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Release of DL- leucine by biomaterials: Apatitic calcium phosphates analogous to bone mineral

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Abstract
The interaction of DL-leucine, an essential amino acid having a hydrophobic character with poorly crystalline calcium phosphate apatites, synthesized at the physiological temperature (37 °C), is studied. These apatites of atomic ratio Ca/P between 1.33 and 1.67 are analogous to bone mineral. Their surface reactivity is indeed linked to the existence of a metastable hydrated surface layer containing labile ionic species. This study shows that the kinetics of release is slow and experimental release profiles corresponding to these phosphates are similar. The chemical composition of the apatites has an influence on the release. The release rate is more important for apatite containing more HPO_4^{2-} ions. It is found that the incubation of apatite after fixation of the amino acid DL-leucine in the de-ionized water solution depends on the R (ml/mg) = volume/mass. The adsorption and release properties of poorly crystalline apatites are essentially dependent on the maturation, the chemical composition of the hydrated layer and the composition of the adsorbate solution. The understanding of adsorption and desorption mechanisms with respect to active molecules can be exploited for the development of drug delivery applications.

Keywords: Release, Adsorption, DL-leucine, Apatitic calcium phosphate.

1. Introduction
Apatitic calcium phosphate has developed considerably in the last few years essentially as bone filling and bone reinforcement biomaterials [1-5].These materials have found various applications especially as bone substitutes because their chemical composition similar to that of the bone [6-11] to their high biocompatibility [12-14] and have been used as drug delivery systems [15-18]. The behaviour of these biomaterials in biological environments is crucial for their use in vivo [19-21]. To date, the biological behaviour of apatitic calcium phosphate has been studied through in vitro tests in Ringer and/or simulated body fluid solutions, with inorganic ion composition and pH similar to biological fluids [22–24]. The high specific surface area of these poorly crystalline apatites allows the high reactivity to the biological medium (table 1). The phenomenon of evolution and maturation of apatites in aqueous suspension [25-28] can be confounded with the reactions of dissolution of these apatites [29-30].The binding and release of active molecules and ions at the interface between calcium phosphate minerals and biological environments are crucial to determine how they perform in vivo [31]. Recently, calcium phosphates are very much studied in the adsorption of proteins [32-35], amino acids [36-37], textile dye [38], fluorides [39]. It is especially important to cellular response studies [40-41]. Other studies on the loading and release of various bioactive molecules in calcium phosphates [42–50] have shown that the release was strongly dependent on the porosity and permeability of the calcium phosphate material. Factors such as solubility, binding capacity and net charge of the bioactive molecules determine the release rate [51-53].

The release properties of DL-leucine by poorly crystalline calcium phosphate apatites, have previously fixed the molecules of this amino acid and the effect of R(ml/mg)= volume/mass are studied. All experiments were performed under near-physiological conditions in the body (temperature 7 ± 0.1°C, solution pH = 7.0 of aqueous media).

2. Materials and methods

2.1. Adsorbents

Apatites used in this study were obtained by precipitation in a water-ethanol (50% ethanol by volume) as previously described [54]. They were prepared by mixing a solution of calcium [A] and a solution containing varying amounts of phosphate and ammonium carbonate [B]. The solution A (30mmol of nitrate calcium Ca $(NO3)_2 \cdot 4H_2O$ in 100 ml of de-ionized water and 100 ml of ethanol (95%)) was immediately poured at 37°C into a solution B (30mmol of diammoniumhydrogenphosphate $(NH4)_2HPO_4$ and ammonium carbonate $(NH4)_2CO_3$ in 250 ml of de-ionized water and 45 ml of a solution of ammonia (d = 0.92) and 295 ml of ethanol (95%)). The precipitates were filtered and washed with a basic solution (180 ml of de-ionized water, 30 ml of ammonia and 210 ml of ethanol) and then dried at 80 °C for 24 hours.

