UNIVERSITY OF MEDICINE AND PHARMACY OF CRAIOVA

DOCTORAL SCHOOL

PHD THESIS SUMMARY

SYNTHESIS AND CHARACTERIZATION OF A COMPOSITE, BIOCOMPATIBLE MATERIAL OF THE TYPE HYDROXYAPATITE - COLLAGEN - ANTIBIOTIC

PHD SUPERVISOR:
PROF. UNIV. Dr. JOHNNY NEAMŢU

PHD STUDENT:

Filip (Ionescu) Oana-Liliana

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Content

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INTRODUCTION

The field of biomaterials research has today reached the fourth generation, but the approach to their evolution should not be followed chronologically but conceptually because each generation was developed according to its properties correlated with the needs of the application for which it was designed. The magnitude reached by progress in biomaterials research expresses the size of the needs identified in medical practice. The medical fields that today successfully benefit from the advantages of biomaterials are: cardiology, orthopedics, dentistry, plastic and reconstructive surgery.

Orthopedics has contributed through research to the development of biomaterials science, more than any other medical or pharmaceutical specialty since the beginning of the 18^{th} century. Even today when we talk about modern medicine, the specialty of orthopedics faces a great challenge, namely the loss of tissue caused by a trauma, injury or disease, as well as the treatment of infections because the therapeutic agents used, raise problems of solubility and toxicity.

The current strategy for the treatment of osteomyelitis includes surgical procedures for the complete debridement of the formed biofilm and necrotic tissues, systemic and oral antibiotic therapy and the clinical use of cements and biomaterials, as bone defect filling materials and as delivery systems for therapeutic agents.

GENERAL CONSIDERATIONS

Bone infection (osteomyelitis) was, and still remains, a difficult risk to manage in orthopedic and trauma surgery. Although there are no exact epidemiological data, the incidence of acute and chronic osteomyelitis is not high, compared to other pathologies, but its implications are disabling, affecting both the quality of life of patients and the health system due to the costs generated. The incidence increases with age, mainly due to the increased prevalence of comorbidities such as diabetes and peripheral vascular disease, but also the increased prevalence of trauma.

Although many organisms can cause skeletal infections, *Staphylococcus aureus*, remains the most widespread pathogen and with the most devastating consequences. This is because A.S (as well as other bacteria) can adhere to bone and surgically implanted devices, express phenotypic resistance to antibiotic therapy and survive intracellularly. This explains the persistence of bone infections and the high failure rate of short-term antimicrobial treatment.

The typical treatment in osteomyelitis involves surgical removal of the affected tissue followed by prolonged administration of antibiotics, which can lead to adverse effects, including kidney and liver damage, but also to the establishment of bacterial resistance to antibiotics. Modern medicine has registered a series of scientific advances in the treatment of bone infection through the development of devices with antimicrobial properties. Starting from the fact that most human tissues, such as bones, tendons, ligaments, skin, teeth, etc., are composites, this later allowed the synthesis of composites capable of imitating these biological tissues, imitating the mechanical behavior to restore a function of a damaged tissue.

Bone tissue is a natural composite with a remarkable proportion of hydroxyapatite (HA) and collagen, essential components for an optimal bone architecture, for processes of attachment, proliferation, migration, intraction and cellular differentiation. Due to the relative low blood vessels network of cortical bone and the low penetrability of antibiotics in bone tissue, the parenteral route of administration is preferred to ensure plasma concentrations that later allow good penetrability at the bone level.

Prolonged treatment with antibiotics, however, involves the risk of adverse effects and antibiotic resistance, as well as high costs for the health system. For this reason, further progress has been made in the use of biodegradable polymeric materials for pharmacological applications, such as controlled and sustained drug release vehicles. The current concern is to design these materials in such a way as to mimic the biological and mechanical properties of the natural composite and at the same time allow the vascularization of the bone.

Starting with the second generation of biomaterials, special attention is paid to the research of ceramics, in order to use them in diseases of the musculoskeletal system that require replacement of bone tissue. Among these, HA is becoming the most used for biomedical applications: metal implant coating, bone tissue graft, drug release agent, wound protection material, cell culture substrate, enzyme immobilization system and bone prosthesis.

