ORIGINAL PAPER

THE CHEMISORPTION-RELEASE AND ANTIBACTERIAL POTENTIAL STUDIES OF CIPROFLOXACIN FROM HYDROXYAPATITE-BASED IMPLANTS

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Abstract. *Implant infections are the result of bacterial adhesion to the implant surface and subsequent biofilm formation at the implant site. A sustained and high antibiotic concentration over minimal inhibitory concentration (MIC) of pathogenic bacteria at the implant site is expected to inhibit bacterial adhesion, colonization, and biofilm formation. In the present study we performed implants based on hydroxyapatite (HAp), HAp reinforced with titanium particles (HAp/Ti) and Hap/Ti with added calcium fructoborate (CaFb) by chemisorption deposition method (HAp/Ti/CaFb). The implants were immersed in ciprofloxacin (CP) solution for 24 hours, then was determined the release profile of antibiotic for 14 days and the antibacterial effect of the three types of composite. The period of antibiotic release may be considered as sufficient to support osteointegration under antibacterial protection.*

Keywords: *chemisorptions; release; ciprofloxacin; hydroxyapatite; implant.*

1. INTRODUCTION

Osteomyelitis comprises a group of infectious diseases characterized by bone infections and / or spinal infections. These diseases can be followed by an acute, subacute, or chronic course. Osteomyelitis is bacterial infections, in which the pathogen was localized to the bone, being brought there by blood, from a distant outbreak of infection. Osteitis is a similar infection, but the pathogen has reached the bone through a direct contamination. Between the osteitis and the osteomyelitis there are not too many differences of manifestation, the infection comprising in fact all the layers of the bone (periosteum, bone, and marrow) [1].

Treatment of bone and joint infections such as osteomyelitis, septic arthritis and infections of the prosthetic joints can be very difficult, requiring long-term administration of antibiotics and additional surgical treatment. The most common cause of acute or chronic hematogenic osteomyelitis in adults and children is *Staphylococcus aureus* infections. Group A *streptococcus*, *Streptococcus pneumoniae*, and *Kingella kingae* are pathogens found in osteomyelitis in children. In chronic osteomyelitis, which may be caused by neighboring infections, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Serratia marcescens*, and *Escherichia coli* were isolated. Osteomyelitis caused by fungal and mycobacterial infections is less common, being reported in patients with functional impairment [2].

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The conventional routes of administration of CP for osteomyelitis are oral and injectable, the challenge being to achieve implants with CP deposited on implants for local delivery at the bone level. Usualy, it was choseen CP as an active substance due to the broad antibacterial spectrum, which acts on both gram positive and gram-negative bacteria [3]. CP is part of the quinolone group, by oral administration it has a good bioavailability, the plasma protein binding is medium, and it has a large distribution volume due to the good tissue diffusion. It achieves higher concentrations than plasma in the kidneys, prostate, lungs, ball, and bones. The mechanism of action of CP is bactericidal and consists in inhibiting AND-topoisomerases, named enzymes, and gyrates, which play a role in the bacterial chromosome superspiration process, thus stopping cell division [4].

Local drug delivery systems, consisting of non-biodegradable or biodegradable and osteoactive materials, such as calcium orthophosphates, bone cement, have proved to be promising alternatives for the treatment of osteomyelitis [5]. These systems allow local delivery of the antibiotic *in situ* with bactericidal concentrations for long periods of time and without the toxicity associated with other means of administration [6].

The aim of this study was to obtain implants based on HAp, which contain CP adsorbed by chemisorption method and to follow the antibiotic release for a period of 14 days. It was also established the MIC of CP on the tested germs: *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853). Subsequently, was determined the therapeutic efficacy of HAp-CP, HAp/Ti-CP and HAp/Ti/CaFb-CP, taking as a reference the MIC of CP (control +) on the standard reference bacterial strains. In carrying out and interpreting qualitative antibiograms, the aim was to respect the American standards, elaborated by CLSI (Clinical Laboratory Standards Institute).