The precipitates were characterized by chemical and physical analysis. The calcium content was determined by complexometry with EDTA, the phosphate ion content by spectrophotometry of phospho-vanado-molybdic acid and carbonate ion by volumetric. The specific surface area was determined according to the Brunauer, Emett and Teller (BET) method using N_2 adsorption (Table 1). Infrared spectroscopy IR was carried out after dispersion of anhydrous KBr (about 1, 5 mg product to 300 mg of KBr) using TENSOR 27 spectrophotometer (Bruker Optics Germany) (Fig. 1). Scanning electron microscopy (JSM-6060LV, JEOL Ltd, Japan) was used to create 3-dimensional images of surfaces (Fig. 2).

These calcium-phosphates, which are defined by a ratio R = $CO_3^{2-}/(CO_3^{2-}+ PO_4^{3-})$ between 0 and 0.15 ($1.33 \leq Ca / P \leq 1.67$), are poorly crystalline apatites [54], deficient in calcium ions in which carbonate ions CO_3^{2-} substitute for HPO_4^{2-} ions [54] of the formula:

$$\mathbf{Ca_{(8+0.57b)}\,[PO_4]_{(3.5+1.64b)}\,[HPO_4]_{2.5\,(1-b)}\,[CO_3]_{0.86b}\,OH_{0.5\,(1-b)}} \quad \mathbf{with\ 0 \leq b \leq 1}$$

For Ca/P = 1.33 (R = 0), the apatite (not containing carbonate ions) is called the phosphate apatitic octocalcium (OCPa) of formula: $\mathbf{Ca_8\,[PO_4]_{3.5}\,[HPO_4]_{2.5}\,OH_{0.5}}$

Table 1. Characteristics of phosphates used

Ca/P	1.33	1.40	1.48	1.67
$R= CO_3^{2-}/(CO_3^{2-}+PO_4^{3-})$	0,000	0,034	0,068	0,150
Specific surface (m^2/g)	58	89	95	108

Previous studies [54] showed that these phosphates are poorly crystalline apatites, the crystallisation degrades progressively when the ratio R= $CO_3^{2-}/ (CO_3^{2-}+PO_4^{3-})$ increases.

The infrared absorption spectra of these precipitates (Fig. 1), confirms the existence of various bands which vary in accordance with the value of the atomic ratio R: the peaks at 472,565 and 603 cm^{-1} are due to P-O bending vibration, the peaks at 962 and 1038 cm^{-1} are due to P-O stretching vibration. The peak at 875 cm^{-1} was assigned to the HPO_4^{2-} and CO_3^{2-} common ions, which is slightly more intense for most carbonated apatite (Ca/P=1.67). The bands at 634 and 3571 cm^{-1} belong to the stretching vibrations of hydroxyl OH. The band observed at1093 cm^{-1} is due to the phosphate stretching vibration. The infrared peaks at 3000–3430 cm^{-1} are due to the adsorption water. Moreover, the band at 1638 cm^{-1} is due to symmetric vibration of water molecules. Our previous studies [36] confirm by SEM micrographs that the morphology of precipitated apatites (Fig. 2) was flat and porous, which can confer to these apatites interesting biological properties when placed in contact with a cellular medium [55]. The agglomeration of particles decreases when the ratio Ca/P increases, this results in the increase of specific surface area. This latter changed from 58 m^2/g for OCPa (Fig. 2 (a)) to 95 m^2/g for carbonated apatite (Fig. 2 (b)) [36].

