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Hydroxyapatite-ciprofloxacin delivery system: Synthesis, characterisation and antibacterial activity

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The main objective of this study was to synthesize hydroxyapatite-ciprofloxacin composites using a chemical precipitation method and to evaluate the properties and *in vitro* release profile of the drug from the hydroxyapatite-ciprofloxacin composites. Composite characterization was achieved by FT-IR, XRD and DLS. Ciprofloxacin determination was accomplished by HPLC, resulting in good incorporation efficiency of the drug (18.13 %). The *in vitro* release study (Higuchi model $C = K\,t^{1/2}$ and Ritger-Peppas model, $C = K\,t^{0.6}$) showed a diffusion-controlled mechanism. The antibacterial activity showed that the bacterial growth inhibition zones were approximately equal for the synthesis composites and for the mechanical mixture on the *Staphylococcus aureus* germ.
The use of hydroxyapatite, which is a biocompatible, bioactive and osteoconductive material, with ciprofloxacin, which has good antibacterial activity in this composite, makes it suitable for the development of bone grafts. Furthermore, the synthesis process allows a slow local release of the drug.

Keywords: hydroxiapatite-ciprofloxacin composites, wet precipitation synthesis, *in vitro* release profile, antibacterial activity

Despite recent advances in operative techniques and therapies based on antibiotics, chronic osteomyelitis management still remains a challenge in orthopedic surgery, the costs of these treatments being high due to the need for long-term therapy (1–3). Osteomyelitis is a bone infection caused mainly by *Staphylococcus aureus*, which adheres to the bone surface, developing a biofilm that ensures its protection against antibiotic treatment (4).

The standard surgical procedure is based on the removal of the infected bone and soft tissues, a procedure described in literature as »debridement«, followed by antibiotic ther-

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apy administered systemically, orally or intravenously over an extended period of time (at least 4 or 6 weeks) with possible side effects and also the need to extend the hospitalization period (5).

Local release, using bone cement impregnation or PMMA chains, has been introduced in orthopedic surgery since 1970 (6). There are many studies, reported in literature, regarding the advantage of using antibiotic impregnated strands in the treatment of chronic osteomyelitis; therefore, this release system represents the gold standard in local therapy (7, 8). The main disadvantage of using impregnated strings is the necessity to remove them before bone grafting surgery. Therefore, improved solutions in order to develop new biodegradable materials are extensively studied.

Calcium phosphates, used in the applications that attract most interest are: hydroxyapatite (HA) and its combinations, tricalcium phosphate (α-TCP and β-TCP), octocalcium phosphate (OCP), amorphous calcium phosphate, *etc*. Biodegradation rate control is achieved by forming biphasic systems of these compounds (9). In the category of these compounds, special attention is given to HA, due to its biocompatibility, bioactivity and osteoconductivity, which make it also suitable for bone graft development (10). This compound is intensely studied as a release system and as well as a vector drug (11). An ideal antibiotic release system will have to ensure burst release during the first 24 hours, followed by sustained release, above the MIC, over an extended period of time (days to weeks). A disadvantage of calcium phosphates, when used as vector drugs in the treatment of bone infections is the burst release of antibiotics. Various techniques for improving the dissolution of drugs have been developed over time (2). This process occurs because, in most cases, the drug loading mechanism is adsorption from solutions. This mechanism leads to rapid drug release over several days.

In order to avoid this phenomenon and to control the drug loading mechanism, in our studies we preferred the synthesis of a HA-ciprofloxacin compound, obtained by the precipitation method. Ciprofloxacin is a fluroquinolone derivative, commonly used in osteomyelitis treatment due to its bactericidal effect on most common osteomyelitis pathogens. By modifying synthesis parameters and using a factorial experimental design, we managed to control the concentration within the HA-ciprofloxacin system and to extend the releasing time.

EXPERIMENTAL

Materials

Chemicals used both in the synthesis and characterization were of analytical reagent (AR) grade and were purchased as follows: calcium nitrate tetrahydrate (≥ 99 %), ammonium hydrogen phosphate (≥ 99 %, ammonium hydroxide (30 %), calcium hydroxide (≥ 95 %), phosphoric acid solution (w = 85 %) and hydroxyapatite were purchased from Sigma-Aldrich (USA). Ciprofloxacin was obtained from Fluka (Switzerland), HPLC Water – LiChrosolv® and solvents (acetonitrile, methanol, buffers) of HPLC grade from Merck (Germany).

Synthesis methods for hydroxyapatite-ciprofloxacin (HA-CIP) composites

In methods I and III, we used as reactants two aqueous solutions of calcium nitrate tetrahydrate 1.08 mol L^{-1} and ammonium hydrogen phosphate 0.65 mol L^{-1}, whose pH was

adjusted to 10 with ammonium hydroxide. In method I, 12.5 mL of 30 mmol L^{-1} aqueous solution of the antibiotic were prepared. To facilitate the solubilization of the antibiotic, the pH was brought to 11 with NH_4OH. For the third method, however, 625 mg of antibiotic were inserted into the flask immediately after the addition of $(NH_4)_2HPO_4$. In both methods, calcium nitrate solution was initially heated to 90 °C. The rate of addition of ammonium phosphate was 0.5 mL min^{-1}. The reaction mixture was stirred at 600 rpm for 5 hours. The obtained product was washed with ultrapure water and centrifuged for 10 minutes at 10.000 rpm. The remaining white powder was dried in the oven at low temperature (< 40 °C) for 24 hours.

