the VMD of nanoparticles. Nanoparticles, which contain vitamin E TPGS in internal phase (npBlank_TPGS(i.p.), npMino_TPGS(i.p.)), had higher VMD than those nanoparticles, which had TPGS incorporated in organic phase (npBlank_TPGS(o.p.), npMino_TPGS(o.p.)). The inclusion of TPGS in the internal phase significantly increased particles size ($p = 0.037$ for npBlank1 vs npBlank_TPGS(i.p.)). From the Figures 22 it was possible to confirm the mathematic data (VMD and PI) on Table XI of npMino_TPGS(o.p.). VMD was higher (374nm) and PI was wider (0.327) compared to the plain nanoparticles npMino1 (Figure 20), even if the difference is not significant ($p > 0.05$). Similarly, the incorporation of minocycline in TPGS containing nanoparticles did not influence their size ($p > 0.05$).

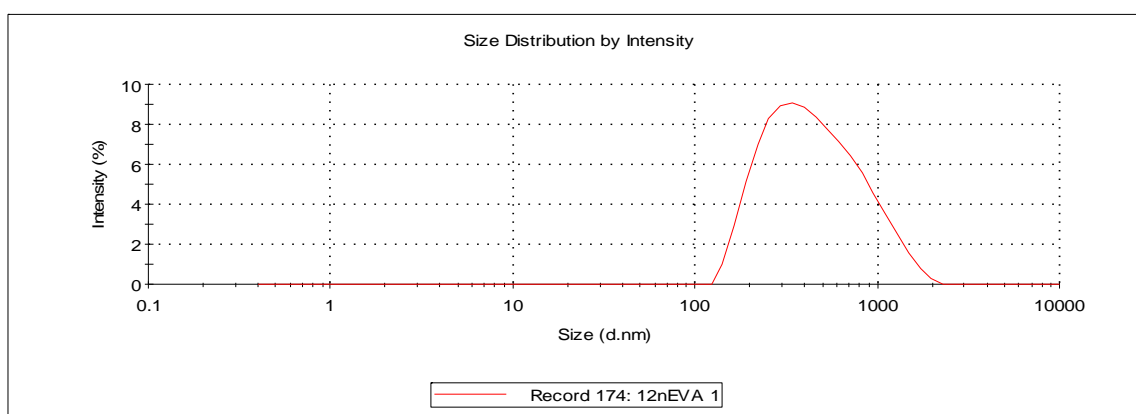


Figure 22. Size distribution of npMino_TPGS(o.p.) nanoparticles.

To conclude, the average particle size of the nanoparticles that were formulated in this research work is 356 nm. As regards nanoparticles (npMino1) that had incorporated 2% (w/w_{PMMA}) of theoretical minocycline, their VMD is 353 nm. Nanoparticles npMino0.75 with 1.5% (w/w_{PMMA}) of theoretical minocycline incorporated, their size average is 344 nm. The impact of incorporated sucrose solution to the size average was lower than we expected. On the other hand, the incorporation of TPGS increased VMD of particle, which was most likely to be dependent on the molecule size of vitamin E TPGS (Figure7).

Comparing micro- and nanoparticles (Table X and Table XI), the most obvious difference was in the VMD. Microparticles had VMD of 787 nm for, while nanoparticles had VMD of 356 nm. These results are expectable and desirable as mention earlier. During the formulation process of nanoparticles the quantity and homogenization variables were changed compared to microparticle formulation process (Tables II, III, IV and V, Section: Materials and methods); these had an impact on particle size and moreover on their size distribution. However, PI of nanoparticles was wider compared to microparticles. It was difficult to formulate extremely small particles as micro- and nanoparticles, but even more difficult was to formulate particles that are within nanometric range and have approximately the same size. It was expected that the smaller the particles are the wider can be the PI.

4.2. Quantitative determination of minocycline

The E.E and L.E are essential parameters for their characterization, since they identify the amount of drug (minocycline) that is actually incorporated into the particles. During the research, there were used two different determination methods: indirect and direct method.

- Identification of the maximum wavelength (λ_{\max})

The standard solutions were essential for the identification of the wavelength of maximum absorption for the antibiotic minocycline, which was needed for its quantification. According to the literature, minocycline wavelength of maximum absorption (λ_{\max}) can be found between 270 nm and 350 nm [43]. Standard scans were prepared to determine the λ_{\max} of minocycline. It was found out to be 343.0 nm. One peak appeared at the λ around 270 nm, but at this wavelength could not be considered for its quantification as many substances, such as PVA, also absorb at this region.

