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Applications to growth factors and drugs delivery

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Farid Errassifi, Anas Menbaoui, H  l  ne Autefage, Lhaj Benaziz, Saadia Ouizat, et al.. Adsorption onto nanocrystalline apatitic calcium phosphates. Applications to growth factors and drugs delivery. 8th Pacific Rim Conference on Ceramics and Glass Technology (PACRIM-8), May 2009, Vancouver, Canada. pp.159-174.
<hal-04087659>

HAL Id: hal-04087659

<https://hal.science/hal-04087659v1>

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Errassifi, Farid and Menbaoui, Anas and Autefage, Hélène and Benaziz, Lhaj and Ouizat, Saadia and Santran, Véronique and Sarda, Stéphanie and Lebugle, Albert and Combes, Christèle and Barroug, Allal and Sfihi, Hocine and Rey, Christian *Adsorption onto nanocrystalline apatitic calcium phosphates. Applications to growth factors and drugs delivery*. (2010) In: 8th Pacific Rim Conference on Ceramics and Glass Technology (PACRIM-8), 31 May 2009 - 31 June 2009 (Vancouver, Canada).

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ADSORPTION ON APATITIC CALCIUM PHOSPHATES. APPLICATIONS TO DRUG DELIVERY

F. Errassifi^{1,2}, A. Menbaoui^{1,2}, H. Autefage¹, L. Benaziz^{1,2}, S. Ouizat^{1,2}, V. Santran¹, S. Sarda¹, A. Lebugle¹, C. Combes¹, A. Barroug², H. Sfihi³, C. Rey¹

¹University of Toulouse, CIRIMAT, UPS, INPT, CNRS, ENSIACET, Toulouse, France

²Departement de Chimie, Faculté des Sciences Semlalia,

³LPEM, ESPCI, and CSPBAT-CNRS, Université Paris Nord, Paris, France

ABSTRACT

Interfacial properties of apatitic calcium phosphate play a crucial role in calcified tissues and biomaterials. Generally the adsorption of molecules on apatitic calcium phosphates is considered to obey to electrostatic interactions. The study of the adsorption of various active molecules such as albumin, heparin, bisphosphonate, and growth factors like BMP-2, VEGF and FGF-2 on such surfaces seems to follow a similar pathway. In all cases the adsorption process can be well described using Langmuir isotherms, although the adsorption process appears generally as irreversible. Further studies of the adsorption reaction reveal, in most cases, an ion exchange mechanism involving the replacement of mineral ions of the apatite surface by molecular ions from the solution. Considering biomimetic apatite nanocrystals, considerable variations of the adsorption parameters are observed depending on the maturation time of the nanocrystals. As the maturation time increases, the adsorption affinity constant increases and the maximum amount adsorbed decreases. The understanding of the adsorption process provides fundamental tools for the development of drug delivery system using apatite materials. Applications to the release of active molecules are examined in the case of apatite coatings.

INTRODUCTION

Adsorption of proteins and drugs on apatites are considered to play a determining role in mineralized tissues and substitutive biomaterials based on calcium phosphate apatites¹⁻³. Several specific bone proteins such as osteocalcin, osteopontin, as well as generic proteins and biomolecules like albumin, phospholipids and others have been shown to adsorb on and interact strongly with apatite⁴⁻⁹. These phenomena are considered to control apatite crystals nucleation, growth and dissolution in vivo⁵⁻⁸. Many macromolecules have been classified as initiator or inhibitor of crystal growth although for a given substance the role is not so clear and could depend, for example, on the local concentration or on the state of solute or solid fractions of this given substance^{10,11}. In the biomaterial field it has been suggested that molecules interacting with calcium phosphates play a crucial role in the recruitment of cells and their expression and that they are strongly involved in the biointegration of implants^{12, 13}. Considering the treatments of bone diseases, some bone adsorbing molecules, like bisphosphonates, have been used to control bone turnover⁹, although their effect on bone cell behavior has been largely documented, the biological activity has been shown recently rather well correlated with their adsorption properties¹⁴. Surface properties of apatites are also largely used in separation chromatography of proteins and, in fact, preparatory and development studies for these applications have started the researches on adsorption on apatite surface¹⁵. The models proposed initially are still widely present in the actual explanations of the adsorption behavior of apatites, essentially base on surface charge considerations^{16, 17}. The aim of this report is to give several, non-exhaustive examples of different types of adsorption reactions on apatites and to present some possible applications especially for growth factors delivery. We will first describe adsorption experiments of small molecules stressing the influence of functional groups on adsorption properties, we will then analyze the effect of the adsorbent characteristics, especially the type of apatite used, and we will finally describe the adsorption process of molecules of biological origin and their applications.

