PHYSICO-CHEMICAL CHARACTERIZATION AND ANTIBACTERIAL ACTIVITY OF A CONTROLLED COLLAGEN-HYDROXYAPATITE-CIPROFLOXACIN RELEASE SYSTEM

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Abstract

The aim of our study was to synthesize a biocomposite material using a natural polymer (collagen), hydroxyapatite and ciprofloxacin. We used Fourier-transform infrared spectroscopy (FT-IR), dynamic light scattering (DLS), high performance liquid chromatography (HPLC-DAD) and scanning electron microscopy (SEM) for the physico-chemical characterization and we determined its antibacterial activity against Staphylococcus aureus (S. aureus), Pseudomonas aeruginosa (P. aeruginosa), Klebsiella pneumoniae (K. pneumoniae) and Escherichia coli (E. coli). The release profile of the ciprofloxacin chemisorbed in the material was also studied, and we found that the release was sustained for 30 days.

Keywords: collagen-hydroxyapatite-ciprofloxacin, biocomposite, physico-chemical characterization, antibacterial activity

Introduction

In the last half century, progress in the development of new biomaterials and surgical implantation techniques has led to an explosive increase in their use in the form of implants or medical devices that play a role in restoring bone tissue integrity [17, 18]. The biomaterials market uses over 300 billion dollars annually, with an increase of 20% annually [12]. A solution that is the focus of many research groups, in the field of biomaterials, is the local delivery of the antibiotic [4] by including it at the surface of the implant or by obtaining structures with local yield, which are implanted in the bone defects that result from infections. Thus, local antibiotic therapy improves cure rates in osteomyelitis [16], ciprofloxacin being one of the most commonly used antibiotics for Staphylococcus aureus infections. Hydroxyapatite and its derivatives (calcium phosphates) exhibit superior mechanical characteristics, having an increased biocompatibility [15], thereby contributing intensively in the bone regeneration process [6, 11]. The association of hydroxyapatite or its derivatives with collagen leads to significant changes that are reflected in the biological properties of the implant, respectively on the simulation of the osteogenesis process [3]. The biomimetic function of these biomaterials is also given by interactions of a supramolecular nature (hydrogen bonds, electrostatic forces, organic matrix packing effects etc.) or molecular assembly phenomena, explained as a result of the
The purpose of this study was the development of a collagen-hydroxyapatite-ciprofloxacin (Coll-HA-CIP) composite that would allow the production of bone allografts, with applications in the treatment of osteomyelitis.

Materials and Methods

Materials

All chemicals were of analytical grade and were purchased from Merck (Darmstadt, Germany). Coll-HA-CIP bio-composite, distilled water (1 mL), ciprofloxacin 15 µg, bacterial strains: isolated from hospitalized patients: Pseudomonas aeruginosa MDRO (Multi Drug Oral Resistance), Klebsiella pneumoniae MDRO, Klebsiella pneumoniae MSS (Multi Drug Sensitivity), Escherichia coli ESBL (Bacteria producing betalactamase), Escherichia coli MSS, Staphylococcus aureus MRSA (S. aureus methicillin resistant), Staphylococcus aureus MSSA (Staphylococcus aureus methicillin sensitive).

Bacterial strains were isolated from haemocultures, collected from patients with haematological diseases, identified by current laboratory methods (Gram smear, oxidase test, coagulase test, citrate test, TSI-test, MIU-test) and automated methods (BD Phoenix and VITEK 2 SYSTEMS).

Sensitivity to therapy was tested using a huge number of antibiotics (Amoxicillin/Clavulanic Acid, Ceftriaxone, Cefepine, Cefazidine, Cefazidim/Avibactam, Imipenem, Meropenem, Ertapenem, Colistin, Gentamicin, Trimethoprim/Sulfametoxine, Cefametoxine, Sulfametoxine, Vancomycin, Linezolid, Erythromycin, Clindamycin, Rifampicin and Tertacyclin), by diffusimetric method (Kirby Bauer) and by automatic method (automatic systems).

Synthesis of collagen-hydroxyapatite (Coll-HA) bio-composite

The synthesis of the Coll-HA biocomposite consists of the in situ generation of hydroxyapatite in the collagen matrix. For this purpose, a Coll-HA mixture was prepared with the mass ratio collagen:inorganic mp = 1:1. The collagen used is stable in acidic pH, containing 2.26% pure (dry) substance.

