UNIVERSITY OF MEDICINE AND PHARMACY

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DOCTORAL SCHOOL

PhD THESIS SUMMARY

STUDIES ON SYNTHESIS AND PHYSICO - CHEMICAL CHARACTERIZATION OF A HYDROXYAPATITE - CIPROFLOXACIN COMPOSITE WITH BONE THERAPY APPLICATIONS

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INTRODUCTION

GENERAL CONSIDERATIONS

In the last half century, the progress in development of new biomaterials and surgical implant techniques led to an explosive growth of their use as implants or medical devices involved in rebuilding bone tissue integrity [1-3].

Movable parts of the prosthesis generates, however, a number of wear particles, "debris", that stimulate local monocyte-phagocyte system. These cells, in an attempt to remove debris from wear, issued a number of pro-inflammatory mediators and proteolytic peri-implant enzymes forming a granuloma and causing a cascading series of biochemical processes that lead to peri-implant bone loss or osteolysis [4].

In addition to these complications, which falls within the so-called aseptic loosening, the removal of the implant is able to intervene as a result of complications due to infections that although are less likely to apear are characterized by high morbidity and high hospital costs [5,6].

Given that bone is a weak perfused organ, systemic antibiotic therapy is long, this therapy is often insufficient to achieve a high local concentration needed to eradicate bone infections. On the other hand, this treatment is often accompanied by toxic effects, while achieving relatively low concentrations in the infected area increases the risk of developing bacterial resistance to antibiotics.

A solution taken under consideration for several research teams in the field of biomaterials, is local antibiotic disposal by including into the implant surface or by obtaining local structures, to be implanted in bone defects resulting from infections [7].

The purpose of this study is the synthesis of a hydroxyapatite - antibiotic composite which allows the achievement of bone allograft, with applications in the treatment of osteomyelitis.

At the same time this composite could be deposited on metal surface of orthopedic prostheses components used in total hip and knee arthroplasty.

Of the most common antibiotics, fluoroquinolones are used successfully in the treatment of bone infections. Of these, we prefer to use ciprofloxacin because it is often used fot its antibacterial activity against most specific pathogens appearing in such infections, but also because of the very small minimal inhibitory concentration (MIC) (0.25-2 μg/ml). It penetrates the bacterial cell wall and inhibit a number of enzymes involved in the replication of bacterial DNA.

PROPOSED OBJECTIVES

For this composite synthesis we prefer to use chemical precipitation method, the main advantage of the method being the low probability of contamination and low costs.

Physico-chemical characterization of this composite was made by X-ray diffraction (XRD), spectrophotometric methods, differential thermal analysis, chromatographic methods, while the morphological analysis by was obtained using SEM.

For composite deposition on prosthetic metal surface, we chose a method frequently lately used in the literature under the acronym MAPLE (matrix assisted pulsed laser evaporation).

In order to determine whether the inclusion of the antibiotic in the hydroxyapatite composite changes antimicrobial activity, we have conducted studies on a number of bacterial germs, frequently present in bone infection such as: *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*.

At the same time we tested also *in vitro* release of ciprofloxacin from obtained allografts by compressing the composite and from the synthesized films and deposited by MAPLE on titanium metal structure.

Chemical synthesis process optimization and determination of experimental conditions that lead to the inclusion of a maximum antibiotic amount in the composite structure was achieved by using experimental design software.

CURRENT STATE OF KNOWLEDGE

CHAPTER 1. THE MANAGEMENT OF BONE INFECTIONS. THE NEED FOR LOCAL MEDICATION DISPOSAL ON BONE TISSUE

Chapter 1 presents the etiology of bone infections associated with joint arthroplasty. In this chapter are presented the main methods of treatment in bone infections characteristic for total hip replacements, methods that typically requires removal of the implant components, surgical removal of the affected bone, followed by a long lasting antimicrobial treatment.

We insisted on local failure of antibiotic implant-bone interface, thus being presented the current treatment schemes. At the same time the new trends on local antibiotic release are presented by antibiotic inclusion on orthopedic prostheses surface through polymer films and bioceramic materials (hydroxyapatite, bioglass) deposited on the metal parts of dentures.

Also in this chapter are presented specific features of osteomyelitis and the treatment of these bone infection using local therapy.

CHAPTER 2. MATERIALS USED FOR LOCAL DISPOSAL OF ANTIBIOTICS

Chapter 2 presents the main biomaterials used in local release of antibiotics. Thus even if the ideal system for local release of antibiotics to the bone wan not yet found, there are some materials in the attention of many research teams in the field (hydroxyapatite, bioglasses).

CHAPTER 3. GENERAL STRUCTURE AND PROPERTIES OF CALCIUM HYDROXYAPATITE

Chapter 3 presents a bibliographic study of the literature describing the properties of calcium hidroxilapatite. Crystallographic structure is described which allows the substitution of the substituents in the crystal lattice, without modifying the bioactivity of this composite.

