**UNIVERSITY OF MEDICINE AND PHARMACY OF CRAIOVA**

**DOCTORAL SCHOOL**

# DOCTORAL THESIS

## THE FORMULATION AND PHYSICOCHEMICAL CHARACTERIZATION OF POLYMERIC NANOPARTICLES FOR LOCAL DRUG DELIVERY IN ORTHOPEDIC DISORDERS

**ABSTRACT**

**Doctoral supervisor:**
**Prof. univ. dr. Johny Neamțu**

**Doctoral candidate:**
**Andreea Gabriela Deca**

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**Key words: orthopedic infections, PLGA nanoparticles, ciprofloxacin, drug delivery systems, experimental design**

# Introduction

There is an increased interest towards tissue engineering and regenerative therapies considering the high number of patients with various trauma or musculoskeletal conditions. More over the necessity to accelerate the fracture healing process and to treat various fractures led to the evaluation of several drugs such as bisphosphonates for bone tissue regeneration.

Infections may occur as a complication due to trauma or orthopedic surgery. These complications represent only 5% of the cases but their treatment is rather complicated. This is a result of biofilm development and limited access of the antibiotic at the infection site when systemically administered.

Chronic osteomyelitis is an inflammatory chronic condition determined by an infection. It may affect only one single part of the bone or multiple sites such as bone marrow, periosteum or cortical bone. Furthermore despite new medical and surgical advances osteomyelitis remains a challenge, as it requires a prolonged and complex management involving both surgical and antibiotic treatment.

The standard surgical treatment involves removing the infected bone and connective tissue, procedure called debridement. This procedure is followed afterwards by an antibiotic treatment administered orally or intravenous over an extended period (at least 4 to 6 weeks). This may lead to side effects that require extensive hospitalization. Therefore local treatment of bone infections represents a good alternative. Considering recent developments in biotechnology, local treatment of bone infections has become a focus point. This led to a local, biofilm targeted, delivery of antibiotics.

Drug delivery systems (DDS) such as polymeric nanoparticles are considered for allograft manufacture since they ensure a controlled drug release. Furthermore the drug is released locally at a therapeutic concentration, therefore avoiding side effects.

Micro/nanoparticles are formulated using biomaterials and an active compound. Microencapsulation may be used to protect the drug from degradation and to ensure the proper concentration considering that some drugs might be rapidly hydrolyzed in vivo or have a low half time. Depending on the nature of the polymer (biodegradable or non biodegradable), it may disappear from the administration site or remain there during the patient life span.

## Current stage of knowledge

### 1. Orthopedic infection management

The bone is one of the most interesting nature created materials. It is light but highly resistant, adaptable and renewable.

Despite recent progress in surgical techniques and antibiotic treatment, chronic osteomyelitis management remains a challenge. Furthermore the cost of these treatments is high due to the required prolonged therapy.

Staphylococcus Aureus is the main causal agent involved in developing osteomyelitis, which is a bone infection. It adheres onto the bone surface and develops a biofilm that protects it against antibiotics. Therefore staphylococcus Aureus causes 20-30% of orthopedic infections, whereas negative coagulase staphylococcus determines 20-40%.

Biofilm develops on certain surfaces. The biofilm bacteria are resistant to antibiotics and the immune system. Low sensibility of biofilm bacteria is due to slow development, resistant subpopulations and an incorporated microenvironment that protects the biofilm from antibiotics.

To this day the treatment for osteomyelitis implies surgery, as antibiotics cannot solely eradicate the biofilm. The surgical treatment implies the removal of the infected area, procedure that goes by the name debridement. Systemically administered antibiotics follow the surgical treatment for an extended period that varies from several weeks to months. Systemic treatment is associated with a series of side effects that may include nephrotoxicity or ototoxicity. This may be an issue when a higher local

concentration is required to treat the developed biofilm.

Therefore local treatment has certain advantages such as local delivery of the antibiotic. Furthermore there is no requirement for an intact vascular system to deliver the antibiotic. This applies especially to trauma patients.

The main antibiotics used in drug delivery systems are aminoglycosides, beta-lactams and quinolones.

Ciprofloxacin is a broad spectrum antibiotic. It is a second-generation quinolone that is currently used to treat bone infections due to a low minimal inhibitory concentration against most pathogens.

## 2. Tissue engineering. Directions for the use of nanoparticles in tissue engineering

Tissue engineering seeks tissue reconstruction by combining biodegradable materials, target tissue cells and pluripotent stem cells.

Total hip or knee replacement (arthroplasty) is a standardized surgical procedure in orthopedic surgery. It is used to reduce pain and to improve patient compliance. Its success relies on a rapid integration of the prosthetic parts.

Osteolysis is the main cause of prosthetic components deterioration. Deploying antiresorptive drugs or newly discovered drugs in different treatment regimens may avoid bone loss. Furthermore bisphosphonates (BP) are antiresorptive drugs that inhibit osteoclasts.