MATERIALS AND METHODS

Adsorbents

The adsorption substrates were prepared according to published methods¹⁸⁻²¹. Briefly, nearly stoichiometric apatite was prepared by double decomposition at boiling temperature and alkaline pH, between a calcium nitrate solution and an ammonium phosphate solution.

Apatite nanocrystals were also prepared by double decomposition but at room temperature and physiologic pH¹⁹.

After filtering and washing the samples were lyophilized. They were all controlled by X-ray diffraction, chemical analyses and FTIR spectroscopy. The specific surface area was determined by BET method (nitrogen adsorption).

Adsorption experiments

Adsorption experiments were made in batches at constant temperature and known pH. A given amount of adsorbate was dissolved in aqueous solution at constant ionic strength (in KNO₃ solution). The apatite powder was added in the solution. After an equilibration period, determined from previous adsorption rate studies, the solutions were analyzed. The adsorption isotherm was determined from the equilibrium concentration in solution. The calcium and phosphate content of the solutions were measured by atomic absorption spectroscopy and spectrophotometry of the phospho-vanado-molybdcic acid respectively. Details are given in the different publications¹⁹⁻²¹.

RESULTS

Adsorption of small molecules

- Serine and o-phosphoserine

The adsorption of these aminoacids on apatite nanocrystals gives a good example of the effect of functional groups using simple molecules²¹. These aminoacids are involved in matrix proteins of bone and it has been shown that it exists a relationship between the phosphorylation ratio of bone tissue proteins and the mineralization process. The adsorption isotherms of serine and phosphoserine on a nanocrystalline apatite are shown in figure 1.

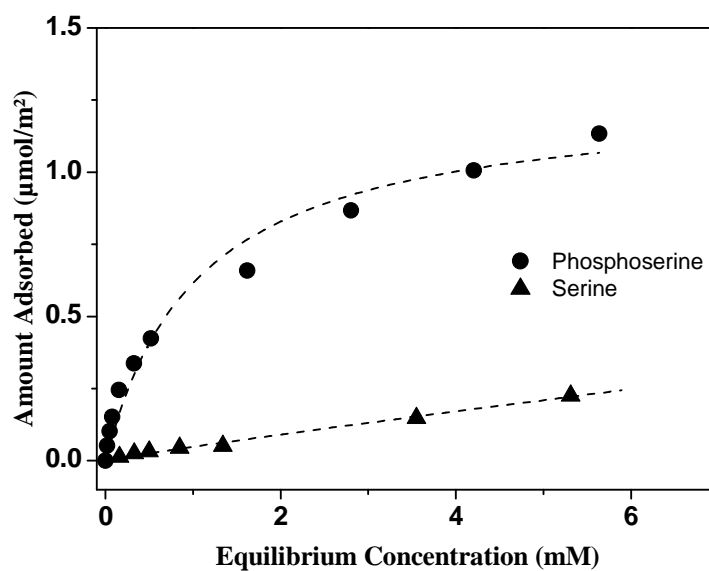


Figure 1: Example of adsorption isotherm of serine and phosphoserine on nanocrystalline apatite (24 hours maturation time, Ca/P ratio: 1.42; Specific surface area 155 m².g⁻¹, pH 7.0, T=25°C).

The adsorption of o-phosphoserine is well described by a classical Langmuir type isotherm and it is characterized by a rather high affinity constant. Comparatively, the adsorption of serine appears much weaker and a plateau was not reached in the domain of concentrations explored. The adsorption of serine can be described by a Freundlich type isotherm like that of other aminoacids or simple molecules weakly interacting with apatite surface such as glycine or acetic acid for example²².