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Thus, in the collagen mixture maintained at low temperatures (to prevent denaturation), a suspension containing Ca^{2+} ions is added dropwise, the mixture being stirred for 30 min at 400 rpm. Subsequently, under careful temperature control, a PO_4 anion-generating suspension is added gradually, the mixture being kept under mechanical stirring for another 30 minutes until the mixture is completely homogenized. The process is accompanied by precipitation of HA directly into the collagen matrix.

The inorganic precursors used were Ca(OH)_2 and (NH_4)_2HPO_4. The obtained final mixture had a white, viscous appearance. For crosslinking, a dilute solution of Glutaraldehyde (1%) was added to the mixture, and then stored at 4°C for 24 h. After crosslinking, the composite was washed with ultrapure water repeatedly until neutral pH was established, then subjected to the lyophilisation process.

The gel thus obtained was subjected to the freeze-drying process (freezing at 55°C for 12 hours, vacuuming at 0.001 mbar for 12 hours and heating under vacuum for 24 hours up to 35°C), to obtain porous composite materials.

Chemisorption of ciprofloxacin

In order to achieve the chemisorption of ciprofloxacin in the Coll-HA composite, a solution of ciprofloxacin with a concentration of 3.33 mg/mL was prepared. Since ciprofloxacin is very slightly soluble in water, having a minimum solubility at pH 7.4 (30 mg/mL at 20°C), the pH was adjusted to 10.5 with NH_4OH and ciprofloxacin was added while stirring (500 rpm). It was chosen to solubilize the drug in a basic medium, because an acidic pH would affect the hydroxyapatite in the composite.

The optimum contact time is the time required for the maximum adsorption of ciprofloxacin within a minimum contact time. Initially there is a rapid adsorption in the first hours after the start of the experiment, then the initial rate of adsorption decreases gradually, with increasing time and reaching equilibrium at about 48 hours.

FT-IR analysis

FT-IR spectra were recorded in potassium bromide, on a Fourier Avatar Nicolet spectrophotometer in the range of 500 - 4000 cm\(^{-1}\).

DLS analysis

DLS analysis was accomplished with a Brookhaven 90 plus/bi-mas multi angle particle sizing option with the following characteristics: size range: 2 nm to 3 µm, diffusion coefficient range: 10^{−10} to 10^{−8} cm²/sec, accuracy: ± 1% to 2% with dust free samples, laser: 15 mW solid state laser, red temperature control: 5 to 75°C, sample volume: 0.5 to 3 mL.

The zeta potential was measured with a Zeta potential Analyzer (Brookhaven) with the following characteristics: zeta potential Range: -150 to +150 mV, size range: 10 nm to 30 µm, accuracy: ± 2%, repeatability: ± 2%, laser: 35 mW solid state laser, red (660 nm wavelength), temperature control: 6 to 74°C, sample volume: 1.5 mL.

HPLC-DAD loading efficiency

The determination of ciprofloxacin was performed by HPLC on a Thermo Finnigan Surveyor chromatograph with a DAD detector and a Thermo Finnigan Xcalibur software. Work was carried out on a Hypersil GOLD C18 (Thermo Scientific) column, 250 mm long, 4.6 mm inside diameter, with a particle size of 5 µm stationary phase. The mobile phase consisted of a mixture of 20 mM citrate buffer solution (containing 16.7 mM citric acid dihydrate and 3.3 mM sodium citrate di-
Pseudomonas aeruginosa strain test was performed (using 3 microorganisms: Klebsiella pneumoniae, Escherichia coli, and E. coli in 1 mL physiological serum sowed on the standard Muller Hilton placemat Agar (MHA). From the suspension of each test product, 0.20 mL of each inoculum of bacterial strain was inoculated. After incubation at 37°C for 24 h, the results were read. We previously tested the CIP action of known concentrations on the bacterial strains studied, comparing the results of the antimicrobial action with the CLSI standard guideline (Clinical and Laboratory Standards Institute).