- Linearity

According to the guidelines of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) [44], the linearity is one of the essential parameters in the validation of an analytical method. The linearity of a method is defined as the range of concentrations when linear relationship between measurement (absorbance) and concentration is present (Beer- Lambert Law) [44]. Consequently, a linear method can lead to a mathematical equation that expresses the ratio between the absorbance and the concentration of the analyte within the detection limits of the device.

In this case, the linearity of the quantification method was verified through the development of two curves for each particle type (micro- and nanoparticles).

Eight different standard solutions with a final volume of 2 ml and final concentrations of 30 $\mu\text{g/ml}$, 25 $\mu\text{g/ml}$, 20 $\mu\text{g/ml}$, 15 $\mu\text{g/ml}$, 10 $\mu\text{g/ml}$, 5 $\mu\text{g/ml}$, 2.5 $\mu\text{g/ml}$ and 1 $\mu\text{g/ml}$ (Table 8, Materials and methods) were prepared, and their absorbance was measured with the UV spectrophotometer at the wavelength 343.0 nm (λ_{maximum} of minocycline). It was used for quantitative determination for both particles. From the obtained results we could plot one calibration curve for both types of particles (micro- and nanoparticles).

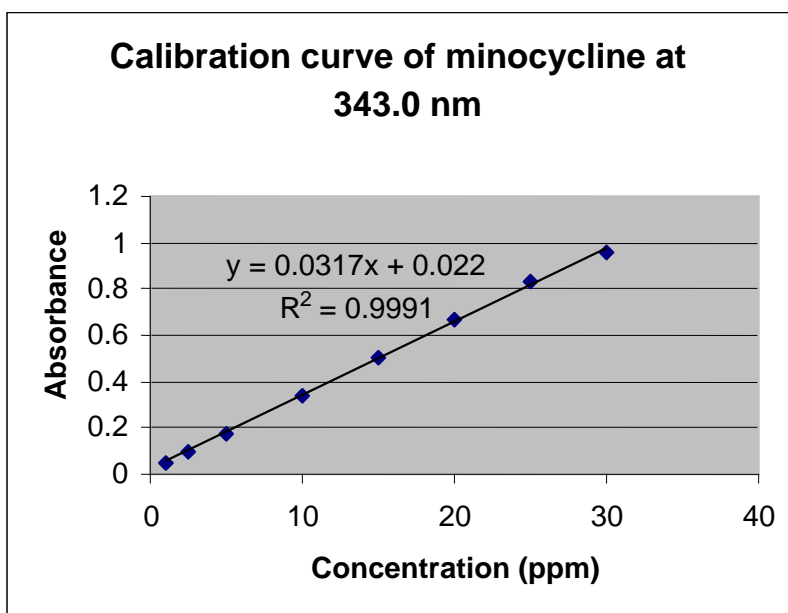


Figure 23. Calibration curve of minocycline at λ_{maximum} 343.0 nm.

The calibration curve was obtained by linear regression using the least squares method. The curve was characterized by two parameters: the least squares coefficient of determination (R^2) and the linear equation.

The calibration curves indicated the existence of a linear correlation between the absorbance at λ_{maximum} of minocycline and its concentration in standard solutions, with R^2 value 0.9991 (Figure 23). The closer this value is to 1.0, the better the data fit to a straight-line. It is considered that a method is linear when the value R^2 of calibration curve is greater than 0.99, which means that over 99% of the experimental points coincide with the values estimated by linear regression.

4.2.1. Minocycline encapsulation and loading efficiency

The ability of PMMA particles to incorporate the antibiotic minocycline was determined as previously described (Section: Materials and Methods, Indirect determination and Direct determination). It should be noted that this procedure should have been done at least in triplicate in order to be able to perform reliable statistical correlations.

4.2.1.1. Indirect method

The amount of minocycline that was not incorporated into micro- and nanoparticles was determined by the quantification of minocycline in particle supernatants.