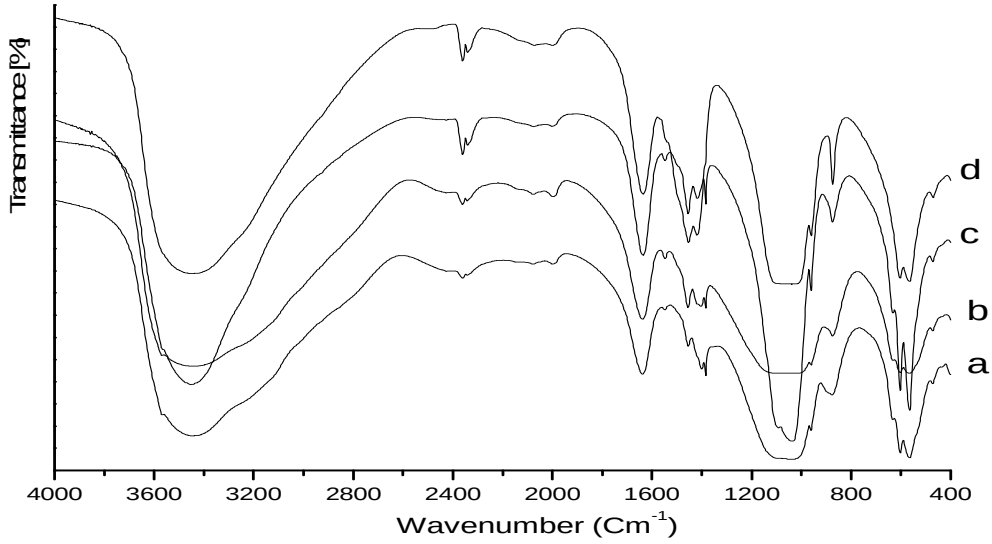
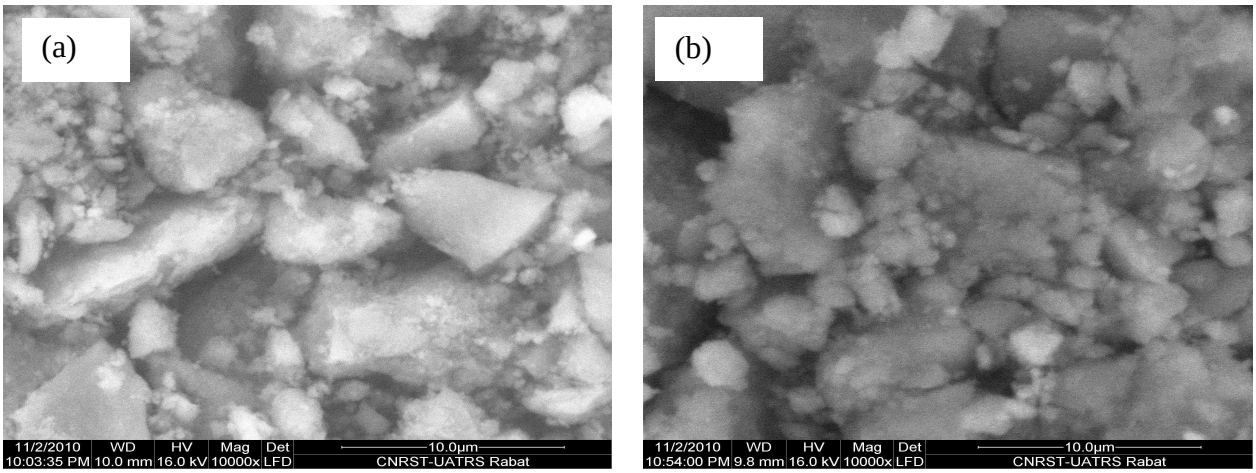


Fig. 1. Infrared absorption spectra of phosphates: (a) Ca/P=1.33, (b) Ca/P=1.40, (C) Ca/P=1.48, (d) Ca/P=1.67

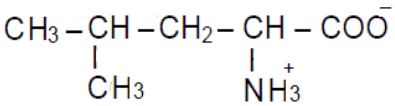


Ca/P=1.33 Ca/P=1.48

Fig. 2. Scanning electron micrograph of the calcium phosphates (a) Ca/P=1.33 and (b) Ca/P=1.48

