

Biomimetic Ca-P Coatings Incorporating Bisphosphonates Produced on Starch-Based Degradable Biomaterials

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Abstract: In this study, sodium clodronate, a well-known therapeutic agent from the family of bisphosphonates (BPs), is incorporated in a biomimetic calcium phosphate (CaP) coating, previously formed on the surface of a starch-based biomaterial by a sodium silicate methodology, as a strategy to develop a site-specific drug delivery system for bone tissue regeneration applications. The effects on the resulting CaP coatings were evaluated in terms of morphology, chemistry, and structure. The dissolution of Ca and P from the coating and the release profiles of sodium clodronate was also assessed. As a preliminary approach, this first study also aimed at evaluating the effects of this BP on the viability of a human osteoblastic cell line since there is still little information available on the interaction between BPs and this type of cells. Sodium clodronate was successfully incorporated, at different doses, in the structure of a biomimetic CaP layer previously formed by a sodium silicate process. This type of BPs had a stimulatory effect on osteoblastic activity, particularly at the specific concentration of 0.32 mg/mL. It is foreseen that these coatings can, for instances, be produced on the surface of degradable polymers and then used for regulating the equilibrium on osteoblastic/osteoclastic activity, leading to a controlled regenerative effect at the interface between the biomaterial and bone. © 2009 Wiley Periodicals, Inc. *J Biomed Mater Res Part B: Appl Biomater* 92B: 55–67, 2010

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INTRODUCTION

Although remarkable progresses have been made to produce superior bone-like materials, the right balance between surface and bulk properties is still to be achieved. At present, one of the most interesting solutions to this problem is to use calcium phosphates (CaPs) as coatings on the surface of biomaterials. In addition, due to their osteogenic potential, CaP layers can be used as drug-carrier systems in bone tissue engineering applications.^{1,2} Recently, CaP layers were deposited on the surfaces of metal implants under highly unphysiological conditions. Therefore, it was not possible to coat polymeric substrates or to incorporate a bioactive agent. Plasma spraying, for exam-

ple, is a commercially available technique in which high processing temperatures are used.^{3–7}

At present, it is possible to produce CaP layers under physiological conditions of temperature, pH, and pressure, using the so-called biomimetic process.^{8–15} As a result, there are new opportunities for applying this type of coatings in the field of tissue engineering, as a way to enhance cell adhesion and proliferation and extracellular matrix production. On the other hand, new possibilities for incorporating bioactive molecules, such as synthetic drugs or proteinaceous osteoinductive molecules, are being considered.² Because of the slow, but definite, degradation of CaPs, as well as their high hydrophilicity,¹⁶ they can be very suitable to serve as carriers for these molecules.

Research envisioning CaP coatings as carrier systems for the release of therapeutic molecules is still scarce, but its potential have captured the attention of some groups in the recent years. For example, antibiotic loading into CaP

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coatings on bone-replacement materials is a clinically relevant concept in the setting of total joint arthroplasty and has been addressed recently. Radin et al.¹⁷ have accomplished the loading of vancomycin by immersion of ceramic-coated discs in vancomycin-containing simulated physiological solution. In a study performed by Campbell et al.,¹⁸ chlorhexidine was incorporated into an apatite coating by placing the substrate into various chlorhexidine solutions in between mineralization cycles. CaP coatings containing tobramycin were produced by Stigter et al.¹⁹ on titanium alloy (Ti6Al4V) implants, using a biomimetic approach. Although the options for creating a delivery system in these coatings can be numerous, the affinity of a specific molecule to the CaP crystals as well as its size, charge, or conformation, are crucial features for an effective incorporation. In fact, some of these agents, because of their chemical structure or conformation, can only be adsorbed superficially, on preformed layers. Such superficially adsorbed molecules are sometimes released too rapidly within a biological milieu to be effective in their therapeutic/osteoinductive capacity.

Bisphosphonates (BPs) are an important family of drugs in various bone- and calcium-related pathologies, such as cancer,²⁰ hypercalcemia,²¹ Paget's disease,²² or osteoporosis.^{20–26} The affinity of BPs to bone mineral hydroxyapatite (HA) is the basis for their use as inhibitors of bone resorption.^{25,26} They are pyrophosphate (P—O—P) analogs in which the oxygen in the P—O—P has been replaced by a carbon to yield a P—C—P backbone. Substitutions on the carbon give rise to a large family of compounds with different properties and potencies, which are determined by the nature of the side chains.²⁶ The physicochemical effects of BPs include binding strongly to the CaP crystals and inhibiting their growth, aggregation, and dissolution.^{25–27} Their biological effects in calcium-related disorders are due to the direct interaction with osteoclasts²⁸ and/or osteoblasts.²⁹ It is now generally accepted that BPs inhibit osteoclast activity.^{23–25,29} However, the major drawback of the clinically used BPs is their pharmacokinetics and poor oral absorption from the gastrointestinal tract, which is typically less than 1%.^{25,26} In addition, BPs have been associated with adverse gastrointestinal effects in humans.³⁰ The attempts to improve the bioavailability of BPs had only very limited success because of a lack of marked improvement of adsorption and/or side effects.³⁰ The challenge for novel drug delivery systems is to achieve improved bioavailability and safety. In addition to oral administration, different strategies have been proposed over the last years, such as nasal delivery³¹ and subcutaneous^{32–34} or intramuscular injection.³⁵

In addition to being used in the treatment of the pathologies described earlier, it has been also discovered^{36–38} that BPs can be beneficial when administered after bone replacement, decreasing the primary failure mode of total hip arthroplasty, which is aseptic loosening following peri-implant osteolysis. Nevertheless, this effect could be

improved if the time delay for the BP to reach the bone-implant interface could be reduced. In this case, a localized delivery of the drug could have a favorable effect to produce a more stable and integrated interface between the implant and the bone at the early stage of implantation, when a significant bone loss can occur.³⁹ In fact, this was already attempted with success in several studies using different BPs.^{39–42} For example, Kajiwara et al.⁴⁰ have treated calcium ion-implanted titanium implants with pamidronate, which significantly accelerated new bone formation in a rat tibia. Yoshinari et al.⁴¹ have studied the effect of the same BP incorporated in a plasma-sprayed HA-coated titanium dental implant in beagle mandibular bone, increasing the bone-implant contact area. Recently, Peter et al.⁴² have grafted zoledronate to plasma-sprayed HA coating of titanium implants that were then inserted in rat condyles. A positive concentration-dependent effect was observed on the peri-implant bone density and on different histomorphometric parameters.

Because of its reported effects on bone, one can envision that to incorporate a BP in a bone-like CaP-coated biodegradable scaffold can be a very promising strategy for designing a site-specific drug delivery system in a tissue engineering approach, by locally enhancing tissue integration and regeneration. In this study, sodium clodronate, a well-known BP, was physically incorporated at different doses in a CaP coating, previously formed on the surface of a starch-based biomaterial by a sodium silicate methodology. The effects on the resulting CaP coatings were evaluated in terms of morphology, chemistry, and structure. The release profile of the drug and the stability of the coatings were assessed at different physiological environments. As a preliminary approach, this study also aimed at screening the ideal sodium clodronate dose to be incorporated in the CaP coatings, which is able to elicit positive effects on the viability, growth, and function of a human osteoblastic-like cell line. In fact, there is still little information available on the interaction between BPs and this type of cells.