The amount of incorporated antibiotic was calculated by the difference between the total mass of antibiotic used during formulation and the mass of antibiotic, which was not incorporated into the particles (*Equations 1 and 2*).

$$E.E (\%) = \frac{\text{mass of min ocycline incorporated}}{\text{mass of min ocycline used}} \times 100$$

Equation 1

$$L.E (\%) = \frac{\text{mass of min ocycline incorporated}}{\text{mass of PMMA}} \times 100$$

Equation 2

Table XII: Encapsulation efficiency (E.E) and loading efficiency (L.E) of minocycline-loaded microparticles

FORMULATIONS	E.E (%)	L.E (%)
mpMino1	38.5	0.77
mpMino1_S2.5%	63.3	1.26
mpMino1_S5%	67.2	1.34
mpMino0.75	44.1	0.66
mpMino0.75_S2.5%	36.8	0.55
mpMino0.75_S5%	64.3	0.96
mpMino_TPGS(i.p)	58.6	1.17
mpMino_TPGS(o.p)	55.5	1.11

The microparticle E.E was 38.5 % for 2.0 % (w/w_{PMMA}) of antibiotic-minocycline (mpMino1) (Table XII). The L.E of these particles was 0.77 %. The L.E seems to be low, but it is important to mention that 2.0 % w_{MINOCYCLINE}/w_{PMMA} minocycline solution (1 ml) was used to be incorporated in 250 mg of polymer. If the volume of antibiotic would be higher, it is expected that L.E would be higher as well, but some formulation problems could occur, mainly related to the amount of the emulsifier (PVA) and its concentration.

In order to increase the encapsulation and loading efficiencies, salting-out was used in the preparation of other batches: mpMino1_S2.5%; mpMino0.75_S2.5%; mpMino1_S5%; mpMino0.75_S5%. Sucrose was chosen as the salting-out agent. In the first attempt, sucrose concentration was 2.5 % (w/v), the same as PVA concentration in the external phase (mpMino1_S2.5%, mpMino0.75_S2.5%). And in the second batch (mpMino1_S5%,

mpMino0.75_S5%) the concentration of sucrose was doubled compared to the emulsifier amount. From Table XII, it is clear that the E.E and L.E had increased. The E.E in samples mpMino1_S2.5%, mpMino1_S5%, mpMino0.75_S5% was higher than 60 %, except for mpMino0.75_S2.5%, where it was only 36.8 %. L.E was around 1 % for samples mpMino1_S2.5%, mpMino1_S5%, mpMino0.75_S5%, but in sample mpMino0.75_S2.5% it was just 0.55 %.

Probably some mistakes have been done during the formulation process of mpMino0.75_S2.5%. In fact, if we compare the E.E and L.E of microparticle batches without sucrose (mpMino0.75) and microparticles where sucrose was used as a salting-out agent (mpMino0.75_S2.5%), it is possible to state that both were higher for batch mpMino0.75. This is not comparable to other results.

To increase particle E.E and L.E, TPGS, an antioxidant and surfactant, was used during their formulation [35, 36]. In mpMino_TPGS(i.p), TPGS was added into the internal phase and in mpMino_TPGS(o.p) TPGS was added into the organic phase. The E.E and L.E were comparable in both samples; E.E was higher than 50 % and L.E was around 1 % (Table XII). Vitamin E TPGS is a novel surfactant stabiliser, which can be added either in the aqueous phase or in the oil phase, and some has been able to increase of incorporation of different drugs [35, 36, 37]. PMMA particles in which TPGS was incorporated had higher E.E and L.E. In this research work, E.E and L.E of microparticles that contain TPGS were higher comparable to those obtained for mpMino1 (Table XII).

Table XIII: Encapsulation efficiency (E.E) and loading efficiency (L.E) of minocycline-loaded nanoparticles

FORMULATIONS	E.E (%)	L.E (%)
npMino1	30.45	0.60
npMino1_S1.25%	33.7	0.66
npMino1_S2.5%	47.0	0.91
npMino0.75	38.8	0.57
npMino0.75_S1.25%	23.8	0.35
npMino0.75_S2.5%	16.3	0.24
npMino_TPGS(i.p)	36.6	0.72
npMino_TPGS(o.p)	29.6	0.57

As regards nanoparticles, the situation is quite similar. E.E of nanoparticles npMino1 and npMino0.75 was, in both cases, higher than 30 %. The L.E, in both samples, was higher than 0.5 %.

Salting-out method, with sucrose, was used also to increase the E.E and L.E of these PMMA nanoparticles. Sucrose was used in the concentration 2.5% (w/v) and 1.25% (w/v).

The E.E and L.E obtained for npMino1_S1.25% nanoparticles were comparable to those obtained for batches npMino1. Probably the concentration of sucrose was too low and thus salting-out was not efficient.