In method II, the initial reactants used to obtain hydroxyapatite were calcium hydroxide and phosphoric acid. 50 mL of an aqueous suspension of $Ca(OH)_2$ 0.5 mol L^{-1} and 50 mL of phosphoric acid 0.2 mol L^{-1} were prepared, respectively. The pH of both solutions was adjusted to 10.5 with ammonium hydroxide, 2 grams of the drug were added into the synthesis flask after the addition of phosphoric acid. The reaction mixture was stirred strongly at 800 rpm. As calcium hydroxide has low solubility, it is desirable that the suspension should be as homogeneous as possible. The rest of the reaction conditions were maintained constant (time of reaction 5 hours, rate of phosphoric acid addition 0.5 mL min^{-1}, temperature: 90 °C. Also, in this method, the obtained product was washed, centrifuged and oven dried).

Physicochemical characterization

Spectral characterization. – Fourier-Transformed Infrared (FT-IR) spectra of the obtained products were recorded on an Avatar Nicolet spectrophotometer in KBr pellets, within the range 4000–400 cm^{-1}.

Structural characterization. – Acquisition of the X-ray diffraction pattern in order to obtain qualitative phase analysis was performed on a RIGAKU ULTIMA IV diffractometer with a vertical goniometer, and an incident radiation Cu-K_α (1.541 Å) line. Acquisition was performed in the $2\theta \in [20–60°]$ range, with a 0.02° angular step.

Dynamic light scattering (DLS) measurements. – Volume and number size distributions were measured by Dynamic Light Scattering (DLS) using a Brookhaven 90 Plus apparatus equipped with a solid state laser (15 mV, scattering angle: 15°, 90°) used in the range of 1–6000 nm. Measurements were made with the dust filter on.

High-performance liquid chromatography (HPLC) analysis. – HPLC analysis was performed with a Thermo Finnigan Surveyor chromatograph equipped with a diode array detector and data acquisition software Thermo Finnigan Xcalibur. Separation was performed on a C18 reverse phase column (Thermo Scientific) Hypersil GOLD, 250 × 4.6 mm inner diameter with a particle size of the stationary phase of 5 μm. The mobile phase was a mixture of 20 mmol L^{-1} citrate buffer (citric acid dihydrate 16.7 mmol L^{-1} and sodium citrate dihydrate 3.3 mmol L^{-1})/acetonitrile (40:60, *v*/*v*) with a flow rate of 1 mL min^{-1}. All experiments were done at room temperature. The stock solution of ciprofloxacin containing 1 mg mL^{-1} was prepared in acetonitrile and stored at 4 °C. Working solutions were prepared by further dilution with acetonitrile. Analyses were performed at 280 nm.

The sample (5 mg composite of hydroxyapatite-ciprofloxacin) was hydrolyzed in a 10 mL vial with 1 mL of hydrochloric acid (concentrated hydrochloric acid 37 % diluted with ultrapure water 1:1, *v*/*v*) for 15 minutes, and then the pH was adjusted to 7 with 25 % ammonia. The resulting suspension was centrifuged to separate the precipitated hydroxyapatite; hydroxyapatite bound to ciprofloxacin remained in the supernatant. The volume of the supernatant was adjusted to 5 mL with the mobile phase. After filtering through a porous membrane of 0.45 μm, 20 mL were injected into the system. A calibration curve with concentrations in the range from 0 to 20.000 ng mL^{-1} was plotted.

To determine accuracy and precision, concentration levels of 250, 2000 and 20000 ng mL^{-1} corresponding to small, medium and large ciprofloxacin concentrations were chosen. All of these standards were processed on the model shown above.

Experimental design approach

Influence of various synthesis parameters on hydroxyapatite-ciprofloxacin characteristics and on ciprofloxacin concentration in the final product were analyzed using the 9.1 MODDE program.

The following were chosen as parameters involved in the synthesis process: the amount of drug added during the process, the stirring speed and the ammonium phosphate addition flow.

Using experimental design, we tried to understand how the final product quality was influenced by the hydroxyapatite-ciprofloxacin synthesis process and the parameters of this process, and finally we will optimize these parameters to obtain the desired characteristics of the final product.

Ciprofloxacin release profile

Release studies were performed on the HA-CIP powder synthesized by the wet precipitation method III (powder whose content of ciprofloxacin was determined by HPLC to be 18.13 %) in the form of compressed tablets (200 mg). Ultrapure water was chosen as dissolution medium.

At regular time intervals (1, 6, 12, 24, 48, 72, 96 hours and 7, 14, 24 and 30 days), 0.5 mL of solution were taken and the amount of ciprofloxacin was determined by HPLC. The drawn volume was replaced with release environment (ultrapure water) to avoid saturation of the solution in ciprofloxacin.