Results and Discussion

FT-IR analysis

The FT-IR spectrum obtained for the synthesized biocomposite presents several peaks belonging to the hydroxyapatite: thus, at 564 cm⁻¹, 606 cm⁻¹ and 1036 cm⁻¹ sharp peaks characteristic of the vibrations of the PO₄²⁻ group in the HA structure were observed, while between 3500 - 3600 cm⁻¹ a broad band was identified, probably due to the stretch vibration of the hydroxyl group in the HA structure. Comparing the wavelengths at which these characteristic peaks of hydroxyapatite appear in the obtained composite with those of the commercial hydroxyapatite spectrum used as a standard, it was observed that in the Coll-HA-CIP biocomposite they appear at identical wave numbers, which leads us to the conclusion that between the Ca atoms of HA and ciprofloxacin inserted by chemisorption into 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 attraction forces.

Based on this, we can assume that the release is mainly controlled by the diffusion and solubility into the aqueous solution, but, because of the low solubility of Ciprofloxacin in water, the release is sustainable. In the FT-IR spectrum of commercial ciprofloxacin (Sigma Aldrich, Germany), the peak at 1616 cm⁻¹ characteristic to -C=O bond of ciprofloxacin is observed [7]. The peak of Coll-HA-CIP is shifted to about 1630 cm⁻¹, which may be due to the binding of the calcium ions from HA to ciprofloxacin by carbonyl oxygen of ciprofloxacin.

At 2840 - 2845 cm⁻¹, both in the spectrum of ciprofloxacin (higher intensity) and in the spectrum of the composite (lower intensity), a band characteristic to the vibration of the N atom in the piperazinyl group is observed. The lower intensity band in the composite spectrum indicates a zwitterionic form of ciprofloxacin (higher intensity) and in the spectrum of the composite (lower intensity), a band characteristic to the vibration of the N atom in the piperazinyl group is observed. The lower intensity band indicates the presence of the biocomposite in the water.

Antibacterial activity

The composite was weighed to determine the amount of antibiotic to be tested (CIP). The test product was inoculated in 1 mL of distilled water and the incubation was accomplished at room temperature for 24 h. The 0.5 McFarland nephelometer standard bacterial strain test was performed (using 3 - 5 colonies of Pseudomonas aeruginosa, Klebsiella pneumoniae, and E. coli in 1 mL physiological serum sowed on the standard Muller Hilton placemat Agar (MHA). From the suspension of each test product, 0.20 mL of each inoculum of bacterial strain was inoculated. After incubation at 37°C for 24 h, the results were read. We previously tested the CIP action of known concentrations on the bacterial strains studied, comparing the results of the antimicrobial action with the CLSI standard guideline (Clinical and Laboratory Standards Institute).

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The equation of the calibration curve was \[ y = 1014x - 177196 \], having a very good regression coefficient, \( R^2 = 0.99993 \). Detection was performed at 280 nm, with a retention time of 3.2 minutes for standard ciprofloxacin.

After 48 hours of adsorption, a smaller amount of ciprofloxacin remained in solution, the remainder being bound to hydroxyapatite in the composite. Calculating the amount of chemisorbed ciprofloxacin (62.7 mg) and reporting it to the hydroxyapatite-collagen mass immersed in the solution, the percentage of bound ciprofloxacin was found to be 18.027%, similar to other methods of binding ciprofloxacin to hydroxyapatite such as adding the drug during the process of synthesis of hydroxyapatite by wet precipitation. A possible major contribution to the high percentage of drug that can bind to the composite is also the porosity of the composite given by the pore forming capacity of collagen gel, which implicitly leads to an increase of the contact surface as well as of the diffusion rate.

**Figure 1.**

A) SEM images for the Coll-HA composite material; B) Coll-HA-CIP composite material

**Morphology**

Figure 1 shows the SEM images for colloidal porous composites, obtained by lyophilisation. In the left images for each sample, made at small magnifications, of 100×, one can see the three-dimensional structure of the collagen-based composite suprastructure. The lyophilized collagen-based composite matrix has interconnected pores, making it an ideal candidate for bone regeneration support, the pore size being large enough to allow cells in growth without altering very much the mechanical properties, the size of the pores being below 150 µm [2, 10].

At higher magnifications, one can notice the collagen fibres [14], interlaced, having the appearance of a folded sheet, in the case of all the analysed materials. In all 3 samples the collagen matrix is mineralized with hydroxyapatite particles, evidenced 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 Coll matrix [7]. Adding drugs does not
substantially alter the morphology of the material, the active substance being most likely absorbed inside the pores or adsorbed on the surface of HA.