For npMino1_S2.5%, it was obvious that salting-out increased E.E and L.E. E.E and L.E of nanoparticles npMino0.75_S2.5% were lower, compared to results of npMino0.75. These results can be probably justified as its vial was broken at -20 °C and therefore was separately freeze-dried. From these results, it is possible to conclude that the freeze-drying process interferes with particle formulation process.

Nanoparticles containing TPGS in the internal phase (npMino_TPGS(i.p)) had an E.E of 36.6 % and L.E of 0.72 %, in contrast to nanoparticles containing TPGS in organic phase (npMino_TPGS(o.p)), which presented an E.E of 29.6 % and L.E of 0.57 %. From these results we could notice that TPGS in the internal phase contributed for a higher amount of antibiotic loaded in these particles, than its presence in the organic phase.

4.2.1.2. Direct method

The direct determination method was based on the complete dissolution of lyophilised PMMA particles in DCM and in the quantification of the antibiotic minocycline by UV spectrophotometry. We could not achieve complete dissolution of particles in DCM. In fact, all batches of micro/nanoparticles resulted as a suspension and not as solutions, when particles were dissolved in DCM. The formation of such suspension could be related to the presence of sucrose and PVA in the freeze-dried powder (micro- and nanoparticles). PVA in theory should have been eliminated during the washing with purified water before freeze-drying the particles, but can be found in the literature that the PVA is not completely eliminated [45]. Due to the presence of substances insoluble in organic solvents, it was not possible to quantify the minocycline embedded in particles by direct reading the absorbance of the dissolved particles. Therefore, it was necessary to develop an alternative approach. Consequently, a double extraction was performed using purified water as a solvent for PVA and sucrose, since they are water soluble (Table 1, Materials and Methods) and almost insoluble in DCM [45]. Extraction had no success, because again it was not possible to obtain clear solutions of PMMA particles in DCM, which would allow measuring the absorbance of minocycline with spectrophotometer at a wavelength maximum, without the presence of interferents.

The same problem appeared at the double extraction with ethanol and DCM; there was no possibility to obtain clear solution of PMMA.

As a final alternative method, the mixture of DCM and acetone was used. But the final result was not that promising, the suspension did not dissolve into a clear solution. As a result, the measured absorbance with UV spectrophotometer was not accurate.

Most likely the new bonds had been formulated between PMMA and antibiotic-minocycline in the phase of particle preparation. Therefore, the particles are practically insoluble in DCM, acetone, ethanol (polar solvent).

To conclude, with the direct determination method it was not able to determinate L.E and E.E of minocycline in PMMA micro- and nanoparticles. In most cases, when PMMA is used as a polymer substance, the direct method is not a suitable determination method. Because of PMMA dissolution problem, indirect determination method is commonly used [6, 7, 45]. In our case the direct determination of incorporated minocycline would be necessary, because it is possible that one part of this antibiotic had chemically react with polymer and had create covalent binds. In future study, this part of minocycline will not contribute to the antibacterial efficiency. One of possible methods would be polymer swelling in lipophylic solvent under continuously conditions.

4.3. *In vitro* minocycline release study

4.3.1. Minocycline release from lyophilised PMMA micro- and nanoparticles

The drug release profile of minocycline from micro/nanoparticles depends on: drug solubility, drug diffusion through the polymeric membrane and matrix degradation [21, 22].

Cumulative release of encapsulated minocycline from micro- and nanopartilces was determined under *in vitro* conditions in PBS pH 7.4 (Chapter Materials and methods).

Although for most polymers used in nanotechnology the method with PBS 7.4 is successful, in the case of polymer PMMA it did not work properly. None of the supernatant samples that were taken out from the screw-capped tubes at pre-defined time

intervals (2 h, 4 h, 6 h, 24 h, 48 h, 72 h, 7th day, 9th day, 14th day and 16th day) had a curve with a peak at the wavelength 343.0 nm (λ_{maximum} of minocycline), compared to the baseline PBS 7.4. It was expected that the antibiotic minocycline would not diffuse easily through polymer, due to PMMA properties previously mentioned.