2.2. Adsorbate

DL-leucine or 2-amino-4-methylpentanoic, of the chemical formula $C_6H_{13}NO_2$, is an essential amino acid-like branched, very hydrophobic and no-polar, higher homologue of valine. It represents about 8% of the amino acids of proteins in the human body. DL-leucine helps regulate the blood sugar, growth and building of muscle tissue (bone, muscle, skin) and the production of hormone growth [56-58]. DL-leucine can also stimulate the release of insulin and help to stabilize or reduce blood sugar. It is slightly soluble in water (24.26 g/l at 20 °C). In solution, it is under zwitterionic form, its pH isoelectric is of the order of pHi = 6.04. The infrared spectrum of the amino acid DL-leucine is shown in Figure 3:



The structural formula of the amino acid DL-leucine

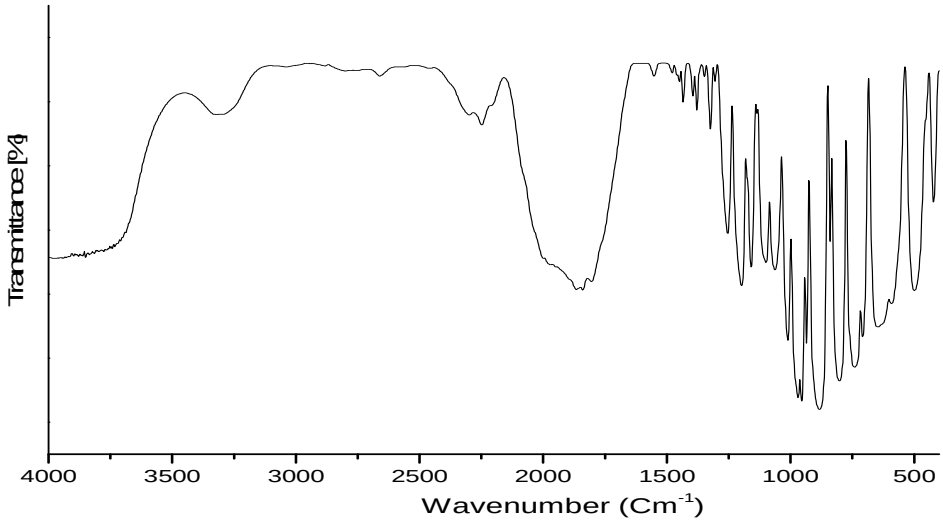


Fig. 3. Infrared absorption spectrum of the amino acid DL- leucine

2.3. Experimental protocol

Adsorption

Adsorption experiments were performed using amino acid DL-leucine in the presence of different phosphates of the atomic ratio Ca/P=1.33, 1.40, 1.48 and 1.67. The solid was dispersed in the adsorption medium at the physiological temperature (37 °C). After some incubation time, the suspension was centrifuged and the supernatant obtained was assayed for DL-leucine [36]. The adsorbed amount of DL-leucine was determined by a ninhydrin colorimetric dosage [59], using a UV/VIS spectrophotometer type GBC 911A 570nm, according to the relation:

$$\mathbf{Qads = V\,(Co\text{-}Ceq) / m.S}$$

Where: **Qads**: adsorbed quantity of leucine (µmol/m^2), **Co** and **Ceq** are respectively the initial and the equilibrium concentration of the leucine (µmol/l), **V**: volume of solution (l), **m**: mass of adsorbent used (g) and **S**: Specific surface (m^2/g).

The kinetic study was performed with a solution of 10^{-3} mol/l the DL-leucine. The results obtained show that the kinetics of adsorption is rapid; the adsorption equilibrium is observed after 30 minutes only (Fig.4 (a)).