*Antibacterial activity*

By reading the sensitivity diameters after 24 h it was observed that all test products had antibacterial action (Table 1) on the following strains: *Klebsiella* MDRO, *Klebsiella* MSS, *E. coli* ESBL, *E. coli* MSS, *S. aureus* MRSA, *S. aureus* MSSA, and resistance on *Pseudomonas* MDRO strains (for all products tested) and *Klebsiella* MDRO for standard ciprofloxacin.

**Table 1**

| Strains       | Inhibition diameter of standard ciprofloxacin |
|---------------|---------------------------------------------|
| *Klebsiella* MDRO | Contact resistance | Contact resistance | Contact resistance |
| *Klebsiella* MSS | 40 mm | 40 mm | Resistant |
| *Escherichia coli* ESBL | 41 mm | 46 mm | Resistant |
| *Escherichia coli* MSS | 38 mm | 32 mm | Resistant |
| *S. aureus* MRSA | 31 mm | 30 mm | Resistant |
| *S. aureus* MSSA | 31 mm | 32 mm | Resistant |

**Figure 2.**

Chromatograms obtained at the release of ciprofloxacin from the Coll-HA-CIP composites: A) at 1 day; B) 21 days; C) 30 days.
Release of ciprofloxacin from the collagen-hydroxyapatite-ciprofloxacin composite

Prolonged release of ciprofloxacin from ciprofloxacin-based compositions was observed by others [1, 3, 15], who successfully synthesized a composite with a 60-day antibiotic release period. Rauschmann et al. studied the release of ciprofloxacin from a compound based on hydroxyapatite and calcium sulphate and found a release period of only 10 days [11], while from the usual drug tablets, ciprofloxacin is released within 4 - 6 hours after administration [5]. Figure 2 shows the chromatograms (diluted sample 1:10) related to ciprofloxacin release at 1 day, 21 days and 30 days respectively.

According to our results, in the first 10 days ciprofloxacin is released by the Coll-HA-CIP composite (which contains 18.027% ciprofloxacin) in a percentage of 32%. The amount of antibiotic released reached 89% by the end of the study.

Figure 2
Release of ciprofloxacin from Coll-HA-CIP

This slow release profile (Figure 3) for the first part of the study may be due to the links that are formed between ciprofloxacin and hydroxyapatite in the synthesis process [19]. The faster release after 20 days is most probably induced by the moderate degradation of the collagen matrix which means that the release rate increases because of the symbiosis of diffusion and bioerosion.

Conclusions

The biocomposite material Coll-HA could stimulate the osteogenesis process due to the special characteristics that both collagen and hydroxyapatite possess (biocompatibility, osteoconductivity, lack of toxicity). In the present paper we chose ciprofloxacin as an antibiotic due to the antibacterial spectrum against most of the specific pathogens found in bone infections, with a very low minimum inhibitory concentration (MIC) (0.25 - 2 µg/mL). Its release from the composite material is slow, over a long period of time.

Conflict of interest
The authors declare no conflict of interest.