It was obvious; that the method which works on other polymers does not work on PMMA polymer, although that it was mention in some articles that PBS was used as media in release study from PMMA [21, 22, 36]. To get some result from the release study, we should have done some changes, maybe using a different working medium or performing changes in pH. This assay needed at least 1.8 ml of the supernatant and therefore most of it was replaced by new buffer, after each measurement of absorbance with UV spectrophotometer. Probably, buffer volume that was replaced was too high considering the amount of antibiotic that has been released. Therefore, the method should be supplemented with some changes. One of the possibilities is that the supernatants, to which the absorbance was determinate at pre-define time points, could be put back into the screw-capped tube after each determination. As a consequence, the cumulative release would be an outcome of this modified method. This alternative would be the most suitable, but it could not be accomplished due to a limited research time. Alternatively, instead of using one eppendorf tube per batch, per assay, a different approach could have been applied; for example, the experiment could have been designed preparing one eppendorf tube per time point. By doing so, the dilution problem would not exist. Another possibility was the formulation of acrylic bone cement plates, which would then be used to perform the *in vitro* release study. In this case the release study method would be radically changed. This last option was selected to be carried out in these experiments.

4.3.2. Minocycline release from acrylic bone cement plates

Drug release study under *in vitro* conditions from acrylic bone cement plates had a successful outcome, compared to the drug release from lyophilised PMMA micro- and nanoparticles. Both of drug release methods were made under similar conditions; PBS with pH 7.4 was used as a working media and, in both cases, *in vitro* conditions were comparable; tubes were placed in an orbital shaking water bath, which was maintained at 37 °C and shaken horizontally at 110 min⁻¹.

We observed two major differences. The fundamental difference was the polymerization *in situ* in the case of drug release from acrylic bone cement plates. Most likely, radical polymerization of acrylic bone cement (PMMA) had a main contribution on drug release profile. Another difference is the working media, which was put back into the screw-capped tube after each determination.

In case of acrylic bone cement plates containing microparticles, the minocycline (% w/w) released at similar time intervals was lower compared to plates containing nanoparticles. This may be a consequence of particle size. Smaller particles have larger surface area; therefore, most of the drug would be at or near the particle surface, leading to a fast drug release. Whereas, larger particles have large cores which allow more drug to be encapsulated and slowly diffuse out. These statements are represented on Figures 24 and 25 [1].

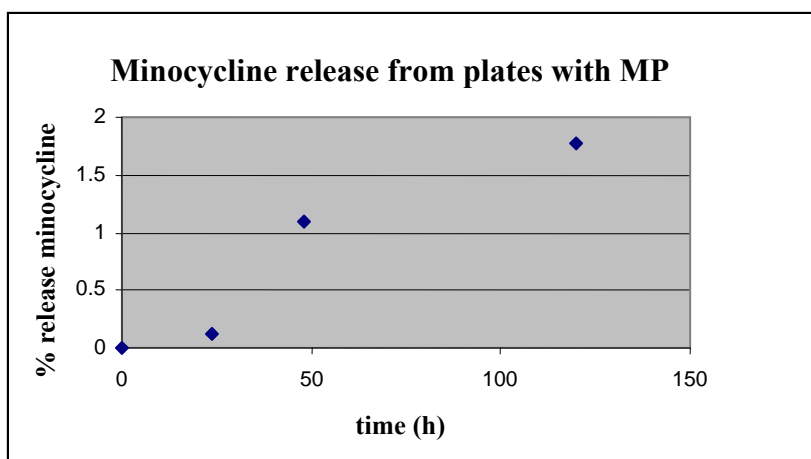


Figure 24. *In vitro* drug release from acrylic bone cement plate (PmM1), containing 2.0% (w/w_{PMMA}) minocycline-loaded microparticles (mpMino1).

As regards plates containing microparticles (mpMino1), drug release is obviously delayed, compared to that obtained for plates containing nanoparticles (npMino1) (Figure 24 vs Figure 25). The minocycline released 24 hours after the beginning of the assay was only 0.13% (w/w) for PmM1 and 0.95% (w/w) for PnM1. After 48 hours, the difference in drug release was even more obvious, being 1.10% (w/w) for PmM1 and 2.21% (w/w) for PnM1. On the fifth day of the assay, 1.8% (w/w) of minocycline was released from the PmM1 and 4.1% (w/w) from the PnM1 (Figures 24 and 25).