2. MATERIALS AND METHODS

2.1. DESCRIPTION OF IMPLANTS

The implanted biocomposite samples are made using the powder metallurgy technology. The matrix material is HAp ($Ca_5(PO_4)_3(OH)$) powder particles (Merck, Germany; average 200 nm particle size) which is reinforced by the titanium hydride (TiH_2) powder particles (Merck; ~100μm; water atomized). The mixing content between the components is 75% wt. HAp and 25% wt. TiH_2. The first step of the biocomposite samples preparation consists in the HAp drying. One calcination cycle is developed in air at 900^0C for 1 hour. Then the calcinated matrix and reinforcement powder particles are mixed in a planetary ball mill for 30 minutes. The ratio between the milling balls (stainless steel, 5 mm diameter) and the powder mixture is 2:1. The milling environment is ethanol (1 mL/1 g powder mixture). the obtained mixture is dried for 1 hour at 200^0c in a conventional furnace, followed by the extraction of the milling balls out of the powder mixture. The biocomposite samples are shaped using the cold compaction operation at 120 MPa in a metallic die of 10 mm as inner diameter on A009 electromechanical-computerized 100kN testing machine, equipped with TCSoft 2004 Plus software. Then the compacts are submitted to the two steps sintering (TSS) heat treatment using a laboratory Nabertherm chamber furnace, type L5/12, at 1200°C maximum temperature. A protective gas atmosphere (pure argon 99.98%) is provided along the entire treatment through the rear wall connection of the furnace [7]. During the first step of TSS the compacts heating reaches a peak temperature (T_1 = 900°C) for a very short time (1 minute) to achieve an intermediate density by the initiation of the diffusion process between HAp and TiH_2 powder particles. Then the second step of TSS develops by means of a rapid

cooling to T_2 = 800°C and the dwell time is 10 hours. The final densification process occurs without the grain growth of the nanosized HAp particles [8]. In order to study the possibilities to improve the osseointegration of the biocomposite samples into the genuine bone, some sintered samples were immersed into a CaFb based solution 0.4 g/10 mL for 48 hours.

2.2. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

The HPLC analysis was performed with a Surveyor(TM) chromatograph (Thermo Finnigan, Maastricht, Netherlands) equipped with a diode array detector and data acquisition software Thermo Finnigan Xcalibur. Analysis was performed on a C_{18} reverse phase column (Thermo Scientific, Waltham, MA, USA) 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 citrate buffer/acetonitrile (40:60, *v/v*) with a flow rate of 1 mL/ min.

2.3. CP CHEMISORPTION

All implants, previously weighed, were completely immersed for 48 hours in CP solution (0.3 mg CP / 10 mL ultrapure water). Subsequently, the implants were deposited on filter paper, dried for 48 hours at room temperature and then weighed.

2.4. CP RELEASE

Release studies were carried out on implants of HAp, HAp/Ti and HAp/Ti/CaFb being chosen ultrapure water as dissolution medium. The temperature was maintained in the oven at $37^oC \pm 0.5^oC$. Bottles with screw cap were used to ensure tightness during the determinations. Thus, 5 mL of HPLC ultrapure water (LiChrosolv, Merck) was initially added and then the test implants were immersed. At regular time intervals (i.e., 1 h, 6 h, 12 h, 24 h, 48 h, 72 h, 6 days, 9 days, 12 days, and 14 days respectively), 0.5 mL solution was taken for quantitative determination of CP by HPLC technique. The volume was replaced with the yield medium (ultrapure water) to avoid saturation of the solution in CP.

2.5. DETERMINATION OF CP AMOUNT IN RELEASE MEDIUM BY HPLC

The mobile phase (100 mL) was prepared by mixing acetonitrile, methanol and water (64:2:34, *v/v*), in which we dissolved 0.12 g of citric acid and 0.033 g of monosodium citrate. 50 μL of the taken sample were brought to 5 mL with the mobile phase and 20 μL there of were injected into the HPLC system.

2.6. PREPARATION OF THE CULTURE MEDIUM AND BACTERIAL SEEDING

The nutrient agar (Mueller-Hinton) was poured in Petri dishes with a diameter of 100 mm, a uniform layer of 4 mm. Inoculum preparation was performed by suspending 2-3 standard colonies in physiological serum; the turbidity of the suspension was controlled nephelometrically. The culture medium must have a pH of 7.2 - 7.4 and a composition

suitable for the proper development of the bacterial species to be tested. Sowing was achieved by flooding the nutrient medium with bacterial suspension, followed by removal of excess. The drying of the inoculated plates was performed by maintaining for 10 minutes at room temperature (22 ^{o}C) before the samples was deposited. The microorganisms to be tested came from standard reference strains, purchased from the National Institute of Research & Development for Microbiology & Immunology "Cantacuzino" (Romania), being classified as sensitive to the action of the antibiotic of choice (CP).

2.7. DETERMINATION OF THE MINIMAL INHIBITORY CONCENTRATION (MIC)

To determine the MIC, it was placed the filter paper washers containing different amounts of CP (2, 3, 4, 5, 6, 7, 8 µg) on the culture media sown with *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*. Petri dishes were incubated for 24 hours at 37 ^{o}C. The MIC was considered to be the lowest concentration of antibiotic that caused the inhibition of *in vitro* bacterial growth (Table 1).