Determination of ciprofloxacin amount in release medium by HPLC

500 mL of mobile phase was prepared by mixing 320 mL acetonitrile, 10 mL methanol and 170 mL water, in which we dissolved 0.60 g of citric acid and 0.165 g of monosodium citrate. 50 μL of the taken sample were brought to 5 mL with the mobile phase and 20 μL thereof were injected into the HPLC system.

Antibacterial activity

The final stage of the experimental study sought to determine the antibacterial effect of hydroxyapatite, ciprofloxacin and HA-CIP samples. To achieve this, we used the agar

diffusion technique. We tested the antibacterial effect of samples in the solid state (tablets of HA-CIP obtained by chemical synthesis, HA-CIP obtained by mechanical mixing).

The antibacterial effect was achieved against the microorganisms *Staphylococcus aureus* (ATCC 25923) and *Staphylococcus aureus* resistant to methicillin.

Bacterial culture medium and seeding. – We poured nutrient agar (Mueller-Hinton) in Petri dishes of 100 mm diameter, in a uniform layer of 4 mm. Inoculum preparation was performed by suspending 2–3 standard colonies in physiological saline solution, nephelometric turbidity of the suspension being controlled. The culture medium had a pH of 7.3 and a composition suitable for proper development of the bacterial species tested. Seeding was carried out by flooding the nutrient medium with the bacterial suspension, followed by removal of the excess.

Drying was achieved by keeping the inoculated plates for 10 minutes at room temperature (22 degrees ambient temperature) prior to sample addition. The microorganisms to be tested were classified as susceptible to the chosen antibiotic (ciprofloxacin).

Determination of minimum inhibitory concentrations. – The antibacterial effect of hydroxyapatite (negative control), hydroxyapatite-ciprofloxacin mixture and hydroxyapatite--ciprofloxacin synthesis composite was then tested by preparing microsuspension samples. Determination of minimum inhibitory concentrations (*MIC*) was performed in a polystyrene panel with 96 wells. The panel contained the following microsuspensions: HA synthesis (0, 25,50, 125, 250, 2500 μg/mL), HA-SA (Sigma-Aldrich), (0, 25,50, 125, 250, 2500 μg mL^{-1}), mechanical mixture ciprofloxacin and hydroxyapatite (HA + CPX) 0, 25, 50, 125, 250, 2500 μg mL^{-1}), HA-CPX composite obtained by chemical synthesis (0, 25,50, 125, 250, 2500 μg mL^{-1}).

The same inoculum suspension was added to each well and then the *MIC* panel was incubated at 37 °C. The *MIC* endpoint was determined by spectrophotometric methods as the lowest concentration of the antimicrobial agent that completely inhibited the growth of the bacteria. Mueller-Hinton broth was the microbiological growth medium used for the antimicrobial susceptibility testing. The pH of the medium was adjusted to 7.2 at room temperature. Furthermore, the strain used for *MIC* testing was *Staphylococcus aureus* ATCC 29213.

RESULTS AND DISCUSSION

HA-CIP synthesis methods

Methods I and III

The main methods of obtaining HA are wet precipitation reactions (13), sol-gel methods, hydro/solvothermal processes, multiple emulsion techniques, each with specific advantages and disadvantages. Thus, HA synthesis using chemical precipitation method chosen for this experimental study is the most commonly used technique, the main advantage of this method being low probability of contamination and lower relative costs. Using this method, it is also easy to maintain the experimental factors constant (14), any slight modification of reaction conditions being able to significantly affect the properties of synthesized compounds (15). As mentioned in literature, changing the temperature and the stirring time can lead to hydroxyapatite with a small size (16). Our previous studies (in

which we succeeded in including another drug, alendronate, in HA structure) confirmed that the chemical precipitation method with starting reagents calcium nitrate and ammonium hydrogen phosphate is the best choice (17).

Choosing the reactants in methods I and III was also judicious, given that both calcium nitrate and ammonium phosphate are easily soluble in water.

Method II

The second method started the synthesis with calcium hydroxide and phosphoric acid. Calcium hydroxide has low solubility in water, needing stronger stirring speed to facilitate hydroxyapatite formation. Another great disadvantage is the addition of H_3PO_4, which lowers the pH of the reaction environment, required for a pure HA without secondary Ca-deficient hydroxyapatite or calcium phosphate (18). Yet, in this method, one of the major benefits is that only water and HA can be found among reaction products.

Physicochemical characterization

FT-IR characterization. – For the resulting compound in method I, characteristic bands of HA were absent, indicating that the HA in that method was not properly formed. We propose that one of the reactants, $Ca(NO_3)_2$ and ciprofloxacin formed a complex in a competitive reaction with the one between $Ca(NO_3)_2$ and $(NH_4)_2HPO_4$.