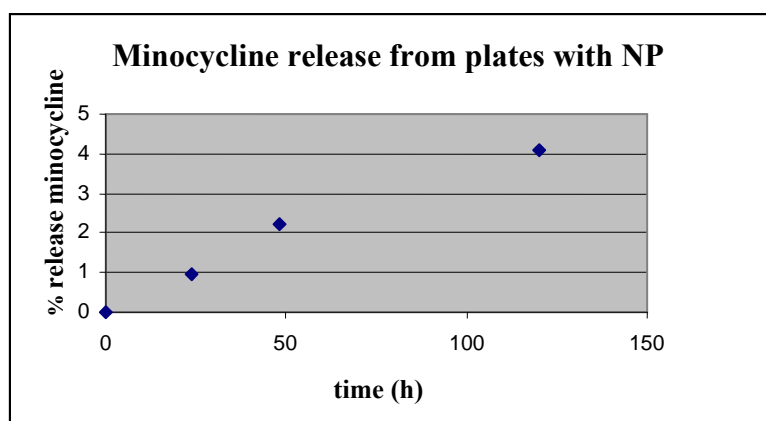


Figure 25. *In vitro* drug release from plate (PnM1) containing 2.0% (w/w_{PMMA}) of minocycline-loaded nanoparticles (npMino1).

Further information could not be obtained due to a limited research time. But it is expected that in the next few days the amount of antibiotic released would grow exponentially until it reaches maximum concentration, which lasts until the consumption of all antibiotics.

5 CONCLUSIONS

In this thesis work PMMA micro- and nanoparticles were designed. To modify particle size into the micro and nano scale, excipients were tested in various proportions.

For the microparticles we used 250 mg of PMMA, 5 ml of DCM and 2.5% PVA. VMD of microparticles was 787nm and PI 0.223. Variations in minocycline concentration were also studied. In formulation of mpMino1, the theoretical incorporation of antibiotic-minocycline was 2% (w/w_{PMMA}). On the other hand, in particles mpMino0.75, the theoretical loading was 1.5% ($w_{MINOCYCLINE}/w_{PMMA}$). From the results obtained with photonic correlation spectroscopy we can conclude, that the quantity of minocycline in the internal phase did not significantly influence the VMD of the particles ($p = 0.09$). Sucrose as a salting-out agent was used in different concentrations 2.5% (w/v) and 5% (w/v) in formulation of microparticles. Sucrose did not significantly augment the VMD ($p > 0.05$). Sucrose had main influence on particles E.E and L.E. Both of these parameters had increase after the sucrose was used. Based on the results, it was possible to conclude that the incorporation of TPGS into the internal phase had significantly increased particle size (mpMino1 vs mpMino_TPGS(i.p.) $p = 0.043$) and also E.E and L.E.

On the other hand nanoparticles were composed of 100 mg of PMMA, 6 ml of DCM and 1.25% PVA. Nanoparticles had VMD 356nm. We found out that in the case of nanoparticles, where amount of PMMA was lower, PI was wider (0.246) and deviation was higher. In case of nanoparticle, sucrose was used in following concentrations; 1.25% (w/v) and 2.5% (w/v). There were none significant difference between VMD of plain batch and batches with sucrose ($p > 0.05$). Incorporation of TPGS into the internal phase had significantly increased particle size (npMino1 vs npMino_TPGS(i.p.) $p = 0.037$).

Minocycline release study was conducted under *in vitro* conditions in PBS pH 7.4 with two different methods. No drug release from PMMA micro- and nanoparticles was determined. On the other hand, minocycline release from acrylic bone cement plates had a successful outcome. After 5 days plate with microparticles PmM1 released 1.8% (w/w) of minocycline and 4.1% (w/w) was released from the plate with nanoparticles PnM1.

For future work, it would be relevant to optimize the formulation of micro- and nanoparticles of PMMA in order to ensure a uniform particle size distribution. It would also be important to characterise the release profile of micocycline from plates for a longer

time. As it was earlier mentioned in this thesis, the mechanical properties of the acrylic bone cement are vital for its successful clinical use. Therefore, it is important to evaluate the influence of minocycline, sucrose and TPGS on the mechanical properties of this biomaterial.