In the last decade the use of micro/nanoparticles as functional components for bone regeneration was highly regarded. Rather than classic implant, micro/nanoparticle implants offer a series of advantages such as an improved prolonged release of the drug or better mechanical implant properties.

## 3. Nanoparticles. Characteristics and formulation

A well-formulated DDS may improve conventional treatments downsides and raise drug efficiency.

Local delivery of the drug with an optimal concentration at the required time is necessary to achieve maximum efficiency and also minimal toxicity and side effects.

There are different approaches for local controlled delivery of a drug. One approach is the deployment of micro/nanoparticles as DDS.

The formulation of micro/nanoparticles can by obtained by one of these methods:

- Coacervation
- Interfacial polymerization
- Solvent evaporation method
- Spray drying
- Solvent extraction method

Certain criteria have to be met when considering nanoparticle formulation such as high concentrations of the drug encapsulated, stability, controlled release and biocompatibility/ biodegradation.

Polymers used for pharmaceutical formulations have to be biocompatible and biodegradable. Biodegradable refers to the quality of being decomposed into non-toxic components that may be metabolized or eliminated. Biocompatible refers to those compounds that are well tolerated and do not determine local or systemic side effects following administration.

## 4. Laser deposition techniques

Pulsed laser deposition (PLD) is an efficient method used for the deposition of thin films of the desired compounds. PLD uses a pulsed laser beam to depose a thin film of an inorganic solid compound onto a substrate.

PLD is not always the better choice for the deposition of materials like polymers or organic compounds, therefore other laser deposition methods were considered. Matrix Assisted Pulsed Laser Evaporation technique (MAPLE) succeeded in the deposition of thin and homogeneous films of organic compounds such as carbohydrates and polymers. MAPLE is a rather less aggressive technique compared to PLD.

Both MAPLE and PLD methods allow control over the width of the deposed film opposed to less expensive methods like drop casting. The advantage of the MAPLE technique is that it better protects the structure of the deposed material. This is essential because micro/nanoparticles are fragile.

## Original contribution

## 5. Synthesis and physicochemical characterization of alendronate PLGA nanoparticles

The method chose in this study for the alendronate PLGA nanoparticles formulation is solvent evaporation. Following degradation these nanoparticles release slowly at a certain rate the incorporated alendronate.

The alendronate PLGA nanoparticles were investigated using Fourier-transform infrared spectroscopy (FTIR). Both PLGA and nanoparticles spectra show the peaks characteristic to the stretching of C-OH and (CO)-OC groups between 1300-1000 $cm^{-1}$.

The presence of the peaks characteristic to esters groups suggests that the formulation process did not affect the polymer structure. Furthermore there is no modification in the nanoparticles spectral bands of the two compounds. This suggests that there is no interaction between PLGA and alendronate in the synthesized nanoparticles.

The encapsulation efficiency was determined after the extraction of the alendronate from the nanoparticles. It was determined by HPLC analysis to be 13.24%.

To study the release profile of the alendronate we obtained bone graft like delivery systems by compressing 100 mg nanoparticles.

The release profile of the alendronate encapsulated in the PLGA nanoparticles is characterized by a burst release the first 48 hours, followed by a constant and slow release up to 20 days. This allows a prolonged contact near the vicinity of the implant. This release profile characterizes the DDS with

prolonged release where the release is driven by diffusion and polymeric matrix erosion mechanism.

## 6. Synthesis and physicochemical characterization of alendronate PLGA nanoparticles

There are several methods that can be used to encapsulate the drugs by solvent evaporation method. The hydrophilic and hydrophobic nature of the drug is critical when deciding the encapsulation method.

In this study we chose two methods for the formulation of the ciprofloxacin nanoparticles:

- Double emulsion method water/oil/water (W/O/W)
- Dispersion method solid/oil/water (S/U/A)

After FTIR analysis we found in both PLGA and nanoparticle spectrum the intense peak at 1750 $cm^{-1}$ that is characteristic to the carbonyl group absorption. Furthermore in both spectra the pecks characteristic to the stretching of C-OH and (CO)-OC groups are present between 1300-1000 $cm^{-1}$. These pecks that are characteristic to the esters groups of the polymer suggest that the polymer structure was not modified during the formulation process.

Encapsulation efficiency was determined after ciprofloxacin extraction from the nanoparticles by HPLC. Therefore the encapsulation efficiency was determined to be 9.32% for the nanoparticles obtained by the W/O/W method whereas it was 27.78% for the nanoparticles obtained by the S/U/A.