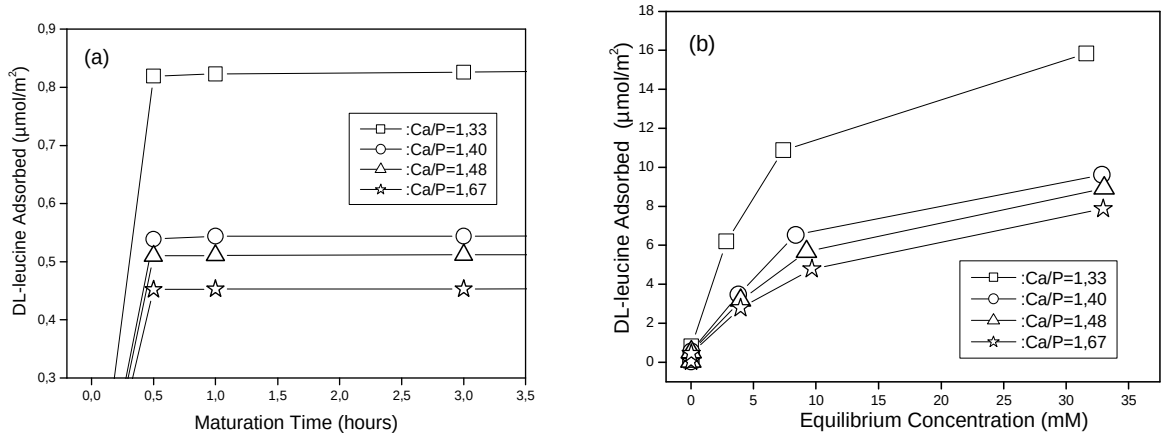


Fig.4. (a) Adsorption Kinetics and (b) Adsorption isotherms of DL-leucine on apatitic calcium phosphates

The amount adsorbed is much higher as the Ca/P ratio is low [36]. The adsorption isotherms obtained were Langmuirian in shape (Fig. 4(b)). The adsorption parameters, maximum amount adsorbed and interaction constants were determined [36]. The isotherms obtained showed a distinct initial increase related to the affinity and adsorption plateau generally considered to represent the saturation of the adsorption sites. This study reveals that the chemical composition of apatites has an influence on adsorption. The high amounts adsorbed at saturation are obtained for compounds containing the more HPO_4^{2-} ions (Fig. 4(b)).

Release

Release was examined considering the used experimental conditions. 200 mg of powder of calcium phosphate previously adsorbed of the DL-leucine is freshly prepared and finely ground. We then add 10 ml of de-ionized water pH=7.0. After stirring for one minute at the speed of 1000trs/mn, the mixture is placed in a thermostatic bath at the physiological temperature (37°C) for a definite time. After treatment, the solid and solution were separated by fritted glass. The supernatants obtained were examined by measuring the pH and determining the amount of the amino acid released. The solid was dried in an oven at 80 °C for 24 hours. The release rate of amino acid is determined by the following relation:

Release rate (%) = Cdes / Cads x 100

Where: **Cdes** and **Cads** are respectively the desorbed and adsorbed concentration of the DL-leucine (µmol/l).

3. Results and discussion

3.1. Study of the release

The kinetic study of the release, performed with a solution at a concentration of 10 mmol/l of the amino acid DL-leucine and apatitic calcium phosphate of atomic ratio of Ca/P= 1.33, 1.40, 1.48 and 1.67, is well illustrated in Fig. 5 (b).The desorption isotherms are performed for the apatitic calcium phosphates (Fig. 5 (a)). The results obtained show that the kinetics of release is slow. Fig. 5 indicates that the release profiles corresponding to these phosphates are similar. There is an increase followed by a decrease beyond 5 days. The release rate is more important when the atomic ratio Ca/P of apatite is lower. It spends 15% for OCPa of Ca/P=1.33 to 10% for the apatite of Ca/P=1.67. One also notes that for all apatites the release rate begins to decrease beyond five days, this suggests the existence of a reverse adsorption reaction between the apatite and the surrounding environment.