6 REFERENCES

- [1] Anderson JM, Rodriguez A, Chang DT: Foreign body reaction to biomaterials. *Semin Immunol* 2008; 20: 86-100.
- [2] Cui Q, Mihalko WM, Shields JS, Ries M, Saleh KJ: Antibiotic-impregnated cement spacers of the treatment of infection associated with total hip or knee arthroplasty. *J Bone Joint Surg Am* 2007; 89: 871-82.
- [3] Anagnostakos K, Fürst O, Kelm J: Antibiotic-impregnated PMMA hip spacer. *Acta Orthopaedica* 2006; 77(4): 628-637.
- [4] Nzihou A, Attias L, Sharrock P, Ricard A: A reological, thermal and mechanical study of bone cement-from a suspension to a solid biomaterial. *Powder Technology* 1998; 99: 60-69.
- [5] Boesel LF, Reis RL: A review on the polymer properties of hydrophilic, partially degradable and bioactive acrylic cements (HDBC). *Prog Polym Sci* 2008; 33: 180-190.
- [6] Walker GM, Daly C, Dunne NJ, Orr JF: Liquid monomer-powder particle interaction in acrylic bone cement. *Chem Engineering Jurnal* 2008; 139: 489-494.
- [7] Bettencourt A, Calado A, Amaral J, Alfaia A, Vale FM, Monteiro J, Montemor MF, Ferreira MGS, Castro M: Surface studies on acrylic bone cement. *Intl J Pharm* 2004; 278: 181-186.
- [8] Total Hip Replacement <<http://www.the-health-pages.com/images/CEMENT2.jpg>> [Consult: 10th of June 2009].
- [9] Nussbaum DA, Gailloud P, Murphy K: The chemistry of acrylic bone cements and implications for clinical use in image-guided therapy. *J Vasc Interv Radiol* 2004; 15: 121-126.
- [10] Liacouras PC, Owen JR, Jiranek WA, Wayne JS: Effect of pigmentation on the mechanical and polymerization characteristics of bone cement. *J Arthroplasty* 2006; 21(4): 606-611.
- [11] Ries MD, Young E, Al-Marashi L, Goldstein P, Hetherington A, Petrie T, Pruitt L: *In vivo* behavior of acrylic bone cement in total hip arthroplasty. *Biomaterials* 2006; 27: 256-261.

- [12] Bettencourt A, Calado A, Amarel J, Vale FM, Rico JM, Monteiro J, Castro M: The influence of vacuum mixing on methylmethacrylate liberation from acrylic bone powder. *Int J Pharm* 2001; 219: 89-93.
- [13] Bettencourt A, Calado A, Amarel J, Vale FM, Rico JM, Monteiro J, Lopes A, Pereira L, Castro M: *In vitro* release studies of methylmethacrylate liberation from acrylic cement powder. *Int J Pharm* 2000; 197: 161-168.
- [14] Mascio CT, Alder JD, Silverman JA (December): Bactericidal action of daptomycin against stationary-phase and nondividing *Staphylococcus aureus* cells. *Antimicrob Agents Chemoth* 2007; 51(12): 4255–60.
- [15] Davey PG, Ledingham JGG, Warrell DA: Antimicrobial chemotherapy, *Concise Oxford Textbook of Medicine*, 2000: 1475-1476.
- [16] Olson MW, Ruzin A, Feyfant E, Rush TS, O'Connell J, Bradford PA: Functional, biophysical, and structural bases for antibacterial activity of tigecycline. *Antimicrob Agents Chemoth* 2006; 50(6) 2156–66.
- [17] Williams DA, Lemke TL: Foye's Principles of Medicinal Chemistry. 5th edition, Lippincott Williams & Wilkins, 2001: 857-860.
- [18] Zakeri B., Wright G. D: Chemical biology of tetracycline antibiotics. *Biochem Cell Biol* 2008; 86: 124-136.
- [19] Harvery RA, Champe, PC: Lippincott's Illustrated Reviews: Pharmacology, 4th ed. Lippincott Williams & Wilkins, 2009.
- [20] Sean C Sweetman: Martindale: The complete drug reference, Thirty-sixth edition, Pharmaceutical Press, 2009, 300-302.
- [21] Mohanraj VJ, Chen Y: Nanoparticles – A Review. *Tropical J Pharma Res* 2006; 5(1): 561-573.
- [22] Kumar M: Nano and microparticles as controlled drug delivery devices. *J Pharm Sci* 2000; 3(2): 234-258.
- [23] Kennedy JG, O'Grady P, McCarthy DR, Johnson SJ, Hynes D, Walsh M: An investigation into the role of oxygen free radical scavengers in preventing polymethylmethacrylate-induced necrosis in an osteoblast cell culture. *Orthopaedics* 2000; 23; 481-485.
- [24] Zhao B, Tham S, Lu MH, Lee LKH, Moomchala SM: Simultaneous determination of vitamins C, E and β -carotene in human plasma by high performance liquid chromatography with photodiode-array detection. *J Pharm Sci* 2004; 7(2): 200-204.