Table 1. Classification of the sensitivity of the germs tested to CP, according to the diameter of the bacterial growth inhibition zone.

Microorganism test	**Antibiotic (control +)**	**R [mm]**	**IS [mm]**	**S [mm]**
Staphylococcus aureus	CP	≤15	16-20	≥21
Escherichia coli		≤15	16-20	≥21
Pseudomonas aeruginosa		≤9	10-15	≥16

2.8. ANTIBIOGRAM PREPARATION

To test the antibacterial effect, the diffusimetric method on nutrient agar (*Kirby-Bauer*) was used. The deposition of the samples was performed approximately 15 minutes after sowing, with the help of an ophthalmological pen, applying each sample to be analyzed on the surface of the culture medium. The samples were deposited 1.5 cm away from the edge of the Petri box and 3 cm apart from each other. Incubation was performed for 18 hours at 37^{o}C, in the inverted position of the Petri dish. The reading of the results was performed with the naked eye, using a graduated ruler, measuring the diameter of the inhibition zone (mm), induced by the test samples. The results were expressed as the average values obtained by performing the arithmetic mean of the diameters corresponding to three measurements. The very small colonies or the subsequent invasion of the inhibition zone and the discrete increases within the inhibition zone was not take into consideration [9]. The final results were expressed in: sensitive, intermediate sensitive and resistant.

3. RESULTS AND DISCUSSION

From Table 2 it can see that the highest amount of CP was adsorbed by HAp and the smallest amount was by HAp/Ti/CaFb. The small amount of chemisorbed by HAp/Ti/CaFb may be due to saturation of the implant surface with CaFb, because the implant during the obtaining step was immersed in a CaFb solution.

Table 2. Weight of test implants before and after CP chemisorptions.

Implant type	Samples mass before chemiosorption [g]	Samples mass after chemiosorption [g]	The amount of CP adsorbed [g]	Percentage of CP adsorbed [%]
HAp	0.3940	0.4120	0.0180	4.5685
HAp/Ti	0.3974	0.4114	0.0140	3.5228
HAp/Ti/CaFb	0.4647	0.4777	0.0130	2.7975

For the quantification of CP, a calibration curve was constructed in the concentration range of 0-2000 mcg / mL. Dilutions were done in ultra-pure water. The equation is: y = 770.47 x - 44499 with a correlation coefficient of 0.9997 (Fig. 1). The retention time for CP was approximately 3.2 minutes, with peak absorption at 280 nm.

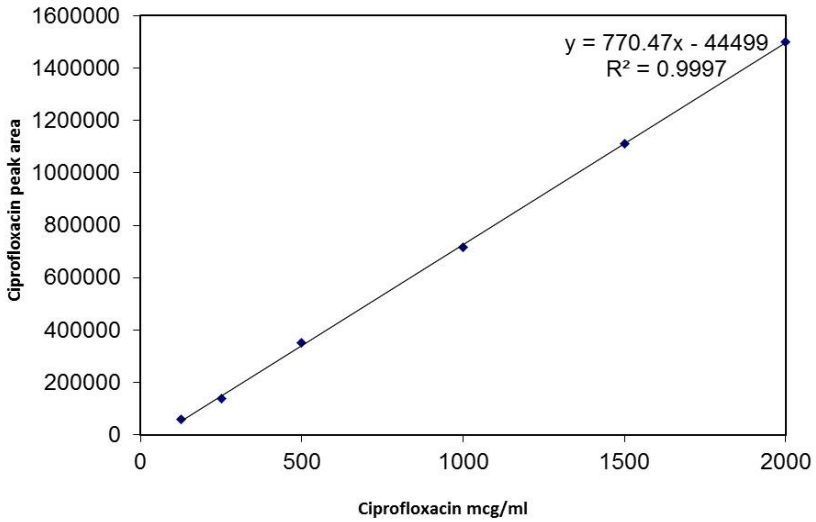

Figure 1. Calibration curve obtained by HPLC for CP determination.

From Fig. 2 it can be observed that for HAp-CP the CP release at two hours is 0, and then the transfer is made in stages, reaching stagnation stages between 4 h and 6 h, 12 h and 24 h and 48 h and 72 h, respectively.