CHAPTER 4. METHODS OF HYDROXYAPATITE SYNTHESIS

Chapter 4 presents the main methods of obtaining this compound by the chemical wet synthesis, dry chemical specific reactions, synthesis in gas phase and various new techniques and it is focusing on the advantages and disadvantages of each method. The synthesis using chemical precipitation method chosen by us in this experimental study is the most commonly used synthesis technique of hydroxyapatite, the main advantage of this method is the low probability of contamination and low costs. It is also important to manain the control of experimental factors; a slight modification of reaction conditions can significantly affect the properties of the synthesized compounds.

CHAPTER 5. SYNTHESIS OF HYDROXYAPATITE USING PRECIPITATION METHOD

Chapter 5 describes the synthesis and characterization of hydroxyapatite using t chemical precipitation method and he method of Hayek Newesely which comprises the reaction of calcium nitrate and ammonium phosphate, with addition of ammonium hydroxide. Using FTIR spectrophotometry, X-ray diffraction, DLS, differential thermal analysis, the influence of the experimental parameters on the chemical structure is presented, the degree of crystallinity, and the synthesized powder particle size.

The main conclusions of this study are:

- FTIR spectra contain all characteristic vibration modes specific for hydroxyapatite. It is also observed a wide band, which appears in the powder spectrum which is due to water molecules that was not completely removed from the precipitate. Even if we worked in an inert atmosphere, the powder spectrum also contains specific bands of carbonates. These bands appear in the spectrum due to trace amounts of carbon, co-precipitated during the chemical synthesis.

- XRD diffraction pattern of the resulting powder diffraction pattern shown very well the characteristics of the HA cristaline. If at 90 °C the characteristic lines of HA from the database overlap, the spectrum acquired at a temperature of 35 ° C the XRD spectrum does not correspond to HA spectrum from JCPDS, thus the powder obtained is a calcium phosphate called monetite, $CaHPO_4$. HA powder obtained by us is stoichiometric (Ca / P = 1.67), in non-stoichiometric synthesis of HA appearing the presence of other phases such as CaO (if Ca/P exceeds 1.67) or tricalcium phospate, TCP (if Ca/P is less than 1.67).

The particle size of synthesized HA by chemical precipitation is of nanometer size and the agglomerates obtained are characterized by a high degree of polydispersity. Thus, by increasing the temperature in the range 35-90˚C, the particle size measured by DLS is increased from an average of 1700 nm to 2396 nm; These results are consistent with the literature, which shows that particle size and morphology depend greatly on the temperature at which the reaction occurs. SEM microscopy images highlight the nano-sized particles.

CHAPTER 6. CHEMICAL SYNTHESIS OF HYDROXYAPATITE-CIPROFLOXACIN COMPOSITE (HA-CPX)

Chapter 6 shows the results of the synthesis of a composite of hydroxyapatite-ciprofloxacine. For the synthesis of this compound three methods were used. Method I is identical to that used by us in the synthesis of hydroxyapatite, with the calcium nitrate reagent and ammonium phosphate. Immediately after the addition of ammonium phosphate, ciprofloxacin was added to the solution of whose pH was adjusted to 11 with NH_4OH to facilitate its solubilization.

The experimental conditions (pH value, reaction temperature, rate of addition of the reactants) were chosen taking into account that the synthesis of a crystalline compound was desired, without other impurities, and considering the results obtained in the synthesis of HA.

Method II is based on another method for the preparation of HA by chemical precipitation, which is based on the $Ca(OH)_2$ and H_3PO_4. Ciprofloxacin was added as a powder just after the addition of orthophosphoric acid.

Method III has the reactants $Ca(NO_3)_2 \cdot 4H_2O$ and $(NH_4)_2HPO_4$ whose pH was initially adjusted to 10 with concentrated ammonia solution. Ciprofloxacin was added immediately after the synthesis of ammonium phosphate, with a flow rate of 0.5 mL/ min. The reaction time was of 5 hours, during which the reaction temperature and the speed of stirring remained constant. The characterization of obtained compounds

was made by spectrophotometry FTIR, XRD diffraction and the dosage of ciprofloxacin was performed by HPLC.

The findings of this study are:

- hydroxyapatite characteristic IR bands appear in the HA-ciprofloxacin spectrum with unchanged frequencies from pure hydroxyapatite, suggesting that the formation of the phosphate group acts as a counter ion. A few days after the synthesis in the supernatant, it was found the occurrence of a compound of needle-like crystalline structure. As a result of FT-IR analysis were observed characteristic bands of a complex formed between the Ca^{2+} and ciprofloxacin.

- from FTIR spectrum it was observed the lack of characteristic IR bands of PO_4^{3-} group (characteristic of hydroxyapatite), indicating that this group does not enter into the complex structure, so the reaction that occurred is a competing reaction, most probably between $Ca(NO_3)_2$ and ciprofloxacin.