MATERIALS AND METHODS

Preparation of the Samples

As a natural polymer with rather interesting properties,^{43–45} starch exhibits a great potential to be used in biomedical applications. The studied material is based on a blend of starch and ethylene vinyl alcohol (SEVA-C). The blend contains around 50% starch by weight, while the copolymer discloses a composition of 60/40 mol/mol. Compact samples were produced by injection molding on a Klockner Desma FM-20, using a nozzle temperature of 170°C. All the samples were shaped into the form of disks with a diameter of 10 mm. Further information on the properties of starch-based polymers and its potential for biomedical applications may be found elsewhere.^{43–46}

Sodium Silicate Methodology

To produce the bioactive coatings, a commercially available sodium silicate gel from Sigma-Aldrich ($\text{Na}_2\text{SiO}_3 \cdot \text{H}_2\text{O}$, containing $\sim 14\%$ NaOH and $\sim 27\%$ SiO_2 , pH 13 was used). The sodium silicate gel was used with concentration and $\text{SiO}_2/\text{Na}_2\text{O}$ molar ratio as received.¹⁵ The viscosity of the gel is $6 \times 10^{-2} \text{ Pa}\cdot\text{s}^{-1}$. This high viscosity allowed for the impregnation other than uptaking the gel into the polymer, which would affect the mechanical and degradation behavior of the material. The materials (SEVA-C disks with a diameter of 10 mm) were “impregnated” for 24 h with the gel, in a controlled atmosphere ($23 \pm 1^\circ\text{C}$; 55% RH). After this treatment, the gel with the samples was diluted with 50% vol. of distilled water and stirred for 5 min. This procedure was aimed at diminishing the viscosity of the gel to produce a homogeneous and thin layer of vitrified sodium silicate in the surface of the materials. The samples were then removed from the solution and dried in a controlled atmosphere ($23 \pm 1^\circ\text{C}$; 55% RH).

Apatite Formation

An aqueous solution was prepared comprising all major inorganic ions present in human blood plasma and presenting its approximate ion concentration, to promote *in vitro* apatite formation. The different salts were dissolved in deionized H_2O , and the pH was adjusted to neutral using tris(hydroxymethyl)aminomethane and hydrochloric acid. This simulated body fluid (SBF) solution was developed by Kokubo et al.¹¹

The treated samples were first soaked in SBF to nucleate the CaP layer, and after 7 days, soaked in another solution, with ion concentrations $1.5 \times$ SBF for allowing this layer to grow. The solutions were renewed every 2 days during the coating process, which lasted for 14 days. The average thickness of the CaP layers formed by this methodology is $\sim 2.5 \mu\text{m}$ (measured from the basis of the CaP nuclei to the interface, according to a previously developed method⁴⁷).

Incorporation of Sodium Clodronate

BPs are stable analogs of pyrophosphate in which the P—O—P structure is replaced by a P—C—P structure. Side chains R^1 and R^2 are attached to the geminal carbon atom. The BP used in this study was a sodium clodronate in which R^1 and R^2 are replaced by chloride. The structure of this drug is shown in Figure 1.⁴⁸

Sodium clodronate (dichloromethylene-BP) is one of the best documented BP that is used effectively in medical applications.⁴⁸ In the literature, the ideal concentration of this BP for inducing a positive effect in bone cells varies from study to study. Therefore, we have chosen two lower concentrations that are considered to be in the therapeutic range⁴⁸ and two higher concentrations to scale up the effects of this BP in the structure of the CaP coating so

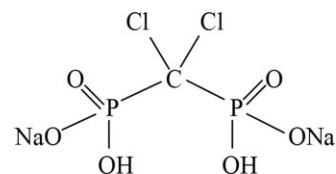


Figure 1. Chemical structure of sodium clodronate.⁴⁸

that could be detectable by infra red and x-ray spectroscopies. Solutions of 0.064, 0.32, 8, and 16 mg/mL of sodium clodronate (Sigma-Aldrich) were prepared with the pH adjusted to 7.4 using NaOH (1 M). Aliquots of 50 μL of each solution were added to the coated disks left for 3 h, followed by drying at 60°C . This procedure has been previously reported elsewhere.⁴⁹ The effective concentration of the BP in the coated samples is approximately as follows: 0.004, 0.02, 0.5, and 1 mg/cm^2 .

Structural and Morphological Characterization

The morphological characterization was performed by scanning electron microscopy (SEM) analysis in a LEICA Cambridge S360, United Kingdom. All the samples were coated with a thin film of carbon, by ion sputtering, before any observation. The electron beam energy was 15 keV. Energy dispersive spectroscopy (EDS) was performed qualitatively in a Rontec spectrometer, to obtain the compositional profiles of the CaP layers incorporating different amounts of sodium clodronate. Fourier transform infrared spectroscopy with attenuated total reflectance (FTIR-ATR) was performed, aiming at detecting any type of chemical modification on the samples chemical structure. The acquisition was performed in transmittance mode from 4000 to 500 cm^{-1} . All spectra were recorded using 64 scans and 2 cm^{-1} resolution in an FTIR spectrophotometer (Perkin-Elmer 1600 Series). Thin-film x-ray diffraction (TF-XRD; Philips X'Pert MPD, The Netherlands) was used to identify the crystalline phases present in the CaP layers formed during the immersion in SBF and after incorporating sodium clodronate. The data collection was performed by 2 θ scan method with 1° as incident beam angle using $\text{CuK}\alpha$ X-ray line and a scan speed of $0.05^\circ/\text{min}$ in 2θ .

Mechanical Behavior

Microhardness tests are usually regarded as a convenient means of investigating the mechanical properties of materials in a localized and nondestructive manner. Microhardness measurements have been used, herein, to study the differences and correlation between the composition and the surface or coatings in different kinds of samples. The motivations of such measurements are to probe the mechanical environment that the cells will feel when in contact with the material, and to evaluate the influence of the presence of the drug in the coating in its

TABLE I. Solutions Used in the Dissolution Studies

Solution No.	Description	pH (at 37°C)
1	Simulated Tris–HCl solution	7.4
2	Acidic buffered solution (simulating osteoclastic activity)	3

mechanical properties. A Leica VMHT30 equipment was used to measure the microhardness of the samples at room temperature ($\sim 20^\circ\text{C}$), using a Vickers diamond pyramid indented (included angle $\alpha = 136^\circ$). The microhardness, H_v , was calculated from the residual projected diagonal impression, using $H_v = 1.854 F/d^2$ equation, where d is the mean diagonal length of the indentation in millimeter and F the applied force in Newton. A load of 9.807 mN was applied during 10 s in all tests. These conditions were chosen for getting measurements only at level of the coating, avoiding the bulk of the substrate samples. Fifteen measurements at randomly selected places around coating of each sample were taken in each condition. The same procedure was followed for the samples' substrate.