The literature shows that a complex (19) with the structure $[Ca(cip)_2](NO_3)_2{\times}H_2O$ between calcium and antibiotic can be easily formed, which was proven to us by the appearance of a nitrate group band at 1383 cm^{-1} (NO_3^- group acting as counterion) and by stretching vibration of the OH group in the same molecule at 3567 cm^{-1}. The pH used for ciprofloxacin solubilization promoted the formation of the above-mentioned structure (Fig. 1b) (20).

Due to these findings, we decided that the first synthesis method did not correspond to the desired goal, the composite HA-CIP had a low yield, while the amount of complex obtained in this undesirable secondary reaction was quite significant. That is why we gave up this method although it offered the advantage of keeping an inert atmosphere that would have made it possible to get a non-carbonated hydroxyapatite.

The FT-IR spectra of the synthesized compounds in methods two and three (Fig. 1c,d) showed sharp peaks due to the PO_4^{3-} group vibration from the hydroxyapatite structure at 560–568 cm^{-1} and 1029–1035 cm^{-1} (21).

In the HA-CIP compound synthesized in the second method, a characteristic peak for acid phosphate group appeared also at 868 cm^{-1}, indicating the possibility of the presence of a second phase of Ca-deficient hydroxyapatite (22).

Regarding the possibilities of ciprofloxacin coordination to calcium ion from hydroxyapatite, we observed the shift of the characteristic carbonyl peak (CIP structure) at higher wavelengths than pure ciprofloxacin from 1616 to 1627 cm^{-1}.

Asymmetric vibration corresponding to carboxyl deprotonated group appeared in HA-CIP at 1579 cm^{-1} (Method II) and 1582 cm^{-1} (Method III) while the peak corresponding to symmetrical vibration of the same group, although present in the ciprofloxacin spectrum (1376 cm^{-1}), disappeared in composite spectra due to coordination *via* the deprotonated hydroxyl group. A bidentate structure was proposed therefore for HA-CIP, the metallic ion being bound to the oxygen atom of the deprotonated carboxyl group and to the carbonyl oxygen atom.

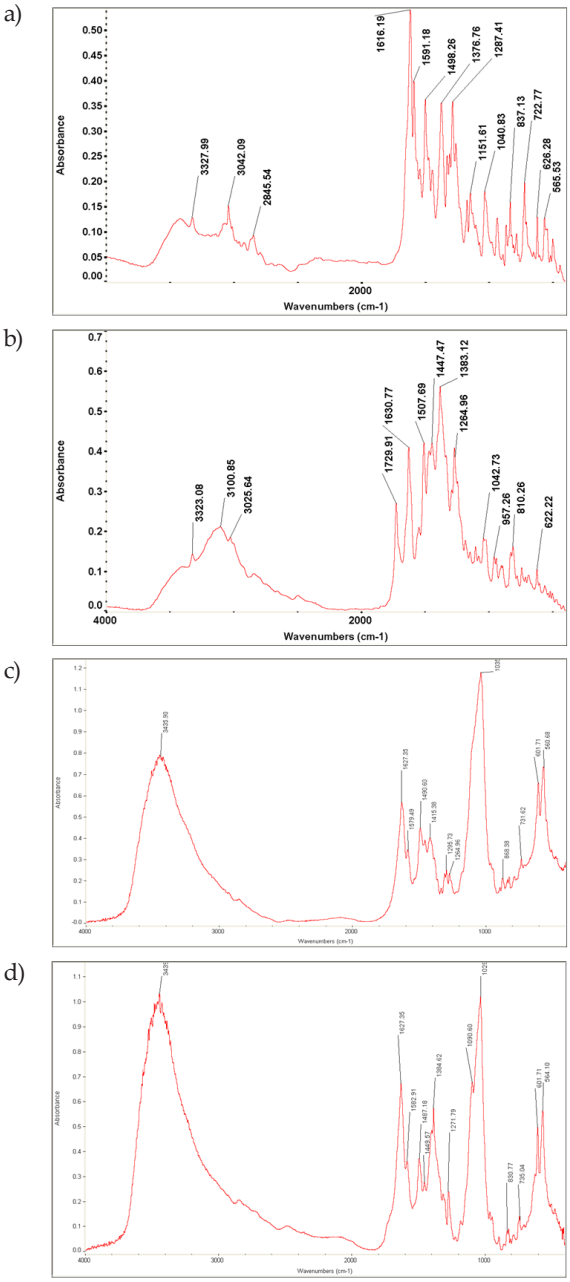

Fig. 1 a) FT-IR spectrum of ciprofloxacin, b) FT-IR spectrum of the HA-CIP obtained by the first synthesis method, c) FT-IR spectrum of the HA-CIP obtained by the second synthesis method, d) FT-IR spectrum of the HA-CIP obtained by the third synthesis method.

Vibration of the nitrogen atom (piperazinyl group) occurred weak at 2845 cm^{-1} in the CIP spectrum (pH = 11, zwitterionic form). In this form, the nitrogen atom did not participate in the coordination process.

An additional argument advocating the zwitterionic form of the drug is the absence of $\nu_{simCOOH}$ (23).