- the amount of complex obtained in this reaction is quite insignificant, soluble ciprofloxacin at alkaline pH is preferential binding to $Ca(NO_3)_2$.

- HPLC analysis confirmed the low degree of incorporation of ciprofloxacin in HA-ciprofloxacin composite, barely of 2,045%.

- FTIR spectrum of the compound synthesized by method II shows a characteristic peak at 868 cm^{-1} characteristic to HPO_4^{2-} group leading to the formation of a second phase of Ca-deficient hydroxyapatite.

- analyzing the X-ray diffraction patterns for hydroxyapatite-ciprofloxacin compounds obtained by the method III, it was found that they have the characteristics of pure hydroxyapatite.

After quantitative determination of ciprofloxacin from the obtained compounds in Method II and Method III, it was found that in terms of ciprofloxacin amount bound to hydroxyapatite, Method III is more efficient, where the percentage is 18.13% as compared to 12 55%, as obtained for Method II.

Also when starting from $Ca(OH)_2$, this compound has a low solubility in water and by adding H_3PO_4 the pH of the reaction medium must be checked rigorously because there is a sharp drop of it, leading to secondary phases formation such as Ca-deficient hydroxyapatite.

As a result of these findings, it was considered that of the three presented methods, the most effective is the one where we started from $Ca(NO_3)_2$ and $(NH_4)_2HPO_4$ and ciprofloxacin was added as a powder into the synthesis (Method III).

CHAPTER 7. SYNTHESIS OPTIMIZATION STUDIES BY EXPERIMENTAL DESIGN

The studies carried out by us in Chapter 7 shows the influence of various parameters of the synthesis on the characteristics of hydroxyapatite-ciprofloxacin powder (the amount of drug added in the process, mixing rate and rate of addition of

the ammonium phosphate) on the concentration of ciprofloxacin in the final compound which was formed with hidroxiapatite. This analysis was performed with 9.1 MODDE Experimental Design program. Screening results of experimental design, showed that the most important factors are the amount of drug added to the synthesis and flow of adding ammonium phosphate, and the interaction between these two factors.

CHAPTER 8. 8. THE RELEASE OF CIPROFLOXACIN FROM TABLETS

Chapter 8 presents studies of ciprofloxacin release from tablets and films films deposited on titanium structures. The dosage of ciprofloxacin dissolution medium was performed by HPLC. To release process kinetics of ciprofloxacin tablets were applied two study designs, Higuchi model and Ritger-Peppas model.

We found that:

- During the first 7 days of the study, ciprofloxacin was released from tablets (containing 20% Ciprofloxacin) in a proportion of 46%. The amount of released antibiotic rise sharply, being at 94% until the 14-th day. The remaining amount is slowly transferred to the 30th day.

- with respect to kinetic process of the release of ciprofloxacin it was found that a higher correlation coefficient was obtained when using Higuchi model, thus emphasizing that diffusion is the main process by which the drug is released in the release.

In the case of ciprofloxacin release from deposited films on titanium substrates, obtained by MAPLE deposition technique (matrix assisted pulsed laser evaporation), we found that:

- the percentage of ciprofloxacin released in first 6 days is higher and then grow more slowly, as was observed in all samples. Due to the smaller amount of the drug contained in samples, in this case the release process was carried out only during a maximum of 14 days. The process of ciprofloxacin release is due to desorption of molecules which are on the surface of HA results in a higher initial drug release, the diffusion of the HA inside being realised slower.

Release kinetics which interpolates best experimental results was obtained using Ritger-Peppas model with $c=Kt^{0.6}$.

CHAPTER 9. ANTIMICROBIAL ACTIVITY DETERMINATION

Chapter 9 contains experimental results on the final stage of the experimental study that determined the antibacterial effect for hydroxylapatite, ciprofloxacin and ciprofloxacin-HA compound. We tested such antibacterial effect of samples in the solid state in the form of tablets, in the form of films of HA-ciprofloxacin composite deposited on titanium surface by MAPLE but also as a microsuspension.

These studies followed if the synthesized compound keeps its therapeutic potential in these formulations. To determine whether the inclusion of

ciprofloxacin affects the antibacterial activity, we conducted a mechanical mixture based hydroxiapatyte and ciprofloxacin.

The conclusions of these experimental studies are:

- HA deposition on the titanium substrates are characterized by a pronounced antibacterian effect. This is consistent with the literature, which shows that nanoparticles of HA have a pronounced antibacterial character on the *Escherichia coli* species explained by the relatively thin thickness of the cell wall, characteristic of the bacterial species, and the chemical structure feature.

The inclusion by chemical synthesis of ciprofloxacin in the structure of HA and the deposit of relatively small amount (approximately 1 microgram) on the surface of the titanium substrate increases the antibacterial activity.

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