Dissolution Behavior

To try to predict the behavior of the CaP coating when facing the biological milieu is of extreme importance. However, to design a method capable of fully replicating it is extremely difficult, considering the complexity of the biological fluids, going from minerals to proteins, enzymes, and cells. *In vitro* dissolution tests, although far from the ideal method, can give some insights on the stability of the materials before implantation. In fact, because these studies are performed in solution, they always simulate the worst case low-end service environment.

Dissolution tests were performed on the coated samples before and after loading with increasing doses of sodium clodronate. Two different media were used in this study, according to the International Standard ISO/DIS 10993-14 procedure and are presented in Table 1.

Solution 1 simulates, in a very simplistic way, the *in vitro* physiological environment. Solution 2 is aimed at reproducing the worse predictable scenario in which the bone is resorbed due to osteoclastic activity. The coated disks, incorporating different amounts of sodium clodronate, were immersed in a volume of 5 mL of each solution. Two replicates of each condition were studied. The experiment was performed at 37°C for periods up to 15 days. Aliquots of 2 mL were removed at different periods and replaced by the same volume of fresh solution. The obtained samples were analyzed by induced coupled plasma emission spectroscopy for the quantification of Ca and P. The final cumulative concentration values were calculated according to the following equation:

$$\sum [V_n - (V_{n-1} * 2/5)]_n \quad (1)$$

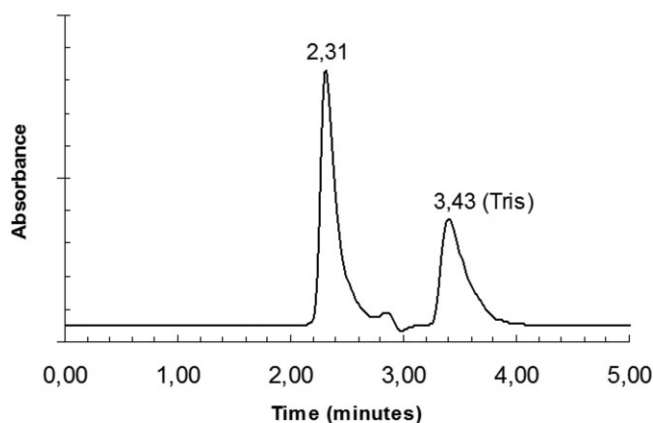
V_n – volume aliquoted;

V_{n-1} – subsequent volume aliquoted;

Sodium Clodronate Release Studies

The release of sodium clodronate from the CaP coatings in the previously described solutions was studied. The analyzed solutions and periods of time were the same in which Ca and P were quantified. The release of the drug was monitored by means of high-permeation liquid chromatography (HPLC). The analysis was performed by using a mixture (60:40) of methanol/aqueous solution of PIC A (a solution of tetrabutylammonium hydrogen sulfate in water, supplied by Waters) as the mobile phase and a flow rate of 1 mL/min. The system consisted of a Perkin–Elmer LC-250 pump, an UV/vis detector Perkin–Elmer LC-95, and a Waters μ Boundapak C-18 column of 3.9×300 mm. Measurements were made at 204 nm. Under these conditions,

a. HPLC profile in buffered solution (pH=7,4)



b. HPLC profile in buffered solution (pH=3)

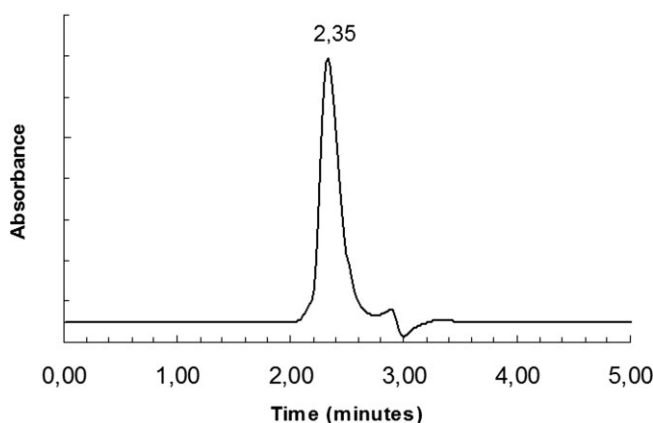


Figure 2. HPLC release profiles of sodium clodronate in (a) a solution buffered to pH 7.4 and (b) a solution buffered to pH 3.

sodium clodronate presented a retention time of 2.31 and 2.35 min in $\text{pH} = 7.4$ and $\text{pH} = 3$, respectively. The obtained profiles for each solution are presented in Figure 2.

Release studies were performed from discs loaded with 8 mg/mL solutions of sodium clodronate, which provided an effective concentration of 0.4 mg of drug per coated disk. Discs prepared with lower concentration of drug (0.32 and 0.064 mg/mL) were not evaluated because they are below the detection limit of the HPLC equipment. The effective concentration loaded was 0.4 mg per coated disk, which is the result of a 50- μL aliquot of a solution with concentration 8 mg/mL. The results were plotted in accordance with Eq. (1).

Preliminary Evaluation of Cell Adhesion and Proliferation

To study the morphology, attachment, and proliferation of osteoblast-like cells over precalcified loaded SEVA-C surfaces, a human osteoblast-like cell line (SaOs-2) obtained from the European Collection of Cell Cultures was selected. Dulbecco's modified eagle's medium (Sigma), supplemented with 10% fetal bovine serum (FBS, Biochrome, Germany) and 1% antibiotic/antimycotic solution (Sigma) was used as cell incubation medium, and growth to confluence in controlled atmosphere conditions (37°C, 5% CO_2 , 100% humidity). Detachment of confluent low passage cells was performed using 0.25% trypsin/EDTA solution (Sigma). Subsequently, osteoblast-like cells were cultured over samples in a concentration of 3.3×10^4 cell/mL and incubated for 3, 5, and 7 days in controlled atmospheric conditions. Tissue culture grade polystyrene

was used as control and replicates were prepared. After each incubation period, samples were washed with phosphate-buffered saline (Sigma) solution and fixed in glutaraldehyde 2.5% (v/v) for observation by means of scanning electronic microscopy. After fixation, samples were dehydrated in crescent ethanol concentrations (50, 70, 90, and 100%), air dried, and sputter coated with gold.

Cell viability was measured through the MTS test, using the CellTiter 96[®] AQueous One Solution from Promega, which is based on bioreduction of a substrate (MTS) into a brown product by dehydrogenase enzymes in metabolically active cells, and it is commonly used for cell viability evaluation. Cells were then incubated for 3 h at 37°C in a humidified atmosphere containing 5% CO_2 . At this time, optical density (OD) was measured with a plate reader at 490 nm. The mean OD value obtained for the positive control (cell seeded on culture plate without any coating) was considered as 100% metabolic activity.