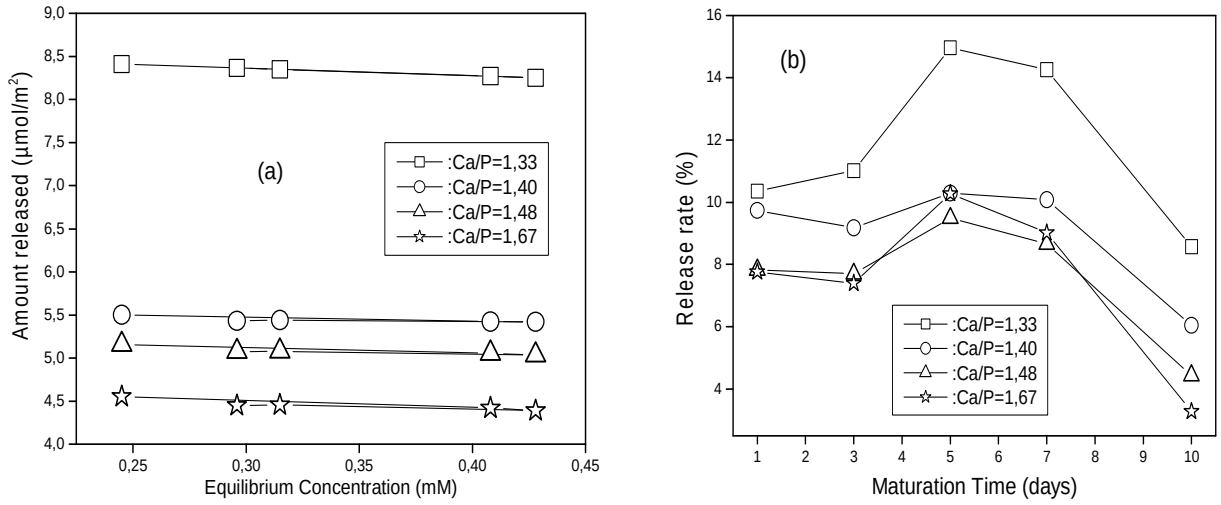


Fig.5. (a) Release isotherms and (b) Release rate (%) of DL-leucine on apatitic calcium phosphates of atomic ratio Ca/P = 1.33, 1.40, 1.48 and 1.67

The pH of supernatant solutions measured after release of DL-leucine at different maturation times and for different apatitic calcium phosphates is between 4,5 and 7,1 (Fig. 6). Also note that, for all apatite, the pH is slightly increased and takes the maximum value after five days. It decreases beyond this period. Figures 5

and 6 indicate that the maximum amount released of DL-leucine correspond to the solution of high pH. This analogy between the release of DL-leucine and pH of the supernatant solutions in maturation time allows us to propose a mechanism of adsorption-desorption (Fig.7). During maturation, other evolution and dissolution of apatite phenomena can occur. The environment may contain released amino acid DL-leucine and ion HPO_4^{2-} and / or CO_3^{2-}at different concentrations depending on the nature of the apatite used.

Apatitic calcium phosphates are covered with a rather fragile but structured hydrated surface layer containing relatively mobile ions [60] (mainly, bivalent anions and cations: $Ca^{2+,}$ HPO_4^{2-}, CO_3^{2-}) (fig.7). The chemical composition of this hydrated layer is still uncertain [61–64]. The consideration of this type of surface state can help to understand and explain the behavior of biological apatite. The amino acid previously adsorbed can be released from these apatites due to the very high specific surface area of these apatites similar of bone and in constituting an important ion reservoir with an availability that depends on the maturation state. Release reaction of DL leucine is carried out on poorly crystalline samples at different stages of maturation (Fig.7).