- [25] Machlin LJ, Bendich A: Free radical tissue damage: protective role of antioxidant nutrients. *Faseb J* 1987; 1: 441-445.
- [26] Brigelius-Flohé R, Kelly FJ, Salonen JT, Neuzil J, Zingg JM, Azzi A: The European perspective on vitamin E: current knowledge and future research. *American J Clinical Nutrition* 2002; 76: 703-716.
- [27] Aguirre R, May JM: Inflammation in the vascular bed: Importance of vitamin C. *Pharmacol Th* 2008; 119: 96-103.
- [28] Sen CK, Khanna S, Roy S: Tocotrienols: Vitamin E beyond tocopherols. *Life Sci* 2006; 78: 2088-2098.
- [29] Wang X, Quinn PJ: Vitamin E and its function in membranes. *Prog Lipid Res* 1999; 38: 309-336.
- [30] Henson PM, Johnston Jr, RB: Tissue injury in inflammation: Oxidants, Proteinases, and Cationic Proteins. *J Clinl Invest* 1987; 79: 669-674.
- [31] Pashkow FJ, Watumull DG, Campbell CL: Astaxanthin: A novel potential treatment for oxidative stress and inflammation in cardiovascular disease. *Am J Cardio* 2008; 101 [suppl]: 58D-68D.
- [32] García-Bueno B, Caso JR, Leza JC: Stress as a neuroinflammatory condition in brain: Damaging and protecting mechanisms. *Neurosci Biobehav R* 2008; 32: 1136-1151.
- [33] Azzi A: Molecular mechanism of α -tocopherol action. *Free Radical Bio Med* 2007; 43: 16-21.
- [34] Reiter E, Jiang Q, Christen S: Anti-inflammatory properties of α - and β -tocopherol. *Mol Aspects Med* 2007; 28: 668-691.
- [35] Mu L, Feng SS: Vitamin E TPGS used as emulsifier in the solvent evaporation/extraction technique for fabrication of polymeric nanospheres for controlled release of paclitaxel (Taxol®). *J Control Rel* 2002; 80: 129-144.
- [36] Mu L, Feng SS: A novel controlled release formulation for the anticancer drug paclitaxel (Taxol®): PLGA nanoparticles containing vitamin E TPGS. *J Control Rel* 2003; 86: 33-48.
- [37] Mu L, Seow PH: Application of TPGS in polymeric nanoparticulate drug delivery system. *Colloids and Surfaces B: Biointerfaces* 2006; 47: 90-97.
- [38] Ferreira IFS: Estudos preliminares de incorporação de partículas polimetilmetacrilato contendo succinato de d- α -tocoferil-polietilenoglicol e acetato de α -tocoferol no cimento acrílico. *Disciplina de Projecto III*, Lisboa, 2009.

- [39] Bettencourt A, Florindo HF, Ferreira IFS, Matos A: Incorporation of tocopherol acetate-containing particles in acrylic bone cement. *J Microencap* 2010; 27(6): 533-541.
- [40] Florindo HF, Pandit S, Gonçalves LMD, Alpar HO, Almeida AJ: *Streptococcus equi* antigens absorbed onto surface modified poly- ϵ -caprolactone microspheres induce humoral and cellular specific immune responses. *Vaccine* 2008; 26: 4168-4177.
- [41] Florindo HF, Pandit S, Lacerda L, Gonçalves LMD, Alpar HO, Almeida AJ: The enhancement of the immune response against *S. equi* antigens through the intranasal administration of poly- ϵ -caprolactone-based nanoparticles. *Biomaterials* 2009; 30: 879-891.
- [42] Michael E: Aulton. *Aulton's Pharmaceutics, The Design and Manufacture of Medicines*, 3rd edition, Churchill Livingstone, Elsevier 2007: 435-438.
- [43] Asiri Ediriwickrema: Novel polymer constructs for controlled release and presentation of topographic cues in support of neuronal cells. Massachusetts institute of technology, 2008.
- [44] Guideline ICH.Q2 (R1): Validation of Analytical Procedures: Text and Methodology, 1996.
- [45] Sheskey PJ, Owen SC: *Handbook of Pharmaceutical Excipients*, 2nd edition, American Pharmacist Association, 1994.
- [46] Lewis G, Janna S, Bhattaram A: Influence of the method of blending an antibiotic powder with an acrylic bone cement powder on physical, mechanical, and thermal properties of the cured cement. *Biomaterials* 2005; 26: 4317-4325.