RESULTS

Morphology, Crystallinity, and Chemical Structure of the CaP Coatings

The effect of the incorporation of different doses of sodium clodronate on the morphology, crystallinity, and chemical structure of the biomimetic coatings was evaluated using SEM/EDS, TF-XRD, and FTIR-ATR techniques. In Figure 3 is presented the morphology of the CaP coatings grown on the surface of SEVA-C samples before and after a treatment with sodium clodronate at 8 mg/mL. The

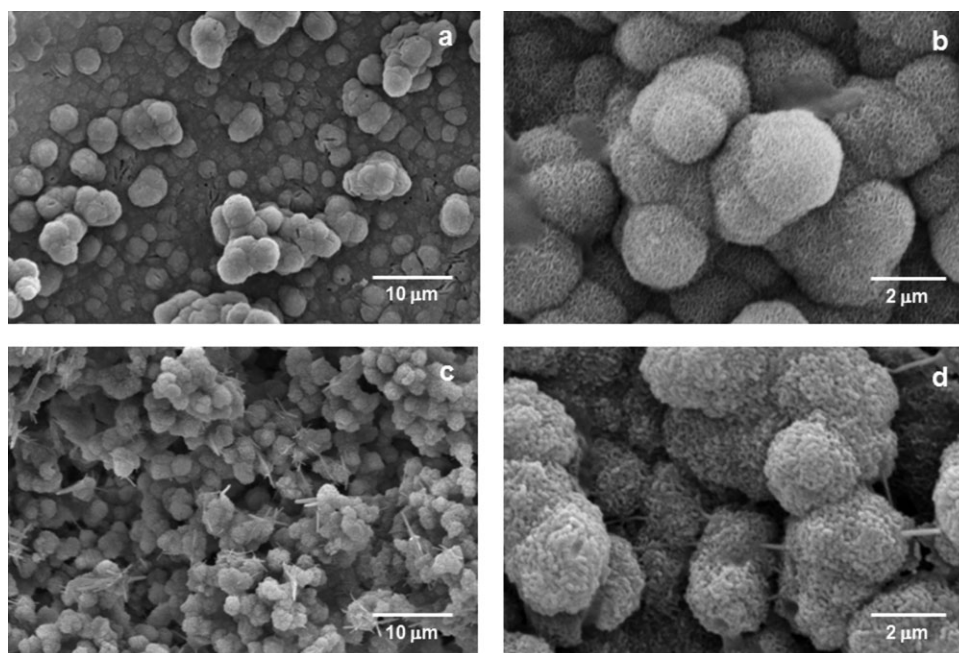


Figure 3. SEM micrographs performed on the apatite coatings grown on the surface of SEVA-C samples (a,b) before and (c,d) after the treatment with 8 mg/mL of sodium clodronate.

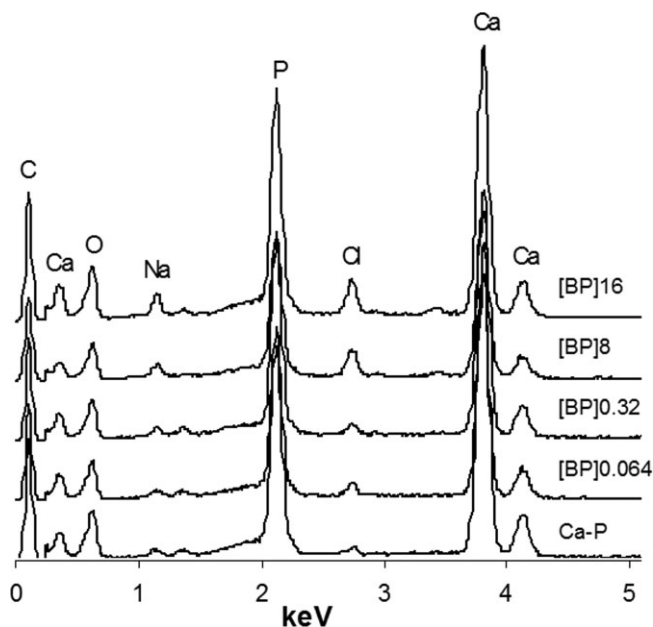


Figure 4. EDS spectra showing the evolution of the elemental composition of the apatite layers with increasing doses of sodium clodronate.

corresponding EDS spectra showing the evolution of the elemental composition are presented in Figure 4.

The use of sodium silicate biomimetic coating methodology resulted in the formation of a CaP layer on the surface of SEVA-C after 15 days in SBF, exhibiting a nanocrystalline needle-like structure, as presented in Figure 3(a, b). After incorporating the lowest concentrations studied of sodium clodronate, that is, 0.064 and 0.32 mg/mL, the CaP crystallites did not present any observable modification in their morphology. Therefore, the micrographs presented in Figure 3(a,b) are representative of the obtained morphologies in these cases. When incorporating the highest studied concentrations of the BP (8 and 16 mg/mL), well-defined spicule crystallites started to grow on the surface of the CaP layer, as it can be visible by examining Figure 3(c,d), for the case where a solution containing 8 mg/mL was used. By performing localized EDS, it was possible to confirm that the elemental composition of these structures exhibited a high content in Cl, which is assigned to sodium clodronate (data not shown). On the other hand, by observing the surface of the CaP layer in greater detail [Figure 3(d)], it is also possible to find a modification in its morphology. The resulting CaP crystals present a more “worm-like” morphology when comparing with the original “needle-like” shape [Figure 3(b)]. This modification can be attributed to a partial dissolution at the surface of the CaP layer in the presence of the solutions containing high concentrations of sodium clodronate (by calcium depletion) and further reprecipitation under these new conditions, which differ from those present in SBF in terms of composition, ionic strength, and buffer capability. On the other hand, because of its high affinity for Ca compounds

(especially to CaPs^{25,26}), the chemical incorporation of the drug onto the CaP structure, to a certain extent, is also predictable, causing dimensional rearrangements.

In Figure 5 is plotted the FTIR spectra performed on the surface of SEVA-C before and after growing an CaP layer and after incorporating the highest doses studied of sodium clodronate. Again, by this technique, it was not possible to detect the presence of BP in the samples treated with the lowest concentrations of the drug. The IR spectrum of sodium clodronate powder is also presented as a reference for identifying its characteristic peaks.