References

1. Akinleye MO, Jolaoso AA, Coker HAB, Comparative dissolution profiles of representative quinolones in different media. Nig Q J Hosp Med., 2011; 21(1): 1-8.
2. Andronescu E, Ficai A, Georgiana M, Mitran V, Sonmez M, Ficai D, Ion R, Cimpean A, Collagen-hydroxyapatite/Cisplatin Drug Delivery Systems for Locoregional Treatment of Bone Cancer. Tech Cancer Res Treat., 2013; 12(4): 275-284.
3. Becker PL, Smith RA, Williams RS, Dukowski JP, Comparison of antibiotic release from polymethylmethacrylate beads and sponge collagen. J Orthop Res., 1994; 12(5): 737-741.
4. Ciocîlteu MV, Podgoreanu P, Delcaru C, Chifiriuc MC, Manda CV, Biţă A, Popescu M, Amzoiu E, Croitoru O, Bleotu C, Bostan M, Neamtu J, PLGA-Gentamicin biocomposite materials with potential antimicrobial applications in orthopaedics. Farmacia, 2019; 67(4): 580-585.
5. Ciocîlteu MV, Mocanu AG, Mocanu A, Duce C, Nicolaescu OE, Manda VC, Turcu-Stoilica A, Nicolaescu C, Melinte R, Balasoiu M, Croitoru O, Neamtu J, Hydroxyapatite-ciprofloxacin delivery system: Synthesis, characterisation and antibacterial activity. Acta Pharm., 2018; 68(2): 129-144.
6. Neut D, Dijkstra RB, Thompson JJ, Kavanagh C, van der Mei HC, Busscher HJ, A biodegradable gentamicin-hydroxyapatite-coating for infection prophylaxis in cementless hip prostheses. Eur Cell Mater, 2015; 29: 42-56.
7. Deacon GB, Phillips RJ, Relationships between the carbon-oxygen stretching frequencies of carboxylate complexes and the type of carboxylate coordination. Coord Chem Rev., 1980; 33(3): 227-250.
8. Head WC, Bauk DJ, Emerson RH, Titanium as the material of choice for cementless femoral components in total hip arthroplasty. Clin Orthop Rel Res., 1995; 311: 85-90.
9. Ionescu (Filip) OL, Ciocîlteu MV, Manda CV, Neacsu IA, Ficai A, Amzoiu E, Turcu-Stoilica A, Croitoru O, Neamtu J, Bone - Graft Delivery Systems of Type PLGA-gentamicin and Collagen - hydroxyapatite – gentamicine. Mater Plast., 2019; 56(3): 534-537.
10. Murphy CM, Haugh MG, O’Brien FJ, The effect of mean pore size on cell attachment, proliferation and migration in collagen–glycosaminoglycan scaffolds for bone tissue engineering. Biomaterials, 2010; 31(3): 461-466.
11. Rauschmann MA, Michelhaus TA, Stirnal V, Dingeldein E, Zichner L, Schnettler R, Alt V, Nanocrystalline hydroxyapatite and calcium sulphate as biodegradable composite carrier material for local delivery of antibiotics in bone infections. Biomaterials, 2005; 26(15): 2677-2684.
12. Santavirta S, Grisita A, Konttinen YT, Cemented versus cementless hip arthroplasty: a review of prosthetic biocompatibility. Acta Orthop Scand., 1992; 63(2): 225–232.
13. Stigter M, de Groot K, Layrolle P. Incorporation of tobramycin into biomimetic hydroxyapatite coating on titanium. Biomaterials, 2002; 23(20): 4143-4153.

14. Schwarz F, Bieling K, Latz T, Nuesry E, Becker J. Healing of intrabony peri-implantitis defects following application of a nanocrystalline hydroxyapatite (Ostim) or a bovine-derived xenograft (Bio-Oss) in combination with a collagen membrane (Bio-Gide). A case series. J Clin Periodontol., 2006; 33(7): 491-499.

15. Suresh KG, Govindana R, Girija EK. In situ synthesis, characterization and in vitro studies of ciprofloxacin loaded hydroxyapatite nanoparticles for the treatment of osteomyelitis. J Mater Chem B., 2014; 2(31): 5052-5060.

16. Tone A, Nguyen S, Deverny F, Topolinski H, Valette M, Cazaubiel M, Fayard A, Beltrant E, Lemaire C, Senneville E. Six-week versus twelve-week antibiotic therapy for nonsurgically treated diabetic foot osteomyelitis: A multicenter open-label controlled randomized study. Diabetes Care, 2015; 38(2): 302-307.

17. Turcu-Stiolica A, Bubulica MV, Nicolaescu OE, Croitoru O, Popescu M, Manda VC, Simionescu A, Neamtu J. A design of experiment approach to the synthesis of alendronate-incorporated hydroxyapatite. Rev Chim., 2018; 69(8): 1944-1948.

18. Muț AM, Vlaia L, Coneac G, Olariu I, Vlaia V, Popoiu C, Hîrjău M, Lupuliasa D. Novel topical chitosan/hydroxypropylmethylcellulose - based hydrogels containing fluconazole and sucrose esters. Formulation, physicochemical characterization, in vitro drug release and permeation. Farmacia, 2018; 66(1): 59-69.

19. Upadiyay SK, Kumar P, Arora V. Complexes of quinolone drugs norfloxacin and ciprofloxacin with alkaline earth metal perchlorates. J Struct Chem., 2006; 47(6): 1078-1083.