The similarity with the inorganic component of natural bone, excellent biocompatibility and osteoconductivity are some of the properties that recommend it as a biomimetic material in medical applications. The fact that HA is a bioactive material means that new bone tissue grows directly on it when used as an implant because HA can establish chemical bonds with living tissue due to its similarity to apatite in its structure. However, it was hypothesized that the development of a biocomposite with a composition similar to bone tissue would present a superior biological performance compared to HA alone.

The use of collagen as a matrix in the field of biomaterials is based on essential characteristics. Excellent biocompatibility due to low antigenicity and safety due to biodegradability, make collagen a perfect natural polymer for implantable medical devices in the form of porous sponges, membranes and threads for surgical applications or cell culture matrices. The merits of the pure collagen matrix are well known, which can serve as excellent platforms in tissue repair and reconstruction, but despite its excellent characteristics, it presents poor mechanical properties and reduced stability.

The strategy of combining a bioactive component (collagen) with other materials such as natural polymers, synthetics and inorganic materials, is frequently used to increase the mechanical strength of collagen matrices.

Hydroxyapatite provides rigidity and hardness and collagen, tensile strength and flexibility. The biomimetic function of the two materials is also given by interactions of a supramolecular nature (hydrogen bonds, electrostatic forces, packing effects of the organic matrix) or molecular packing phenomena due to the functional complementarity between the components of the composite. In addition, HA can form direct bonds with the host bone tissue which may contribute to a faster and better bond between the biocomposite support and the host bone tissue.

In the treatment of osteomyelitis, in order to overcome the problem of bone diffusion, the parenteral route of administration was preferred to ensure plasma concentrations that would later allow good penetrability at the bone level. In the case of bone infections, prolonged treatment with antibiotics is required, therefore the parenteral route has a number of limitations. Whether we are talking about the intramuscular route, which is difficult to approach in prolonged treatment, or the intravenous route, which exposes the patient to infectious, thrombotic events, additional costs are generated. Consequently, special attention is paid to oral antibiotics with high bioavailability. The choice of antibiotic is made taking into account a number of factors: local epidemiology, antimicrobial susceptibility, bioavailability, previous history of infections, contraindications, allergies, adverse reactions, drug interactions, degree of penetrability, etc.

Ciprofloxacin it is not used in daily practice only for its high spectrum of action, but also for good availability and high bone penetrability, due in part to the binding of quinolones to calcium in the bone. It is used to treat a large number of infections due to the spectrum of action that includes most pathogens. In the treatment of infections, it is used both in monotherapy and in combination with other antibiotics.

Aminoglycosides (of which Gentamicin is also a part) have a broad spectrum of action that covers aerobic organisms including gram negative bacteria *(Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa*) and mycobacteria. The bactericidal effect is dose-dependent: high concentrations correlate with greater antimicrobial destruction. An important characteristic of aminoglycosides is the suppression of bacterial regeneration that persists several hours after the antibiotic concentration falls below the minimum inhibitory concentration (MIC). Aminoglycosides can cause nephrotoxicity and ototoxicity, both during the treatment of complicated infections and during prophylactic treatment even if

it is a short-term exposure. Therefore, clinicians must balance toxic potential and therapeutic efficacy, and they are maintained as second-line antibiotic therapy.

In order to further explore the therapeutic potential of aminoglycosides, research continues and tries to develop alternative strategies such as using them in combination with other therapeutic classes, optimizing the active concentration, modifying the environmental conditions, formulating them in biocompatible carrier materials.

PROPOSED OBJECTIVES

The main objective of the research project is to obtain a biocomposite material doped with antibiotics and used as a delivery system to ensure constant therapeutic levels, without reaching toxic levels, over a long period of time, as an advantage over the conventional systemic administration route. In this study, we will use third-generation biomaterials due to their bioactive and bioresorbable properties that cause the implanted material to resorb over time, its place being taken by the newly formed tissue.

RESULTS AND DISCUSSION

The doctoral research consists of a general part (the current state of knowledge) structured in subchapters that represent an update of the existing information in the specialized literature. Here we reviewed the notions regarding the evolution of generations of biomaterials, the properties of biomaterials, the medical and social impact of bone infection on the population and the medical system, as well as the importance of two classes of antibiotics in the treatment of bone infection.

Knowledge of bone physiopathology and recent research presented in specialized literature helps to better understand the disease and its approach by the specialists involved in its management. For this purpose, we accessed the PubMed, Medscape and Google Scholar databases.