The very high affinity of phosphoserine for apatite surface compared to that of serine can be related to the existence of the phosphate group which can interact strongly with the apatite surface. Phosphoproteins for example have been shown to play a key role in apatite nucleation in bone tissues. In the present case, it has been shown by solid-state NMR that phosphorylated amino acids, more specifically, seems to interact with nascent bone crystals in embryonic bone mineralization²³. In the field of biomaterials, the attachment of methylmethacrylates monomers to calcium phosphates has been shown to be considerably enhanced with the esterification of alcohol groups with phosphoric acid²⁴. Among the other functional groups considered to interact strongly with apatite surface are silicate and sulfate. Generally carboxylate group interact weakly except when they are associated in polyacids.

An interesting aspect of the adsorption of phosphoserine is that the maximum amount adsorbed can considerably vary depending on the pH or the presence of mineral ions in the solution. Thus in the presence of phosphate or carbonate ions the maximum amount adsorbed decreases whereas in the presence of calcium one can adsorb much more molecules at saturation.

The adsorption of phosphoserine cannot be reversed by simple dilution of the solution and the corresponding decrease in the equilibrium concentration of the adsorbate. This phenomenon is common to many adsorption processes on apatites.

- Adsorption of a Bisphosphonate: risedronate

Bisphosphonates are among the most prescribed medication for several bone diseases and more specifically osteoporosis. The adsorption isotherm of a bisphosphonate, risedronate, on hydroxyapatite is shown in figure 2.

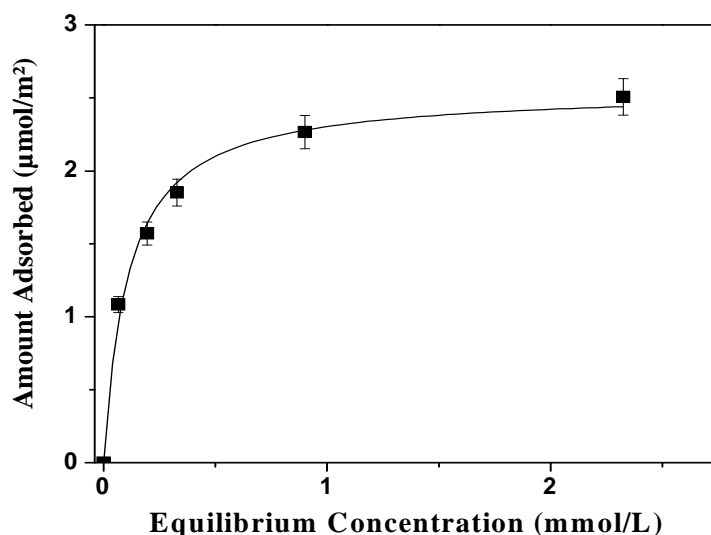
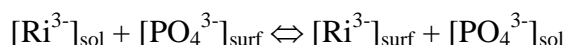


Figure 2: Adsorption isotherm of risedronate on well crystallized hydroxyapatite (Ca/P ratio 1.64, Specific surface area: $59 \text{ m}^2 \cdot \text{g}^{-1}$, pH=7.4, T=37°C)

It corresponds also to Langmuir's isotherms. The follow-up of the variation of the mineral ions content of the solution indicate that the adsorption of risedronate is associated with a release of phosphate ions

(Figure 3) whereas the calcium concentration remains almost unchanged. These findings are analogous to those reported by others concerning the adsorption of another bisphosphonate, zoledronate, on calcium phosphates²⁵.

Considering that the dissolution of hydroxyapatite in our system is congruent (i.e. the Ca/P ratio is the same for the solid and the solution), the calculations indicate that for most of the points of the isotherm there is one phosphate ion released for every risedronate molecule adsorbed. In this case the adsorption reaction corresponds in fact to an ion exchange:



in which the risedronate ions from the solution substitute for the phosphate ions of the apatite surface.