SEVA-C presents —C—O—C— bands at 1015–1140 cm^{-1} , which are characteristic of the glycosidic bonds from starch.^{50,51} After coating the biomimetic layer, it is possible to detect the typical bands of CaPs. The bands at 864, 1406, and 1434 cm^{-1} result from the presence of carbonate (CO_3^{2-}) chemically incorporated in the CaP lattice.⁵² These bands clearly indicate that a carbonated CaP was formed, as it is found in bone apatite and as reported for other biomimetic coating methods.^{13,53} The bands at 578 and 598 are assigned to the ν_4 bending mode of the O—P—O bonds in the apatite, indicating some degree of crystallinity. The band at 1012 cm^{-1} indicates the ν_3 stretching mode of P—O bonds.⁵² When sodium clodronate is present, besides the major P—O bond attributed to the ν_3 vibration of — PO_4 , additional bands attributed to the same

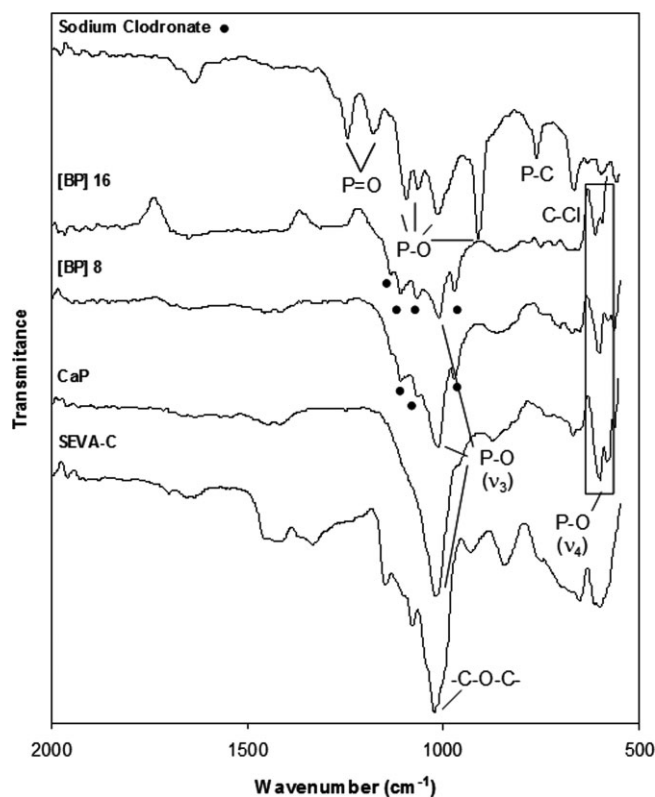


Figure 5. FTIR-ATR spectra of SEVA-C before and after forming an apatite layer and after the incorporation of 8 and 16 mg/mL of sodium clodronate. Sodium clodronate powder was used as reference.

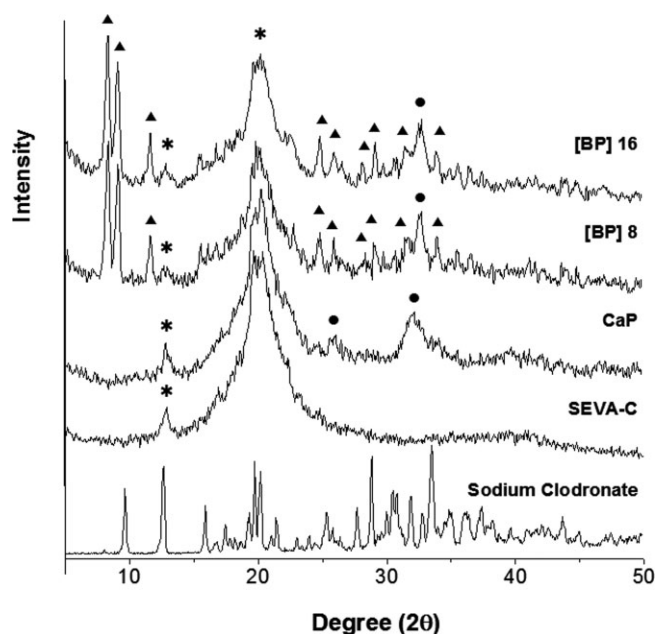


Figure 6. TF-XRD spectra of SEVA-C before and after forming an apatite layer and after the incorporation of 8 and 16 mg/mL of sodium clodronate. Sodium clodronate powder was used as reference. * SEVA-C; ● Apatite; ▲ Sodium clodronate.

type of bonds are detected at 968, 1060, 1102, and 1122 cm^{-1} . The crystallites newly formed on the CaP layer and detected by SEM can then be assigned to the precipitation of the sodium clodronate.

Figure 6 presents the typical XRD patterns for the surface of SEVA-C samples before and after growing the CaP layers and for the same layers after incorporating sodium clodronate at the highest studied doses. The profile of pure sodium clodronate powder was also acquired and is, herein, presented as a reference pattern.

SEVA-C diffraction profile presents two characteristic broad peaks, detected at $2\theta = 12^\circ$ and $2\theta = 20^\circ$, assigned to its crystalline phases.⁵¹ After growing the biomimetic layers on its surface, it is possible to detect the two main characteristic peaks attributed to HA (JCPDS card 9-432). The peak at $2\theta = 26^\circ$ is assigned to (002) plane in the CaP crystalline lattice. Another important diffraction peak is detected around $2\theta = 32^\circ$ and is related to the overlapping of planes (211), (112), and (300). This broad peak indicates that the CaP structure is composed by small crystallites. Similar XRD profile was obtained after adding sodium clodronate at 0.064 and 0.32 mg/mL to the coatings. In this way, XRD technique was not suitable for detecting such low amounts of the drug in the coating. However, when incorporating the drug at concentrations of 8 and 16 mg/mL, several other strong reflections can be detected, as presented in Figure 5. This indicates the presence of a new crystalline phase. These peaks can be well correlated with the diffraction pattern of the drug. Nevertheless, differences between both spectra can be detected, such as the appearance of an intense peak at $2\theta = 8^\circ$ and a shift left of the

majority of the diffraction peaks. This result indicates that sodium clodronate crystals have suffered a dimensional rearrangement during crystallization at the surface of the coating.

Microhardness

Figure 7 shows the microhardness of SEVA-C surface and of the coatings as function of the increasing concentrations of sodium clodronate. For the concentrations of 8 and 16 mg/mL, it was not possible to obtain consistent results because of the high variability of roughness caused by the sodium clodronate crystal spicules at the surface of the CaP coating [as presented in Figure 3(c,d)].

After coating SEVA-C samples with the biomimetic CaP layer, the microhardness increased from 12 to around 15 MPa. When incorporating sodium clodronate at concentrations from 0.064 to 0.32 mg/mL, an increase is observed to values up to 22 MPa. In this way, the microhardness increases significantly with increasing concentration, indicating that the drug will influence the mechanical features of the coating. Sodium clodronate in the form of a salt easily precipitated in the surface and in the pores of the CaP coating, contributing to the structure reinforcement.