Determination of the antibiotic incorporation efficiency by HPLC

In chromatographic analysis, sample preparation is a very important step in achieving ciprofloxacin separation of HA-CIP. Separation of the two compounds requires a hydrolysis reaction and an appropriate adjustment of the pH in order to precipitate HA. The pK_a value reported in the literature for ciprofloxacin is 6.09 (24). At pH = 3.1 (mobile phase), both carboxyl and amino groups are protonated. The reason why we used an acid mobile phase was to obtain symmetrical peaks, easily integrated without "heading" or "tailing".

Ciprofloxacin peaks, both for pure ciprofloxacin (standards) and for samples (corresponding hydrolyzed composite) have the same retention time (3.2 min). Maximum absorption wavelength was also the same for all samples (280 nm), certifying the purity of the obtained peak. The presence of ciprofloxacin was confirmed qualitatively and was quantitatively determined in all analyzed samples by the disclosed method.

Once we had performed quantitative determination of ciprofloxacin from the compounds obtained in method II (from $Ca(OH)_2$ and H_3PO_4) and method III ($Ca(NO_3)_2$ and $(NH_4)_2HPO_4$), we found that from the point of view of the amount of ciprofloxacin bound to hydroxyapatite, method III was more effective, where the percentage was 18.13 % compared to 12.55 % obtained for method II.

Also when starting from $Ca(OH)_2$ and H_3PO_4, we must take into account that calcium hydroxide has a low solubility in water and by adding H_3PO_4 we must check rigorously the pH environment to avoid the appearance of secondary phases such as Ca-deficient hydroxyapatite, as shown by FT-IR characterization.

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

Structural characterization by XRD analysis

X-ray diffraction patterns for hydroxyapatite, ciprofloxacin and two hydroxyapatite-ciprofloxacin compounds obtained by two different routes (method II and method III) were investigated. The qualitative phase analysis was made with PDXL software by Rigaku, and *COD (Crystallography Open Database)* database showed the following: in the synthesized HA sample, the phase identified was $Ca_{10.084}(PO_4)_{5.94}(OH)_{3.39}$ (carbonated hydroxyapatite); in the HA-CIP sample obtained by method II (starting reagents $Ca(OH)_2$, H_3PO_4 and ciprofloxacin) both phases hydroxyapatite ($Ca_{10.084}(PO_4)_{5.94}(OH)_{3.39}$) and ciprofloxacin ($C_{17}H_{18}FN_3O_3$) were identified but also we found some peaks that did not belong to either of them. These peaks could not be identified with certainty as belonging to some known phases from the database. In the HA-CIP sample (Fig. 2) obtained by method III (starting reagents $Ca(NO_3)_2$, $(NH_4)_2HPO_4$ and ciprofloxacin), it was revealed that these

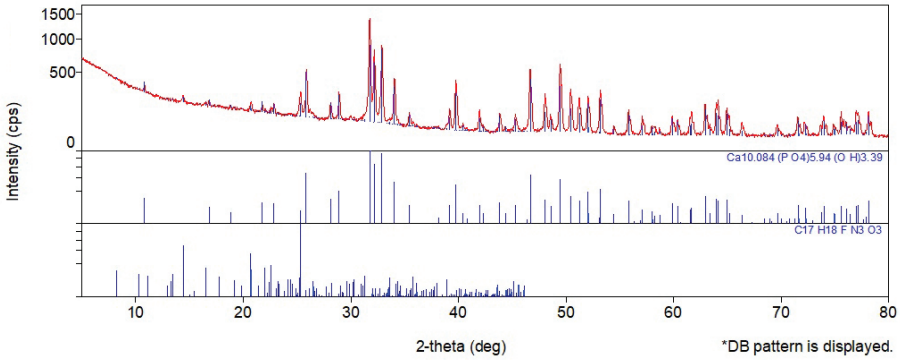

Fig. 2. X-ray diffraction pattern and qualitative analysis for the HA-CIP compound obtained by us by method III (starting reagents $Ca(NO_3)_2$, $(NH_4)_2HPO_4$ and ciprofloxacin).

spectra had the characteristics of pure hydroxyapatite ($Ca_{10.084}(PO_4)_{5.94}(OH)_{3.39}$) and ciprofloxacin ($C_{17}H_{18}FN_3O_3$). Under the synthesis conditions, other secondary phases were not observed (pH = 10, flow 0.5 mL min^{-1}, 600 rpm).