The part of personal contribution (original) can be found in the third Chapter, structured on three important subchapters.

Subchapter 3.1 It summarizes the working hypothesis and the secondary objectives that supported the project in its dynamics.

The working hypothesis was formulated starting from the need to optimize the therapeutic management of osteomyelitis in order to limit the disabling character both for the patient and for the health system. We established the constituents of the composite system (collagen and hydroxyapatite), based on their similarity to physiological tissue (bone), and initiated the synthesis process of the biocomposite. We chose to dope the test material by adding two antibiotics (ciprofloxacin and gentamicin) from different classes (fluoroqinolones and aminoglycosides) due to their spectrum of action and penetrability at the level of bone tissue. Taking into account the type of materials used, their synthesis method, the structural and functional characteristics, the agreed sterilization method but also the type of interaction the system has with the physiological tissue, we established the way to evaluate the cytotoxicity of the composite material. Once the biocompatibility was tested, we considered it absolutely necessary to confirm the effectiveness by evaluating its antibacterial activity.

Subchapter 3.2. establishes the research methodology by addressing the incidence data of osteomyelitis, a statistic still unknown even at the level of developed countries. Despite the existing therapeutic protocols in osteomyelitis, limits were still identified that opened new perspectives for research.

Subchapter 3.2.2 describes the materials and methods used in the synthesis of the biocomposite and the subsequent addition of the antibiotic Gentamicin (Genta) and the chemisorption of the antibiotic Ciprofloxacin (Cipro). The quantitative and qualitative analyzes allowed highlighting the antibiotic and also its percentage expression. For information on the size, shape and tendency of particle agglomeration as well as the porous nature, we chose structural and morphological analysis techniques (scanning electron microscopy, dynamic light scatttering). The spectrometry methods (MTT/MTS, immunofluorescence, ALP activity, SEM) were used to evaluate the biocompatibility parameters.

We preferred to test the antibacterial activity of the composite material using current laboratory methods (Gram smear, oxidase test, coagulase test, citrate test, TSI - test, MIU- test) and automatic methods (BD Phoenix and VITEK 2 SYSTEMS).

Subchapter 3.3 It centralizes the results of each stage in the research, previously established.

Subchapter 3.3.1. As a biocomposite synthesis method, we approached in *situ synthesis*, co-precipitation of hydroxyapatite in the collagen matrix, in a single step. A simultaneous self-assembly occurs between the hydroxyapatite precursors and the collagen gel. The process allows a high degree of interaction between the organic and inorganic components of the composite, an important aspect for the final properties of the composite. Also, the chemical cross-linking to which the material is subjected optimizes the chemical bonds between the constituents. The fact that we chose the laboratory synthesis of hydroxyapatite, starting from precursors, allowed us a better control over the size and morphology of the particles, ion substitution and synthesis parameters, as well as the avoidance of possible impurities. It is a reliable and fast laboratory method. As special recommendations during synthesis: keeping the collagen gel on an ice bath to prevent its denaturation and it is essential to keep the mixture at a pH<9 to produce collagen precipitation and fibrinolysis.

Subchapters 3.3.2, 3.3.3 and 3.3.4 presents the results obtained for the addition, encapsulation and release of ciprofloxacin in/from the structure of the Col-HA biocomposite. Between the adsorbent (biocomposite) and the drug, a chemical reaction takes place as a result of which the hydroxyapatite binds to the drug at the level of calcium and oxygen atoms from the carboxyl group of ciprofloxacin. The percentage of ciprofloxacin bound was 18.027%, similar to other methods, with a slow release profile of 32% in the first 10 days followed by an explosive release of 89% by the end of the study. This in vitro release behavior of Cipro from the Col-HA biocomposite delivery system can be explained by the fact that Cipro breaks more difficult from the bonds formed with the Ca atoms in the HA structure. The release profile of ciprofloxacin was analyzed with the help of kinetic models of order 0 (I) and order 1 (II) but also applying two kinetic models: Higuchi (III) and Rigter-Peppas (IV). In both models, a similar correlation coefficient was obtained, thus suggesting a drug release process, on the one hand by diffusion, but also by surface erosion and respectively desorption.