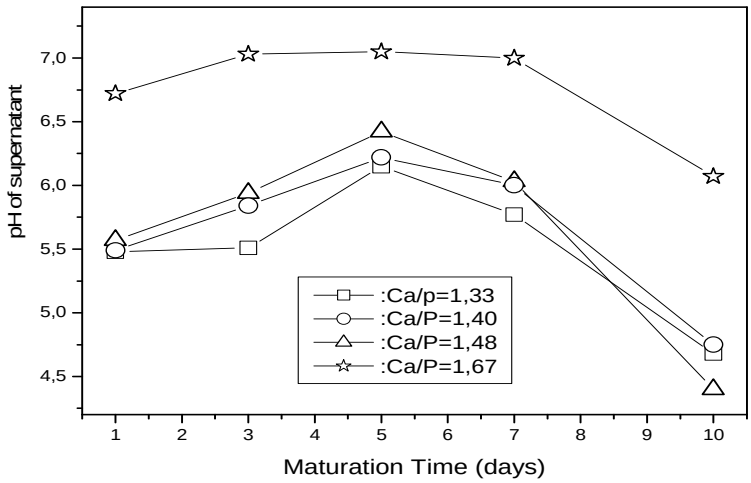


Fig.6. pH of supernatants of DL-leucine released for apatitic calcium phosphates

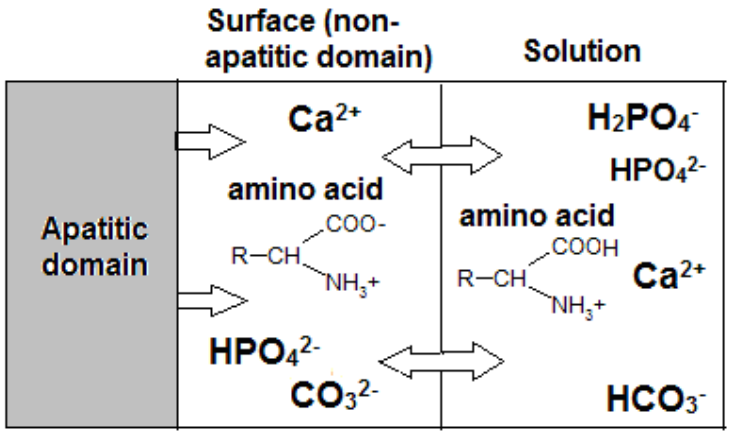


Fig.7. Schematisation of surface reactions adsorption-release the amino acid involving the hydrated surface layer for poorly crystalline apatite

3.2. Effect of ratio R (ml/mg) on the release

In this part, effect of dilution on the release of DL-leucine, by adopting the same experimental procedure as previously described, is studied. We vary the ratio volume of de-ionized water on the R (ml/mg) = volume/mass; R values obtained are between 0.05 and 0.5 ml/mg. The release of DL-leucine depends on R and the initial composition of the apatite Ca/P; the surface properties are initially associated with these parameters. Fig. 8(a) indicates that the release profiles corresponding to these phosphates are similar; the increase in the

ratio R resulted in a decreased release rate. It passes, for example, from 15% (R=0.05 ml/mg) to 6% (R=0.5 ml/mg) for OCPa of Ca/P=1.33: compound of exempt ion CO^{3-} and presents the maximum of HPO_4^{2-}ions, and from 10% to 4% for apatite of Ca/P=1.67: in compounds which present the maximum of CO^{3-} ions. Decrease of the release rate of DL-leucine due to the existence of CO^{3-} and HPO_4^{2-} ions caused by the phenomenon of dissolution of apatitic calcium phosphates.

The pH of the supernatant after release of DL-leucine depends both on R as the initial composition of the solid. So, for different values of the ratio R, the pH of the solution increases when the CO^{3-} ion content increases and the HPO_4^{2-} ion content decreases. The pH values of the supernatant solution show that it is more acidic as the R values are low (Fig. 8 (b)). This observation can be linked to the high release of HPO_4^{2-} ions in the solution. A similar phenomenon was observed in the *in vivo* implantation of apatite materials where, in some cases, the pH of the surrounding environment of the implant can reach the value 3.7 [65].

The results reported in this study indicate that the interaction between the amino acid DL-leucine and the apatitic calcium phosphates of atomic ratio Ca/P between 1.33 and 1.67 is in the form adsorption-desorption reaction, influenced by maturation time and by the existence of mobile ions CO^{3-}, HPO_4^{2-} and Ca^{2+} present on the hydrated surface layer [61] that constitutes a pool of loosely bound ions which can be incorporated in the growing apatite domains and can be exchanged by amino acids or proteins [62] (Fig.8). The release of the DL-leucine is maximal at the isoelectric point of DL-leucine (pHi = 6.04) and varies more or less symmetrically on both sides. The decrease in pH of the solution after five days of maturation may be explained by the adsorption reaction between the released DL-leucine and apatite Ca^{2+}. Incubation of apatite after the fixation of the amino acid DL-leucine in the de-ionized water solution depends on the volume / mass R (ml/mg). The release rate does not exceed 15% when desorption is examined by de-ionized water dilution. Other studies have shown that this rate can be change depending on the nature and concentration of the electrolyte used [31].