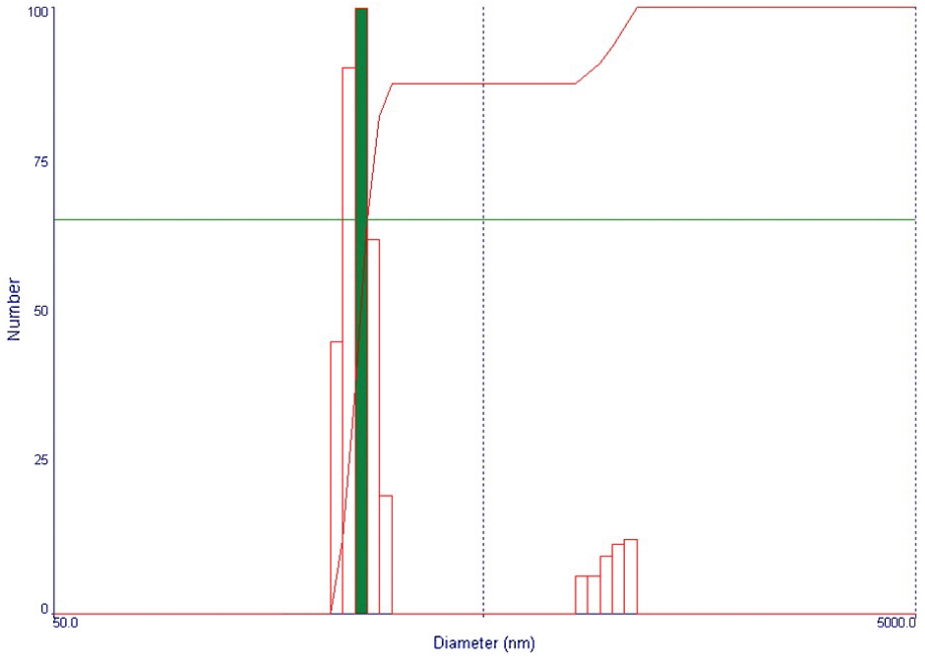

Fig. 3. Particle size distribution (numerical distribution of HA-CIP particle size) using dynamic light scattering shows two intervals: one in which most of the particles are contained (226–294 nm), and one in which small agglomerations are highlighted (838–1089 nm).

Considering the qualitative and quantitative results obtained by FT-IR, HPLC and XRD, we further characterized (by DLS, experimental design approach, release study and antibacterial activity) the HA-CIP composites obtained by method III.

Dynamic light scattering (DLS) measurements

Particle number distribution has two size ranges between 226 and 294 nm and 838 and 1089 nm, respectively (Fig. 3). The same ranges were found in the particle volume distribution, mentioning that the second range was reduced. Analyzing the two distributions (number and volume), it was observed that most of the particles had the diameter around 259 nm.

Minor particle agglomeration was displayed by the second range between 838 and 1089. Compound agglomeration should be avoided because it may hide several impurities.

Experimental design approach

For method III, a full factorial experimental design was used with the purpose of obtaining a mathematical model represented by polynomial regression where the result (ciprofloxacin concentration, Y) depended on the influence of three factors (quantity of the drug used in the synthesis X_1, stirring rate X_2, volumetric flow rate of diammonium phosphate in synthesis X_3):

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_1X_2 + b_5X_1X_3 + b_6X_2X_3 + b_7X_1X_2X_3$$

where: Y is a dependent variable, b_0 is a constant, b_1, b_2, b_3, b_4, b_5, b_6 şi b_7 are regression coefficients, X_1X_2, X_2X_3, X_1X_3 şi $X_1X_2X_3$ are interactions between the main effects.

The values obtained following the eight experiments are detailed in Table I.

Table I. Full factorial design with responses. (+1) is the maximum and (–1) is the minimum value of each parameter

Experiment number	Experiment name	Execution order	Quantity of the drug used in the synthesis (g), X_1	Stirring rate (rpm), X_2	Volumetric flow rate of diammonium phosphate in the synthesis (mL min^{-1}), X_3	Ciprofloxacin concentration (%)
			(X_1)	(X_2)	(X_3)	
1	N1	7	0.5 (–1)	600 (–1)	0.1 (–1)	18.134
2	N2	2	2.5 (+1)	600 (–1)	0.1 (–1)	11.353
3	N3	5	0.5 (–1)	1000 (+1)	0.1 (–1)	16.4966
4	N4	1	2.5 (+1)	1000 (+1)	0.1 (–1)	11.186
5	N5	3	0.5 (–1)	600 (–1)	10 (+1)	4.3346
6	N6	4	2.5 (+1)	600 (–1)	10 (+1)	8.0252
7	N7	8	0.5 (–1)	1000 (+1)	10 (+1)	6.5022
8	N8	6	2.5 (+1)	1000 (+1)	10 (+1)	6.1556

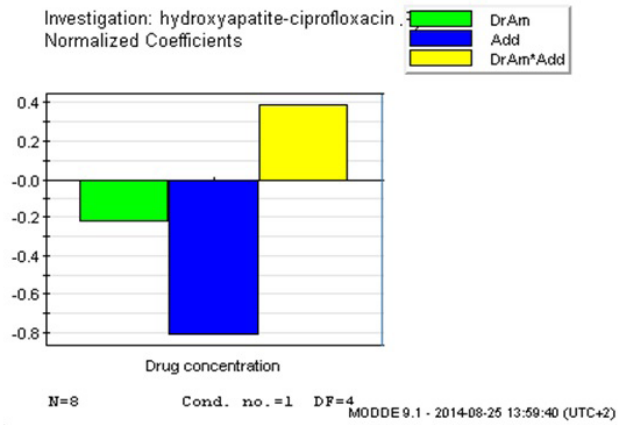

Fig. 4. Influence of parameters on the drug concentration in the sample. DrAm = quantity of the drug used in the synthesis; Add = volumetric flow rate of diammonium phosphate in the synthesis; DrAm* Add = interaction of the two before the mentioned factors.