Dissolution of Ca and P

It is important to investigate the solubility of the CaP layers because they will dictate the release profile of the drug incorporated into it. In Figure 8 are plotted the dissolution profiles of Ca and P from the studied CaP coatings immersed in the different media: a solution buffered to pH 7.4 and another buffered to pH 3. This last solution

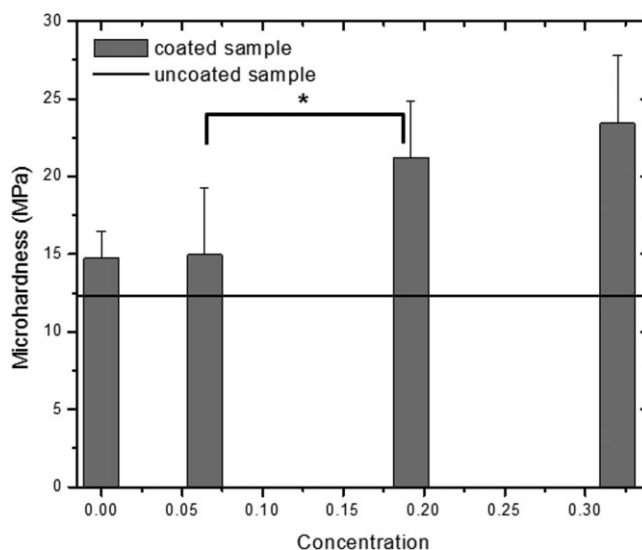


Figure 7. Microhardness of the samples with different concentrations of sodium clodronate in the CaP coatings. The continuous line represents the average corresponding to the microhardness at the level of the bulk substrate (uncoated sample).

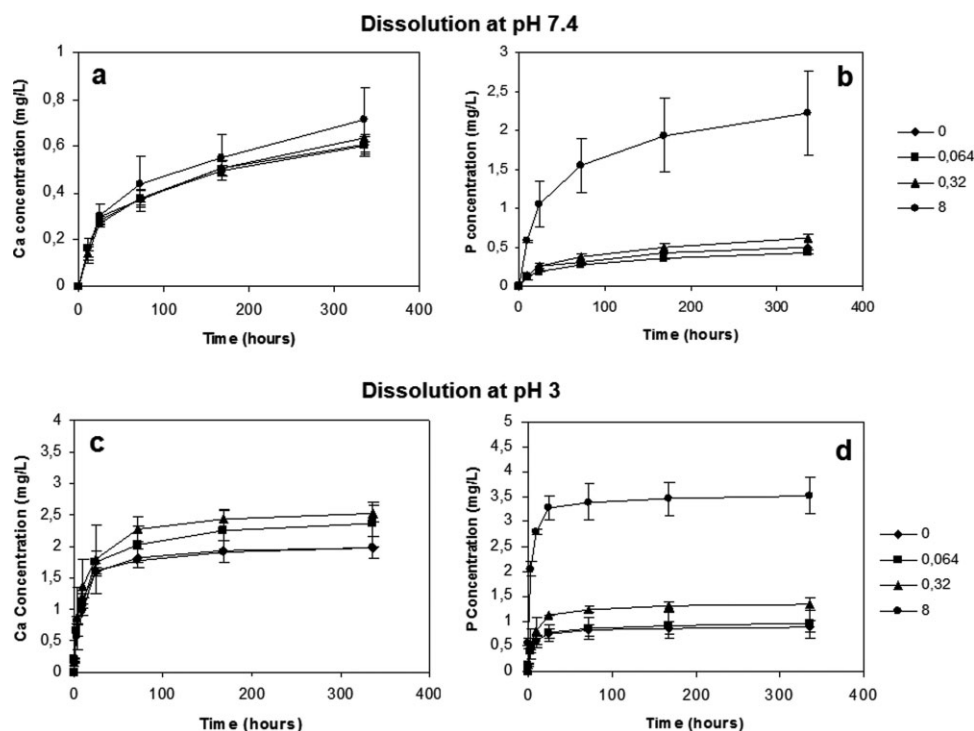


Figure 8. Dissolution profile of Ca and P in standard solutions buffered at (a,b) at pH 7.4 and (c,d) at pH 3.

replicates the worst *in vivo* circumstance resulting from the osteoclasts' activity.

When analyzing the dissolution profiles of Ca and P from the CaP coatings, one can observe that for the studied solutions, different dissolution kinetics are observed. The dissolution rates depend heavily on the pH because CaPs are more soluble in acidic than in neutral conditions.^{27,54} As expected, when comparing the two dissolution media, the CaP coatings dissolve at a higher rate when immersed in the acidic solution, at pH 3. Nevertheless, the dissolution characteristics of CaPs are governed by a large number of other factors that are related either with the *in vitro* solution environment or with the properties of the material. In addition to the pH, the environmental factors include the type and concentration of the buffered or unbuffered solutions, the degree of saturation, ionic strength, and so forth; the material properties include chemical composition, crystallinity, crystal particle size, density, and so forth.⁵⁵

By comparing the dissolution profiles of Ca at pH 7.4 [Figure 8(a)], it is possible to observe that the trend of the curves is quite similar, independent of the amount of drug present in the CaP coating. Because the dissolution kinetics was not significantly altered with the presence of the drug, it means that the coating stability was not compromised. When concerning the dissolution profile of P at pH 7.4 [Figure 8(b)] this trend is also present, with exception to the case where 8 mg/mL of sodium clodronate was incorporated in the coating. In this case, the amount of P detected results not only from the dissolution of the CaP coating but also from the release of sodium clodronate, which has contributed significantly to the total P measured. The dissolu-

tion rate of Ca and P is higher during the first 24 hours. This can be correlated with nature of the CaP coatings. In a dissolution study by Zhang et al.,⁵⁶ using the same Tris-HCl solution, it has been clearly demonstrated that at an early stage of the dissolution, the process is governed by the morphology, crystallinity, and crystal size of the CaP layers. Because the CaP coatings are constituted by small crystallites, there will be a high surface contact area with the solution (the specific area), which will favor the dissolution process. With time, the dissolution rate decreases and other factors became rather important, as for example, the ionic product of the surrounding medium. The increase of dissolved Ca and P in solution will favor some degree of reprecipitation, which can also contribute to the decrease in the dissolution rate.

The dissolution profiles of Ca and P at pH 3 [Figure 8(c,d)] indicate that after 24 hours, the CaP coatings were completely dissolved. Furthermore, in this case, the dissolution kinetics was not significantly altered with the presence of sodium clodronate, which means that the coating initial stability was preserved. Again, in case of the coatings incorporating 8 mg/mL of sodium clodronate, the released amount of P was higher.

Sodium Clodronate Release Profiles

The release profiles of sodium clodronate, incorporated at a concentration of 8 mg/mL, in the two studied solutions are plotted in Figure 9. The release profiles at concentrations lower than this value were below the HPLC detection limit.