The release of DL-leucine is described as ion exchange with orthophosphates ions of the surface. These results are in agreement with our previous studies [36], which revealed that adsorption of DL-leucine on the same apatites is mainly due to the electrostatic interaction between the group $-COO^-$ of DL-leucine and calcium ions Ca^{2+} of apatite. Furthermore, the process was not reversible with respect to dilution. The released molecules can only be displaced by a reverse ion-exchange reaction. The main driving forces at the mineral interface seem to be an ion-exchange process involving the functional groups of the molecules and the ionic groups at the apatite surface.

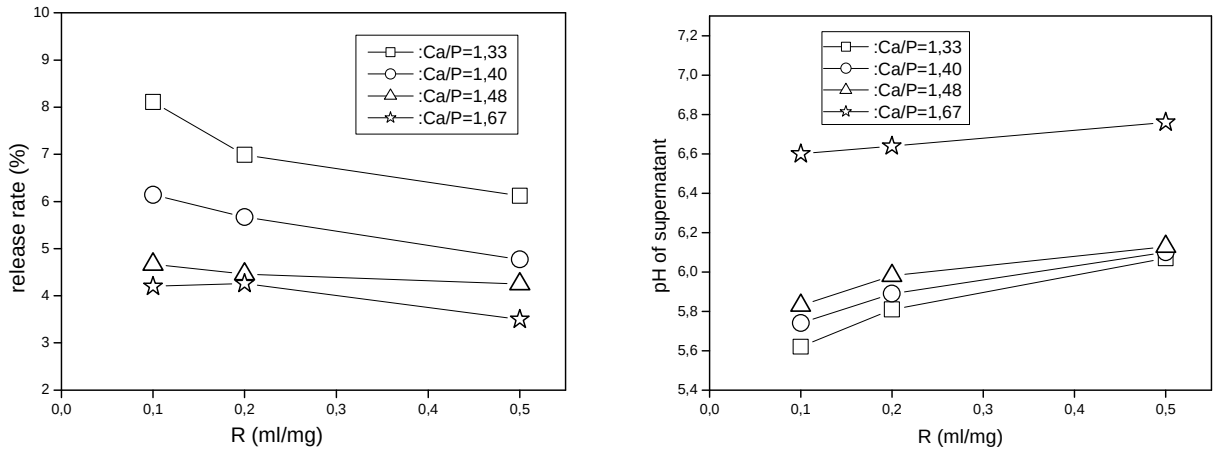


Fig.8. (a) Effect of ratio R (ml/mg) on release rate (%) of DL-leucine and its effect on pH (b) of supernatants

The present study stresses the fact that the release properties of poorly crystallized calcium phosphates of atomic ratio Ca/P between 1.33 and 1.67 are essentially dependent on their surface characteristics, especially the development of the hydrated layer, and on the composition of the adsorbate solution. Exchange reactions highlighted between aptitic calcium phosphate similar to the bone mineral having previously fixed protein molecules or amino acids such as DL-leucine and the surrounding environments, could be analogous to those involved in the regulation of bone metabolism. The flexibility of surface and biological properties of calcium

phosphates such as nanocrystalline apatites suggest that these could be used as potential substrates for drug delivery [66].

Conclusion

The adsorption and release properties of poorly crystallized calcium phosphates appear strongly dependent on the composition of the hydrated surface layer and the surrounding environment and are still a great challenge. The understanding of the interaction mechanisms and the control of the driving forces may provide fundamental tools for the development and application of delivery systems based on the adsorption of pharmaceuticals onto calcium phosphates.

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