When the optimal regression model is reached, the model should be used. We can better understand the system in this way and we can decide if further experiments are required and if this is necessary to find the factors of interest.

The created model showed the negative influence of the two factors (X_1 and X_3) and the positive influence of the interaction between the two factors (X_1X_3, drug amount* flow rate of diammonium phosphate in the synthesis, Fig. 4. yellow). Our model equation became:

$$Y = 10.2734 - 1.09345*X_1 - 4.019*X_3 + 1.92945X_1X_3$$

In conclusion, we cannot obtain ciprofloxacin concentrations higher than 18 % with the synthesis conditions tested for this model.

Ciprofloxacin release profile from HA-CIP composites

Prolonged ciprofloxacin release from HA-CIP composites was also analyzed by other authors in similar studies. Kumar *et al.* (25) synthesized a composite that allowed constant release of the antibiotic over a 60-day period. They concluded that by raising the ciprofloxacin concentration in the composite, the release period also rose.

Rauchmann *et al.* (26) showed that the release of the antibiotic from a hydroxyapatite and calcium sulphate nanocrystal composite was over a period of approximately 10 days whereas the release of ciprofloxacin from a usual tablet was complete within a few hours (27).

According to our experimental results, in the first 7 days of the study, 46 % of the ciprofloxacin was released from the HA-CIP compound (with 18 % ciprofloxacin). Then, the released antibiotic quantity suddenly rose and reached 94 % by day 21 (Fig. 5a). This extended release profile may be due to bonds formed between ciprofloxacin and hydroxyapatite during synthesis.

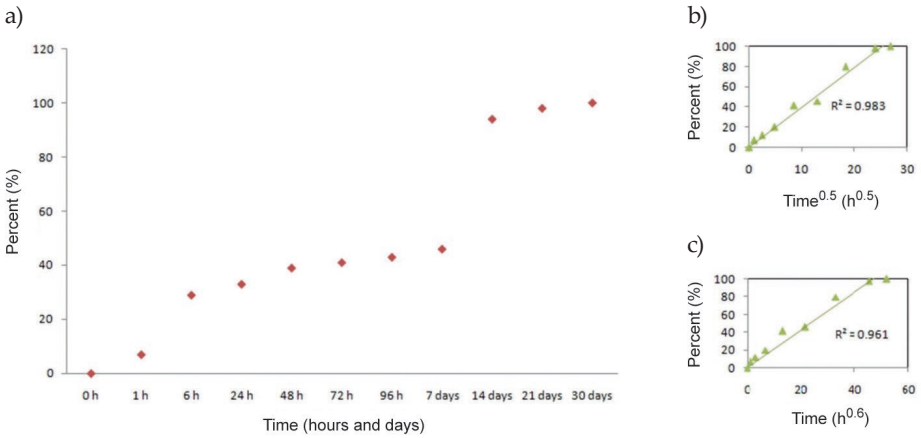

Fig. 5. Release of ciprofloxacin from HA-CIP tablets: a) cumulative release over time, b) linear regression of the release data using Higuchi's model, c) linear regression of the release data using the Rigter-Peppas model.

Figs. 5b and 5c show two ciprofloxacin release kinetic models for the compound: Higuchi model ($C = K\,t^{1/2}$) and Ritger-Peppas with $C = K\,t^{0.6}$ equation. A higher correlation coefficient was obtained when cumulative ciprofloxacin percentage was represented in a graph in relation to the square root of time. This shows that diffusion is the main process for ciprofloxacin release.

Antibacterial activity

In most studies of chemical synthesis of HA, there are no references to antibacterial activity of this compound, most of the articles considering it a negative control (28).

In the synthesis we conducted, HA was tested for antibacterial activity in order to observe whether the synthesis process affected the final product.

In addition to the HA-CIP synthesized compound, we made a mechanical mixture of HA and ciprofloxacin to observe the effect of ciprofloxacin binding to HA on antimicrobial activity. The antimicrobial effect was evaluated by measuring the diameter of the inhibition zones. This diameter (D) was directly proportional to the more pronounced antimicrobial effect.

In our study, it was found that the synthesized HA had no antibacterial activity. While the antimicrobial activity of ciprofloxacin against the microorganism studied in this experiment [29] is well known, the binding of this antibiotic to hydroxylapatite may modify the antimicrobial activity of the HA-CIP synthesized.

Thus, in an experimental study, Heijink *et al.* (30) developed delivery systems by antibiotic impregnation of biodegradable materials used in the treatment of bone disorders. These biomaterials based on calcium sulfate (Osteoset), demineralized bone (DBX) and collagen-hydroxylapatite (Collagraft) were impregnated with a series of antibiotics

(vancomycin, gentamicin) aiming to observe the influence of biomaterial on antibiotic release and antimicrobial activity. Studies have shown that the antimicrobial activity changes depend on the nature of the antibiotic and biomaterial. Thus, the antibacterial activity of the mixture of DBX and gentamicin was not altered but the mixture of the same antibiotic with Osteoset and Collagraft activity dropped to 60 %.