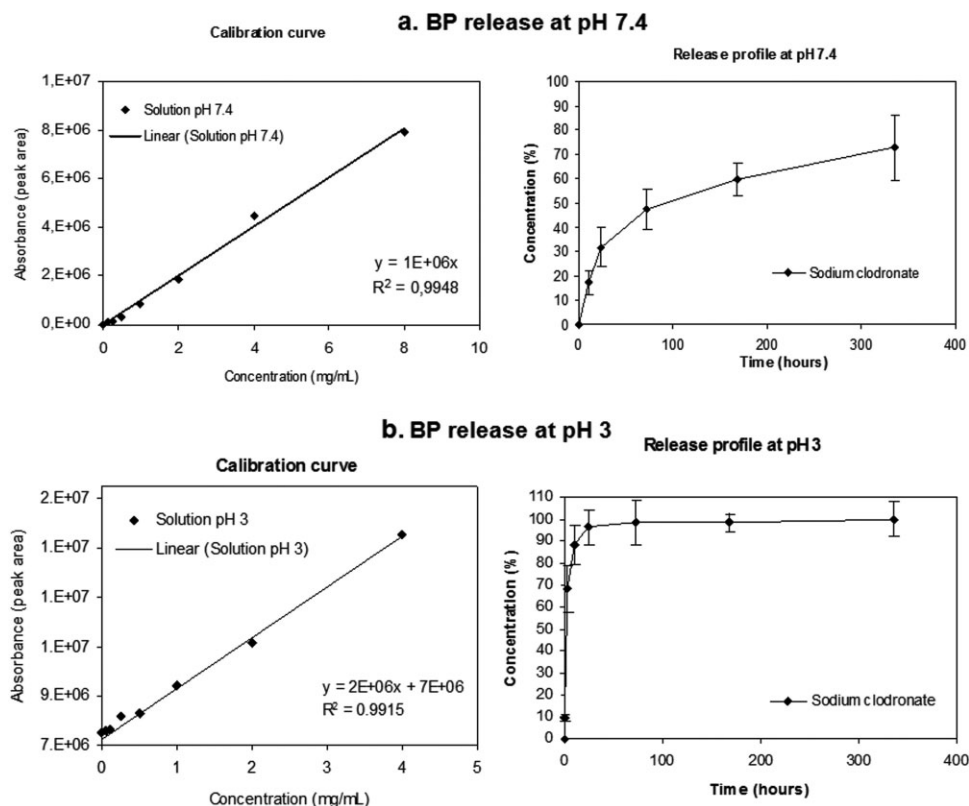


Figure 9. Calibration curves of sodium clodronate and respective release profiles in (a) a solution buffered at pH 7.4 and (b) a solution buffered at pH 3.

The release profile of sodium clodronate obtained in the solution buffered at pH 7.4 [Figure 9(a,b)] follows the same trend of the P release presented in Figure 8(b). Around 30% of the drug is released in the first 24 hours. After 14 days, 70 % of the drug was released, the remainder being incorporated in the structure of the CaP layer, possibly due to a reprecipitation phenomenon.⁵⁶

The sodium clodronate release profile at pH 3 [Figure 9(c,d)] indicates that the entire drug is released in the first 24 hours of the experiment. Again, this result is consistent with the correspondent dissolution profile of P presented in Figure 8(d). At this time period, all the CaP is completely dissolved. The drastic effect of this solution simulates the osteoclastic digestion, which, *in vivo*, would be characterized by the release of citric acid in the surface of the coating and the formation of the so-called resorption pits.⁵⁷ In this extreme situation, the stability of the CaP is very low, leading to total dissolution. Further *in vitro* osteoclasts studied will help to determine the stability of the CaP coatings in the presence of these cells.

Preliminary Evaluation of the Cell Adhesion and Proliferation

Figure 10 presents SEM micrographs of the morphology of the osteoblasts after 3 days of seeding as function of

time and concentration of sodium clodronate. In Figure 11 are plotted the results concerning the cell viability (MTS) for the different times and concentrations of BP studied.

The incorporation of sodium clodronate on the CaP coating had a significant influence on the adhesion of the cells onto its surface. This can be clearly observed in the example presented in Figure 10 after 3 days of culture, when 0.064 and 0.32 mg/mL were present in the CaP coating. With the presence of the BP incorporated in the CaP coating at these doses, the cells presented a more flatten morphology, reflecting higher number of adhesion points to the surface. This behavior indicates a superior affinity to this type of surface compared with the cells that adhered to the CaP-coated surfaces but without the BP. For the higher concentrations of drug used, that is, 8 and 16 mg/mL, a decrease in the cell number was observed, and the cells presented a more round morphology (data not shown), indicating that at these levels sodium clodronate induced a toxic behavior.

Based on the MTS assay (Figure 11), BP stimulated cellular proliferation, and this effect was strongly dependent on the dose and on the coating type. The effect of the drug followed a sigmoid curve, causing inhibition of cellular activity at the highest dose studied and peaked at 0.32 mg/mL. Along the time, it was also observed that a sigmoid curve peaked at day 5 and decreased at day 7.

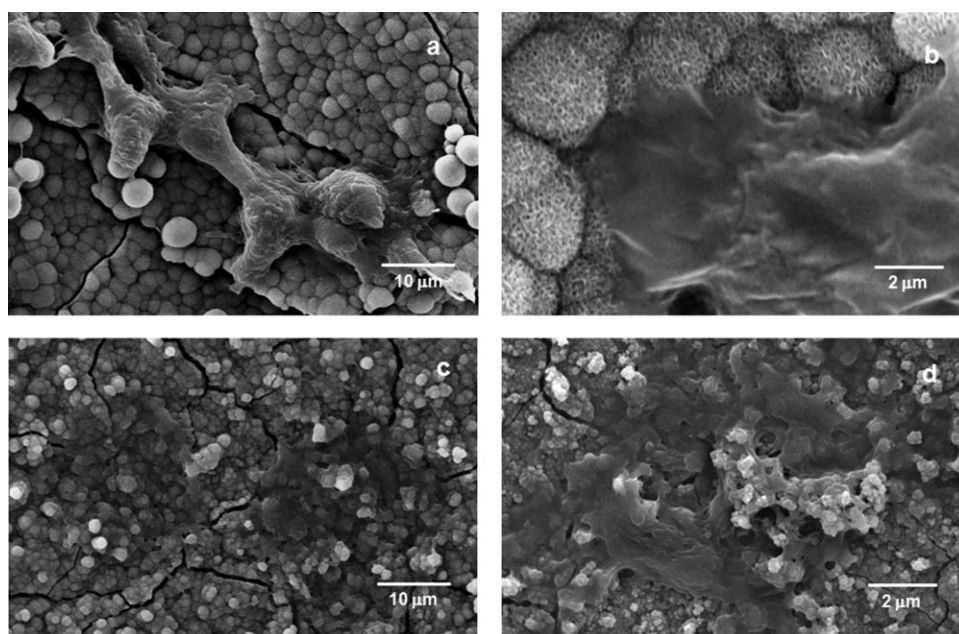


Figure 10. SEM micrographs osteoblast-like cells (SaOs-2) after 3 days in culture of showing the adhesion onto the surfaces of the apatite coatings: (a,b) without incorporation of sodium clodronate, and after incorporation of sodium clodronate at (c) 0.064 mg/mL and (d) 0.32 mg/mL.

DISCUSSION

Sodium clodronate could be successfully incorporated, at different doses, in the structure of a biomimetic CaP layer formed by a sodium silicate process. This initial study was aimed at evaluating the effects of this BP (i) on the structure and *in vitro* stability of the CaP layers; and (ii) on the growth and function of a human osteoblast cell line, because there is still little information available on the interaction between BPs and this type of cells. In addition to defining the minimum dose to be incorporated in the CaP coating, it was also possible to detect a stimulatory effect of the osteoblastic activity when the ideal dose was incorporated.