Subchapters 3.3.5 and 3.3.6 highlights the encapsulation and release efficiency of gentamicin. And in the case of gentamicin, a significant encapsulation coefficient of 15.92% was obtained, which may be due to the porosity of the biocomposite support,

determined by the collagen. The yield profile started explosively at a percentage of 50% in the first ten days and continued with a slow yield of 93.33% in the next twenty days.

The explosive release may be a consequence of the release of gentamicin present on the surface of the biocomposite and the slow release may be described as the release of gentamicin that has diffused within the biocomposite material. In the case of gentamicin, there is no chemical reaction between HA and the antibiotic. This *in vivo* release behavior of Genta is very important both for preventing the risk of infection in the first 10 post-operative days when the risk of infection is high, and for the sustained long-term maintenance of an optimal concentration for its antibacterial action.

In the case of gentamicin, we performed a kinetic study using the Higuchi and Rigter-Peppas models, which showed mixed kinetics with an insignificant difference in the correlation coefficient suggesting that the main processes of gentamicin release from the analyzed biocomposite take place simultaneously, cumulative: surface erosion, diffusion and desorption.

In subchapter 3.3.7 we find the results obtained through structural and morphological characterization techniques.

Analysis by scanning electron microscopy-SEM performed for the three samples (Col-HA, Co-HA-Cipro and Col-HA-Genta), at 100 x magnifications it highlighted the three-dimensional structure of collagen, interconnected pores, with dimensions greater than 100 μm, which makes it an ideal candidate to support bone regeneration. At higher magnifications, collagen fibers can be noted, interpenetrated, having the appearance of a folded sheet, in the case of all the analyzed materials.

In all three samples, the collagen matrix is mineralized with hydroxyapatite particles, highlighted by the presence of large agglomerates, both on the surface and inside the porous materials, demonstrating their homogeneous distribution, due to the *in situ* method of obtaining HA, in the matrix of Col. Following the addition of the inorganic phase, a decrease in the volume of macropores in the collagen matrix is observed, as well as the appearance of smaller pores, of only a few microns. The addition of the drug does not substantially change the morphology of the material, the active substance being most likely absorbed inside the pores or on the HA surface. The synthesis method used to obtain the Col-HA biocomposite, the lyophilization method, contributed to obtaining the spongy structure that exhibits excellent absorption properties, oxygen permeability, which will

later influence the adhesion and cell proliferation phenomena, the rate of dispersion and yield of the antibiotic. Using the dynamic light scattering technique (DLS) we were able to evaluate the granulometric distribution (hydrodynamic diameter) of the Col-HA particles and the zeta potential. The results obtained on the porous biocomposite material indicate a numerical distribution in which the sizes of the powder particles are in the [457-779] nm range with the highest representation (41,84%) for the size 457.2 nm and the smallest representation at the upper limit of the 779 nm range. Regarding the volumetric distribution, it describes a bimodal evolution with two intervals, the first between [457-779] nm and the second interval between [5-9] μm. It is found that in the case of numerical distribution, micron particles are missing or practically undetectable.

The values recorded for the zeta potential are in the negative range -6.5mV and -12mV, they fall within the range [-30,+30] mv, which means that the particles remain stable, with favorable premises for cell attachment and proliferation. Our results find arguments in a series of studies that have shown that a material that has an electronegative surface charge is more accessible for osteoblast attachment and proliferation because negatively charged species (mesenchymal cells and osteoblasts) are attracted when are in intimate contact.

Spectrometric analysis with the Fourier transform- FT-IR provided us, information about the wavelengths at which the peaks characteristic of the vibrations of the PO43- and – OH groups appear in the HA structure .

Comparing the wavelength at which peaks characteristic of the biocomposite hydroxyapatite (Col-HA) appear, with those of the commercial hydroxyapatite spectrum used as a standard, it was observed that in the Col-HA biocomposite, they appear at identical wavelengths, which leads us towards the conclusion that between the Ca atoms of HA and the ciprofloxacin inserted by chemisorption in the biocomposite, there are no strong chemical bonds, which would have led to a shift of the frequencies in the spectrum, but rather weak intermolecular forces of attraction probably act.

In the FTIR spectrum of commercial ciprofloxacin (Sigma Aldrich, Germany) the appearance of the peak at 1616 cm^{-1} characteristic -C= O of ciprofloxacin is observed.

The peak in Col-HA-Cipro is shifted to about 1630 cm^{-1}, which may be due to binding of the calcium ion from HA to Cipro via the carbonyl oxygen of ciprofloxacin.