Therefore in our study, we prepared samples in the form of tablets, obtained both by compressing the synthesized HA-CIP composite and mechanical mixture of HA and cip-

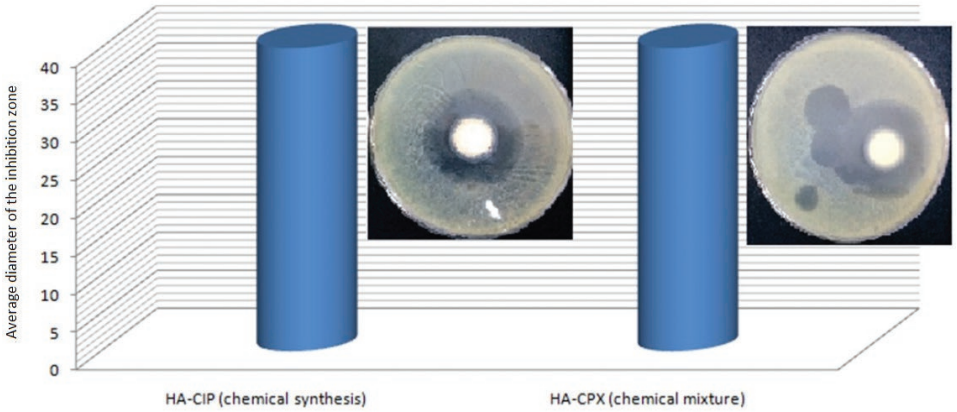

Fig. 6. Average diameter (n = 3) of the bacterial growth inhibition zone measured using the agar diffusion test for samples HA-CIP (chemical synthesis) (39.93 ± 0.24 mm) and HA-CIP (mechanical mixing) (40.1 ± 0.21 mm) against *Staphylococcus aureus* ATCC 25923.

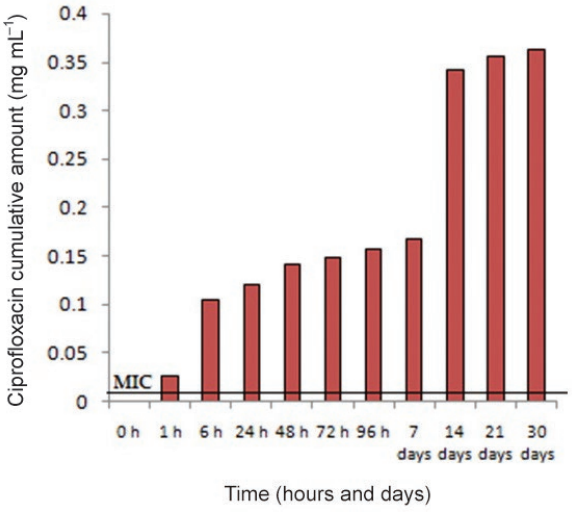

Fig. 7. Cumulative release of ciprofloxacin from HA-CIP composite (m = 10 mg).

rofloxacin. The mixture kept the ratio of the compound synthesized by us (18 % ciprofloxacin).

From the antibiogram results, shown in Fig. 6, it is noted that by binding ciprofloxacin to hydroxyapatite, antibacterial activity was not significantly modified (diameter of zone of inhibition decreased insignificantly).

Determination of minimum inhibitory concentrations (MIC)

Analysis of the antibacterial potential of the *Staphylococcus aureus* species showed the efficacy of the synthesized compound and the mechanical mixture. We can observe that even in this case, in microsuspensions, ciprofloxacin binding of hydroxyapatite did not influence the antibacterial activity, its release of bioceramic being easily achieved. The calculated minimum inhibitory concentration was 1250 μg composite mL^{-1} for both the synthesized composite and the mechanical mixture.

The release of ciprofloxacin from 10 mg of HA-CIP composite was carried out at extremely high concentrations above the minimal inhibitory concentration of the antibiotic over the entire time period, ensuring antibiotic efficacy on *S. aureus* but below the toxicity threshold of the drug (Fig. 7.)

CONCLUSIONS

According to the obtained results, the biocompatible composites obtained by chemical synthesis are pure and crystalline. The extended release time of the antibiotic makes this material usable as extended-release antibiotic delivery system. The drug loading on HA may be controlled by adjusting synthesis parameters. *In vitro* release measurements display an extended release up to 30 days, which is similar to the prophylactic treatment. By comparison, *in vivo* release may be prolonged due to restricted fluid volume surrounding the implant.

The antibacterial activity showed that the diameters of the bacterial growth inhibition zones were approximately equal for the composite obtained by synthesis and for the mechanical mixture on *Staphylococcus aureus* germ. Inclusion of ciprofloxacin in the HA structure did not affect the antibacterial activity on *Staphylococcus aureus*. In conclusion, the results of these tests show that the chemically synthesized HA-ciprofloxacin composite by the precipitation method exhibited antibacterial activity. Further investigations are required to determine *in vivo* effects of the synthesized composites.

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