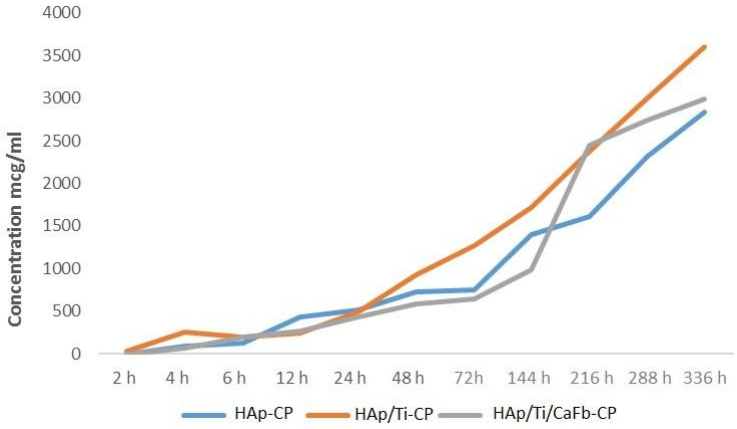

Figure 2. The amount of CP given (mcg / mL) from tested implants in the time unit.

In the case of HAp/Ti-CP the antibiotic delivery begins at 2 h and takes place exponentially for a period of 14 days. In the case of HAp/Ti/CaFb-CP antibiotic delivery is

quantifiable at four hours after immersion, the release profile being slowed up to 144 h (6 days). Between 144 h (6 days) and 216 h (9 days) there is a sudden release of CP, then again slow until 336 h (14 days) (Figs. 2-3).

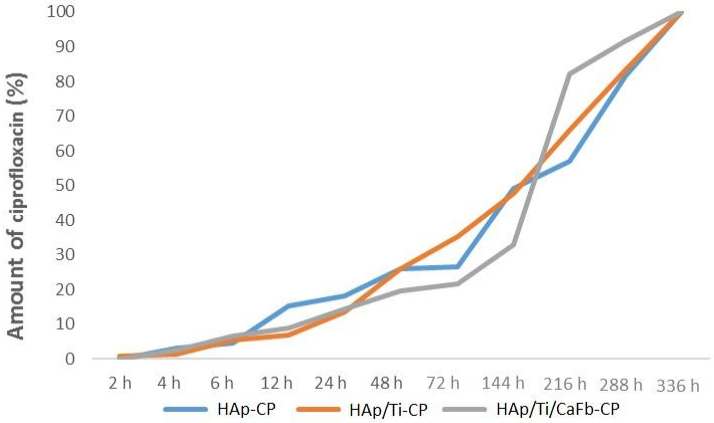

Figure 3. Percentage of CP given as a function of time.

In the specialized literature it has been observed that the release time of an active substance from a composite is done for a maximum period of 4 weeks, varying according to the nature of the constituents of the composite and the method of obtaining it. HAp/alendronate deposited on titanium implants by Matrix-Assisted Pulsed Laser Evaporation (MAPLE) technique released alendronate from the metallic component into the release medium for 10 days [4].

Rauschmann et *al.* showed that the release of the adsorbed antibiotic (vancomycin and gentamicin) from a HAp and calcium sulphate nanocrystal composite was over a period of approximately 10 days [10]. In another experimental studies release profile was performed on the HAp-CP powder synthesized by the wet precipitation method, in the form of compressed tablets. In the first 7 days of the study, 46 % of the CP was released from the HAp-CP composite, then, the released antibiotic quantity suddenly rose and almost completely delivered up to day 21. This extended release profile may be due to bonds formed between CP and HAp during synthesis [11].

Nanocomposite made from HAp nanoparticles with sodium alginate and polyvinyl alcohol release the antibiotic for about 30 days. The layer-by-layer coating of polymers on the HAp nanoparticles leads to the sustained release of drug [12]. According to the current results, in the first 144 h (6 days) of the study, 49.157 % of the CP was released from the HAp-CP compound, 47.583% from HAp/Ti-CP and 32.962% from HAp/Ti/CaFb-CP. Then, the released antibiotic quantity rose and reached 56.893% for HAp-CP, 65.921% for HAp/Ti-CP and 82.061 % for HAp/Ti/CaFb -CP by 216 h (i.e., day 9). The final amount of CP is released at a slow rate between 288 h (i.e., 12 day) and 336 h (i.e., 14 day), being from approx. 80% to 100%.

The insertion of bone implants with HAp-ceftazidime composites for the treatment of experimentally induced osteomyelitis in rabbits has proven its antibacterial efficacy, removing chronic infection [13]. Other recent studies have performed *in vitro* optimization of antibiotic release from HAp implantable compound – ofloxacin [14].