We used diffusion light scattering (DLS) to determine the nanoparticle size using the Brookhaven 90 PLUS. DLS is used to determine the size of the nano and micro Brownian particles in colloidal suspensions. The monochromatic light is send to a solution of spherical particles that are in Brownian motion. This results in a Doppler effect when the light meets the particles in movement, which causes a deviation of the light wavelength. The deviation is directly proportional to the particles size.

We determined the ciprofloxacin PLGA nanoparticle sizes for the nanoparticles formulated with a PLGA concentration of 1%, 5% and 10% stirred at rates of 500 rpm, 1000 rpm and 1500 rpm. Therefore the samples have a bimodal volume distribution with both a nanometer and a micrometer scale range whereas in the number distribution there remains only the nanometer scale range.

According to Mobarak et al the nanoparticles size is essential to their diffusion inside the cell. The nanoparticle diffusion increases while their size decreases. This pattern is followed down to 100 nm because under this value the cells are no longer able to internalize the particles.

The zeta potential was determined using the BI-ZETA accessory of the Brookhaven 90 PLUS. The main value of the zeta potential was -40.8 mV. Smeets et al consider that a negative value of the zeta potential of biphasic calcium composite favors bone regeneration and osteointegration.

## 7. Experimental design study

To control the encapsulated ciprofloxacin quantity we conducted an experimental design study using the Full Factorial (2 levels) design, interaction model using the MODDE 9.1. Program (DoE – Design of experiments).

Experimental design is an approach that leads efficiently to the desired results with better time and cost management.

There are a number of parameters such as dispersed phase viscosity, drug quantity, stirring rate, temperature that can be varied to influence the encapsulation rate, particle size and morphology.

For the current study we chose 3 factors:

- PLGA concentration (%) with values in the [1,10] range
- Ciprofloxacin quantity (mg) with values in the [5,35] range
- Stirring rate (rpm) with values in the [500,1500] range

The dependent variables also called answers are used to characterize the design experiment. The number of independent variables does not affect the

number of experimental determinations. Therefore for a complete characterization we chose two answers:

- Encapsulation efficiency EE % (w/w)
- Nanoparticle size nm

Therefore it is recommended to increase the PLGA concentration and decrease the ciprofloxacin quantity at a high stirring rate in order to obtain a high encapsulation efficiency and lower nanoparticle sizes.

These results are confirmed in other studies where it was concluded that particle size is substantially influenced by increasing the viscosity. Furthermore the drug encapsulation rate is also improved. Yang et al confirm the fact that a high stirring rate determines smaller particles. This phenomenon occurs because the secondary emulsion is divided into smaller drops due to the increased force which simultaneously leads to a lower size distribution.

## 8. Ciprofloxacin release from drug delivery systems

To determine the ciprofloxacin release profile we used the ciprofloxacin PLGA nanoparticles to develop graph like delivery systems. The following DDS were obtained:

- Ciprofloxacin PLGA nanoparticles
- Ciprofloxacin PLGA nanoparticles: hydroxyapatite NP: HA (25:75)
- Ciprofloxacin PLGA nanoparticles: hydroxyapatite NP: HA (50:50)
- Ciprofloxacin PLGA nanoparticles: hydroxyapatite NP: HA (75:25)
- Ciprofloxacin and hydroxyapatite

We also developed implant like drug delivery systems by deposing a thin ciprofloxacin PLGA nanoparticle film on titanium. We used the MAPLE technique to transfer the PLGA-CIP nanoparticles. The nanoparticle deposition was conducted at the Laser-Surface-Plasma Interactions Laboratory from Lasers Department, National Institute for Laser Plasma & Radiation Physics (INFLPR), Bucharest.

We evaluated the release of the ciprofloxacin from the above mention formulations. The DDS that contained only ciprofloxacin and hydroxyapatite showed a complete release in only 24 hours.

The results show that both the samples composed of only nanoparticles and the samples where the nanoparticles are mixed with hydroxyapatite have a release profile split in two stages. Therefore for all samples in the first 72 hours there is a burst release where approximately 60% of the ciprofloxacin is released. This release follows a type 1 kinetics. Next the ciprofloxacin release follows a type 0 kinetics that is typical to these types of drug delivery systems. With all samples the ciprofloxacin was released over a period of 45 days following a mixed kinetics. Therefore the hydroxyapatite addition to the nanoparticles did not influence the ciprofloxacin release profile.

The burst release may be determined by the absorbed ciprofloxacin on the polymer surface or the diffusion of the ciprofloxacin close to the polymer surface. When the samples come in contact with the release environment the ciprofloxacin diffuses rapidly. It is considered that this release stage characterizes the systems with prolonged release.

In the second stage the drug encapsulated in the nanoparticles is released. In this stage the ciprofloxacin is released at a constant and slow rate that permits a prolonged presence of the antibiotic in the implant adjacent area.