The properties and composition of the produced biomimetic CaP layers were tailored in previous studies^{15,58} and have dictated the type of interaction with the drug and its loading efficiency. In fact, the choice of the adequate CaP to be used as a carrier for the drug is an important aspect for an effective incorporation. The processing route for obtaining the CaP is also a relevant aspect. Although known to be stable under most chemical conditions, BPs decompose at relatively low temperatures (200°C),⁵⁹ which makes most coating techniques, as for example plasma-spray or sol-gel, inapplicable for coprecipitation. In these cases, a postloading methodology has been an adopted solution,^{60–62} which showed limited success. Because of the more crystalline and dense nature of the CaP coatings, the BP could only be incorporated at the outermost surface. Some authors^{62,63} have tried to overcome this problem by coprecipitating carbonated CaP layers with the BP over their highly crystalline CaP substrates. In a recent study,

Peter et al.⁶³ proposes to coprecipitate pamidronate with a CaP coating from an SBF solution onto a preformed plasma-sprayed coating. The efficiency of this method was, however, limited. Plasma-sprayed HA presents a less reactive surface and usually a more compact structure when comparing with the currently proposed coating technique. Therefore, the drug was only incorporated in the layer of carbonated CaP, which was more reactive to the drug. In a study performed by Seshima et al.⁶⁰ it was demonstrated that the porosity (specific surface area) and crystal size of a CaP have a profound influence in its solubility and consequently in the ability to incorporate/release the BPs. CaPs with lower crystal size and higher specific surface area will present a higher solubility as well as a higher ability for incorporating the BP. This effect was also experienced by

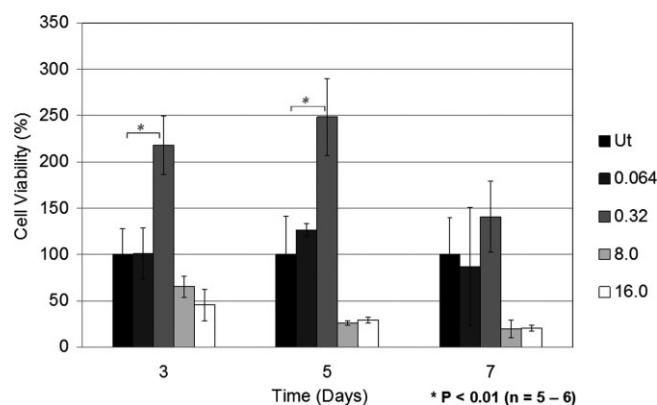


Figure 11. Quantification of the cell viability after different periods of culture on apatite coatings as function of sodium clodronate content.

Josse et al.⁶¹ when incorporating the BP zoledronate onto different CaP compounds. By using mainly ³¹P NMR spectroscopy, two different association modes have been observed, according to the nature of the CaP support and/or the initial concentration of the zoledronate solution. β -Tricalcium phosphate (β -TCP) and mixtures of HA and β -TCP seem to promote zoledronate-containing crystal formation. On the other hand, at low concentrations, calcium-deficient CaPs seem to undergo chemisorption of the drug through a surface adsorption process, due to PO₃ for PO₄ exchange.

In our study, we were able to promote the interaction of different amounts of sodium clodronate with a biomimetic CaP layer because of its partially amorphous and nanocrystalline nature. It was possible to detect the presence of this drug by SEM/EDS, TF-XRD, and FTIR analysis when incorporated at concentrations of 8 and 16 mg/L. However, for these high concentration levels, the majority of the drug detected resulted from the precipitation of crystal spicules of the drug at the surface of the CaP layer during drying. Nevertheless, at higher magnifications, it is possible to detect a modification of the CaP nanocrystalline structure, indicating that a structural rearrangement has occurred probably due to a dissolution and reprecipitation phenomena in the presence of the drug.⁵⁶ However, the extent of this modification did not significantly alter the overall structural stability of the CaP coatings, as it can be clearly indicated by the similar dissolution profiles at pHs 7.4 and 3. When 8 mg/mL of BP is incorporated in the CaP coating, after 14 days of release, around 30% of the drug remained in its structure, indicating that a chemical interaction is most certainly occurring because, like other BPs, sodium clodronate has a great affinity to Ca ions by forming complexes.^{25,26,64} The type of interactions between this drug and CaP layer will be further investigated by NMR studies.

At lower concentrations (0.064 and 0.32 mg/mL), sodium clodronate was not detected by the techniques mentioned earlier. Nevertheless, it was able to influence the mechanical properties of the coating toward an increase of the microhardness of the surface. It can be extrapolated that the presence of the drug at low amounts will influence the mechanical environment that the cells will feel when in contact with the material together with the new chemical environment. In fact, sodium clodronate incorporated on the CaP has influenced cellular proliferation. This effect was strongly dependent on the dose incorporated, being 0.32 mg/mL of BP the ideal concentration for enhancing cell viability.

Ganguli et al.⁶⁵ demonstrated that the synthesis of DNA, protein, and collagen was enhanced in osteoblasts when cultured on CaP surfaces coated with clodronates or pamidronates, proving to have a potent proliferative effect on osteoblasts. Our preliminary studies with SaOs-2 cells seem to indicate a similar response to BPs in terms of cell proliferation. Without minimizing the influ-

ence of other factors, these results, although still nonconclusive, presented a good reproducibility and a good correlation between the presence of sodium clodronate on the CaP coatings and cell morphology (Figure 10) and viability (Figure 11). The stimulatory effect of this drug, together with the potential for inhibiting bone resorption via osteoclasts, without altering the structure of the CaP, can make this approach very effective for improving implant–bone integration, and moreover, in applications where regeneration of the tissue is required, as in tissue engineering TE applications. This preliminary results encourage further studies, namely to test the osteoclastic activity, that will be performed in the near future. Furthermore, because of the physiological working conditions (T, pH) of this biomimetic methodology, new design strategies are currently being considered with the aim of coprecipitating this drug at an early stage of the coating process to obtain BP-containing layers for long-term controlled release. It is foreseen that the coatings incorporating their therapeutic agent can, for instances, be produced on degradable polymeric carriers for the treatment of specific bone diseases regulating the equilibrium on osteoblastic/osteoclastic activity in the direction of a regenerative effect.

CONCLUSIONS

Sodium clodronate could be successfully incorporated, at different doses, in the structure of a biomimetic CaP layer formed by a sodium silicate process. At lower concentrations, sodium clodronate was able to influence the mechanical properties of the coating towards an increase of the microhardness of the surface. The presence of the drug in low amounts has influenced the mechanical environment that the cells feel when in contact with the material together with the new chemical environment. This effect was strongly dependent on the dose incorporated, being 0.32 mg/mL of BP the ideal concentration for enhancing cell viability and osteoblastic profile. This stimulatory effect, together with the potential for inhibiting bone resorption, without altering the structure of the CaP, can make this approach very effective for improving implant–bone integration, and moreover, in applications where regeneration of the tissue is required, as in tissue engineering TE applications.

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