Although the antimicrobial activity of CP on the germs studied in this experiment is well known, binding of this of HAp compound could alter the antimicrobial activity of the chemically synthesized compound. Thus, in an experimental study, Haijink *et al*. developed yield systems, by impregnating some biodegradable materials used in the treatment of some bone diseases. These biomaterials based on calcium sulphate (Osteoset) demineralized bone (DBX) and HAp-collagen (Collagraft) have been impregnated with a series of antibiotics

(vancomycin, gentamicin) following both the influence on the yield but also on the antimicrobial activity. Several studies [15-18] have shown that antimicrobial activity is altered, being dependent on the nature of the antibiotic but also of the biomaterial. Thus, if gentamicin activity by mixing with DBX is not modified, when mixed with Osteoset and Collagraft the same activity decreases to 60%. From the results of the antibiograms, it is observed that the binding of CP to HAp, HAp/Ti, HAp/Ti/CaFb does not influence the antibacterial activity. Following the development of the antibiograms to determine the MIC, it was established that the 5 μg dose of CP is the smallest amount of antibiotic that causes the inhibition of bacterial growth on the tested germs. This concentration of CP, established in the preliminary testing, was used as a positive control in the subsequent antibiograms.

Analysis of the antibacterial potential of *Staphylococcus aureus* showed the effectiveness of all implants is equal to that of pure CP. It was observed that the binding of CP to HAp does not influence the antibacterial activity, its transfer from bioceramics being easily achieved. HAp showed a low antibacterial effect, the species *Staphylococcus aureus* being classified as resistant to it (14.5 cm) (Table 3).

Table 3. Average mean diameters of bacterial growth inhibition.

Sample		***Staphylococcus aureus***	***Escherichia coli***	***Pseudomonas aeruginosa***
DZI *	*CP (control +)*	28.5	32.6	19.5
	HAp (martor-)	14.5	16.5	8.5
	HAp-CP	28.4	32.2	19.7
	HAp/Ti-CP	28.5	32.3	18.6
	HAp/Ti/CaFb-CP	28.6	33.1	19.5

**DZI- Average value of the diameters of the inhibition zones [mm]*

The data regarding the antimicrobial activity of HAp are quite contradictory. Thus, if in the first studies on the chemical synthesis of HAp there are no references regarding the antibacterial activity of this compound, most articles considering this compound as a negative control, lately the idea that this compound would be active is becoming more frequent. Thus, in a recent study by H.S. Ragab *et al*., it is shown that the nanocrystalline powder of HAp has a pronounced antibacterial effect on *E coli*, causing cell wall destruction. The mechanism of this action is explained by the authors through active oxygen species (OH-, H_2O_2, O_2^-) that would be found on the surface of HAp nanoparticles. Another factor that determines this antibacterial effect would be due to the abrasive surface, characteristic of HAp aggregates, which can cause mechanical damage to the membranes. As observed in the results presented in the table, HAp is characterized by a medium antibacterial effect, consistent with the data from the literature, which shows that HAp nanoparticles have a more pronounced antibacterial effect on the *Escherichia coli* species. The effect can be explained by the relatively thin thickness of the cell wall, characteristic of this bacterial species, but also by the characteristic chemical structure. After performing the antibiogram, it was observed that the diameters of the bacterial growth inhibition zones are approximately equal for the three test implants (Table 3). After measuring the diameter of the inhibition zone produced by CP on the growth of *Pseudomonas aeruginosa* in the culture medium, we observed that the germ is resistant to the action of fluoroquinolone. Compared with the positive control, the tested samples had relatively equal antibacterial efficacy (Table 3) [19].

4. CONCLUSIONS

Chemisorption is a kind of adsorption that involves a physicochemical interaction between the surface and the adsorbate, generated at the adsorbent surface. In the present study, the release of the chemisorbed antibiotic onto the surface of the implant is made relatively quickly, especially due to the adsorption method. However, the simplicity of the chemisorption method in relation to the duration of the release constitutes favorable arguments for the introduction of antibiotic implants, made by the chemisorption method. The period of antibiotic release may be considered as sufficient to support osteointegration under antibacterial protection. The HAp-CP, HAp/Ti-CP, HAp/Ti/CaFb-CP implants exhibited antibacterial efficacy substantially equal to that of pure CP on the tested germs (*Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa*). Following the development of the antibiograms we observed that HAp has medium antibacterial properties *Escherichia coli* being classified as intermediate-sensitive to its action, *Staphylococcus aureus* and *Pseudomonas aeruginosa* being resistant.

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