In a previous study we determined the ciprofloxacin release profile from graft like drug delivery systems developed with a HA-CIP composite chemically synthesized. The ciprofloxacin release profile is similar to this study profile, although the release was complete in only 30 days. Furthermore in this study there is the initial burst release, which is desirable as it fast, ensures a high local drug concentration. This is also beneficial as it lowers the possibility of resistance development.

The ciprofloxacin release profile from the thin film deposed on titanium implants is also split in two stages. Firstly, in the burst release stage, 60% of the ciprofloxacin is released rapidly in the first 48 hours. Then as it was determined in the graft like systems, CIP is slowly released. For these samples the release was complete in 10 days. This may be due to the small

quantity deposed to the plates and the fact that the nanoparticles were deposed as a thin film.

The release profile was investigated with the Higuchi and Korsmeyer-Peppas models. Therefore a 0.98 correlation coefficient was obtained after applying the Higuchi model. This suggests that the diffusion process intervenes in the release of the ciprofloxacin from the DDS. Moreover a 0.97 correlation coefficient was determined after applying the Korsmeyer-Peppas model. The ciprofloxacin is released by both diffusion and erosion mechanism as the n value was 0.63.

## 9. Antimicrobial activity evaluation

The purpose of this study is to determine whether the process of CIP encapsulation in the nanoparticles and their deposition on the titanium implants influences the ciprofloxacin antimicrobial activity or not.

In this stage we tried to ascertain the antimicrobial activity of both graft like and implant like delivery systems using the disc diffusion method.

We tested the sensibility of *Staphylococcus aureus* (ATCC 25923) and *S. Aureus methicillin resistant* to the encapsulated ciprofloxacin using the above-mentioned method.

The standard was the ciprofloxacin activity over the reference strains.

The standards developed by the Clinical Laboratory Standards Institute were followed to obtain and interpret the results using the antibiotic susceptibility testing.

To prepare the Mueller-Hinton agar we poured the broth in Petri Discs. To prepare the inoculum we used the direct colony suspension method. The suspensions turbidity was standardized at $1.5x10^{8}$CFU/ml. We used a ruler to measure the diameters of the inhibition zones. The results presented are the mean diameters obtained after three determinations.

The inhibition zone diameter is 32 mm for the susceptibility determination on *Staphylococcus Aureus* and 24 mm for the determination on *Methicillin Resistant S. Aureus*. This suggests that both stains are sensible to

the graft like delivery systems. The implant like delivery systems showed a 22 mm inhibition zone for the determination on *S. aureus* suggesting that the germ is sensible to the film deposed on titanium. The encapsulated ciprofloxacin slowly inhibited the bacteria growth. Furthermore at 24 hours when the reading was performed only 30% of the ciprofloxacin was released.

This results show that by including ciprofloxacin in PLGA nanoparticles the activity of the antibiotic is maintained.

## Conclusions

The purpose of the studies conducted for this PhD thesis is to formulate ciprofloxacin PLGA nanoparticles and use them to develop graft like and implant like delivery systems.

The objectives of this thesis are

- The formulation of alendronate PLGA nanoparticles using the W/O/W double emulsion solvent evaporation method and their physicochemical characterization by FTIR. Furthermore the encapsulation efficiency and the release profile were evaluated using HPLC chromatography.
- Formulation of ciprofloxacin PLGA nanoparticles by two methods: W/O/W double emulsion, solvent evaporation method and emulsion-suspension S/O/W solvent evaporation method.
- Formulating both graft like delivery systems by compressing the ciprofloxacin PLGA nanoparticles and implant like delivery systems by deposing them on titanium using MAPLE.
- Assessing both CIP nanoparticles and the deposed films by FTIR and diffusion light scattering to evaluate the size and the zeta potential. The morphology of the nanoparticles was determined using SEM.
- Completing a design study to control the size and the quantity of the ciprofloxacin included in the nanoparticles using the Full Factorial (2 levels) design, interaction model.

- Assessment of the ciprofloxacin release profile from both graft like and implant like delivery systems.
- Antimicrobial activity evaluation of both graft like and implant like delivery systems over *Staphylococcus Aureus* (ATCC 25923) and *Methicillin resistant S. Aureus* (ATCC 43300). These are the most prevalent stains in orthopedic infections.

Therefore the result show that the graft like delivery systems have a release profile split in two stages. The first part of the curve that corresponds to a burst release is desirable in the treatment of orthopedic infections as it rapidly ensures an efficient local concentration. Furthermore the ciprofloxacin was slowly released at a constant rate up to day 45. This ensures the presence of the drug at the implant site for an extended period of time.

We also determined that including ciprofloxacin in PLGA nanoparticles and deposing them on titanium afterwards does not modify the activity of the antibiotic.

This thesis offers a series of new research directions such as formulating PLGA nanoparticles with other bisphosphonates and antibiotics; incorporating nanoparticles in a HA- Collagen matrix; in vivo test to study the release of the antibiotic on an animal model.

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