At 2840-2845 cm^{-1}, a band characteristic of the vibration of the N atom from the piperazinyl group can be observed both in the spectrum of ciprofloxacin (higher intensity) and in the spectrum of the composite (lower intensity). The lower intensity of the band in the spectrum of the composite indicates a zwitterionic form of the nitrogen atom, so this atom cannot form coordinative bonds with the calcium ion. The fact that ciprofloxacin is found as an amphiion in the biocomposite is also characterized by the absence of the symmetric stretching vibration of the carboxylic group, which is present in the pure ciprofloxacin spectrum at 1375 cm^{-1} along with that asymmetric stretching vibration at 1590 cm^{-1}. Our assumption is that ciprofloxacin is bidentately coordinated.

Subchapter 3.3.8 was assigned to a study to test the antibacterial activity that the three-dimensional support developed by us, doped with antibiotics, exerts on some bacteria incriminated in the formation of biofilms in the case of surgical interventions in the orthopedic field.

The tests demonstrated that the antibacterial properties of the three-dimensional biocomposite support of Col-HA are evident at a concentration of 18.27% Cipro and 15.92% Genta, highlighting zones of inhibition between 30 mm - 40 mm for Cipro and 21 mm - 25 mm for Genta, in the case of all strains tested. Between the inhibition diameters for the standard antibiotics and the inhibition diameters for our antibiotic-doped biocomposite, a slight difference is observed in the direction of the decrease in the diameters on the biocomposite-antibiotic material. This, however, cannot be interpreted in the sense of a lower antibacterial activity, influenced by the biocomposite material because all the values obtained are in the reference range of antibiotics according to the CLSI Guide.

Subchapter 3.3.9 refers to the results obtained in biocompatibility tests on cell cultures. The results of the MTS test showed that the osteoprogenitor cells cultured on the three-dimensional support based on hydroxyapatite and collagen matrix retain their mitochondrial metabolic activity and are relatively evenly distributed on the surface of the material.

Viable cells identified on antibiotic-containing materials are fewer in number. In harmony with these observations, the quantitative signal given by the spectrophotometric measurement of the medium in which we added the MTS substrate shows a tendency to decrease the number of metabolically active (viable) cells on the samples with antibiotics

compared to the support based on HA and simple collagen matrix. A complementary viability analysis was performed, by fluorescence microscopy performed on the three-dimensional support of Col-HA on which MSCs were cultured. This test confirmed that the measured MTS signal is given by the majority of cells grown on the material that maintain their viability. Cells tend to infiltrate the pores of the material and grow along the collagen fibers that provide support for cell adhesion. We repeated the viability test on cells cultured in the presence of the three types of material, in DMEM expansion medium. The fluorescence microscope analysis showed that the cells do not lose their viability; we appreciate that the decrease in the MTS signal may be due to an impairment of cell proliferation or mitochondrial metabolism and not due to the entry of cells into apoptosis.

In order to evaluate the properties of the material to support the osteogenic differentiation of MSCs, we then performed comparative tests in expansion medium (DMEM) and osteoinduction medium (OIM - osteoinduction media).

In osteogenic conditions, the number of cells is slightly reduced, an anticipated fact because during differentiation the cells decrease their proliferation rate. From a functional point of view, an analysis was carried out that highlights the active alkaline phosphatase (ALP) and the results showed that the cells effectively infiltrate the simple three-dimensional supports Col-HA and those containing ciprofloxacin but much fewer cells with Active ALP were identified in Col - HA - Genta samples under osteoinduction conditions. Interestingly, both antibiotics had the property of increasing ALP activity even in expansion conditions (DMEM), in the absence of osteoinductive factors. Due to the intrinsic content of calcium phosphates (HA), an Alizarin Red staining analysis could not be performed to assess the degree of mineralization of the samples.

The material proved biomimetic properties that allowed cell adhesion and proliferation, which does not justify proposing its further development for applications in implantology. We consider the biocomposite eligible for a possible use as an antibiotic delivery system for the treatment of complete, surviving infections and complications following surgery in orthopedic wards.

Chapter 4 is devoted to the discussions generated both by the obtained results and by the research perspectives that our results offer.

However, the discussions were punctuated in the results presented in the previously described sub-chapters to ensure the continuity of the information.

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