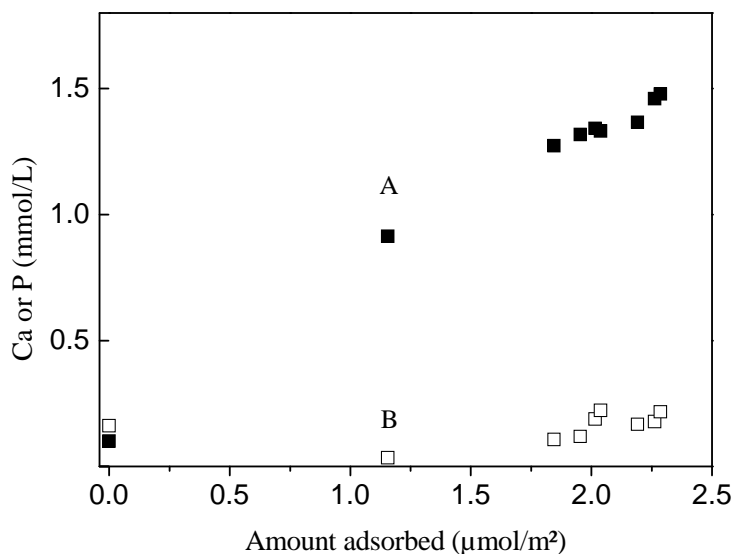


Figure 3: Variation of the calcium (B: white squares) and phosphate (A: black squares) content of the solution with the amount of risedronate adsorbed.

This reaction is responsible for the release of additional phosphate ions and the apparent non-congruent dissolution equilibrium of the apatite. Considering this equilibrium, the calcium concentration should decrease as the phosphate in solution increase. This is not observed and the stability and even the slight increase of the calcium content with the increase of risedronate in solution can be interpreted as an indication for the probable formation of ion pairs or complexes between risedronate molecules and calcium ions in solution.

An interesting aspect of risedronate adsorption is that the amount of molecules adsorbed at saturation can vary considerably depending on different parameters such as the temperature, the pH, the initial phosphate and calcium content in the solution. Such variations cannot be explained by the chemical equilibrium proposed.

As previously noticed the adsorption of risedronate is irreversible and the amount adsorbed is not altered by dilution of the adsorption solution or even on washing of the samples.

The adsorption of biological macromolecules appears more difficult to follow as the possible interactions with a mineral surface are more numerous than for simple molecules. One may regard a biological macromolecule as a charged object which can interact with a charge mineral surface depending respectively on the point of zero charge of the molecule and the surface charge of the mineral surface. Theoretical approaches, however, such as computational modeling, lead to multiple but precise bonding schemes involving identified groups of atoms of the molecule and of the mineral

surface. In addition in many cases the adsorption of biological macromolecules has been shown to be related to phosphate ion release¹⁶, like for small molecules, and some of these molecules can be purified and separated by chromatography on apatite columns using phosphate solutions with a concentration gradient for as mobile phase²⁶. These observations suggest that the existence of specific chemical reactions with apatite surface cannot be discarded in the description of adsorption processes.

Adsorption of biological macromolecules

-Bovine serum albumin (BSA)

Albumin is the most abundant protein in blood serum. It is also one of the major proteins found in bone and it is considered to be involved in enamel formation. Several studies have been made on albumin interactions with apatites^{19, 27,28}. Like small molecules BSA adsorbs on well crystallized, nearly stoichiometric, apatite according to Langmuir's theory. However, the biological apatites are very different from stoichiometric ones: they are nanocrystalline and they exhibit a surface hydrated layer with mineral ions in non-apatitic environments. This layer is related to the precipitation process at physiological pH and its structure as well its extension depend on the ageing time in solution²⁹. The data obtained for BSA adsorption (but also for phosphoserine, and risedronate, data not shown) indicate an increase of the affinity constant and a decrease of the maximum amount adsorbed at saturation with the maturation time (Figure 4). These data illustrate the variability of adsorption on apatite substrates and the need to define and characterize precisely the type of apatite used and its surface.

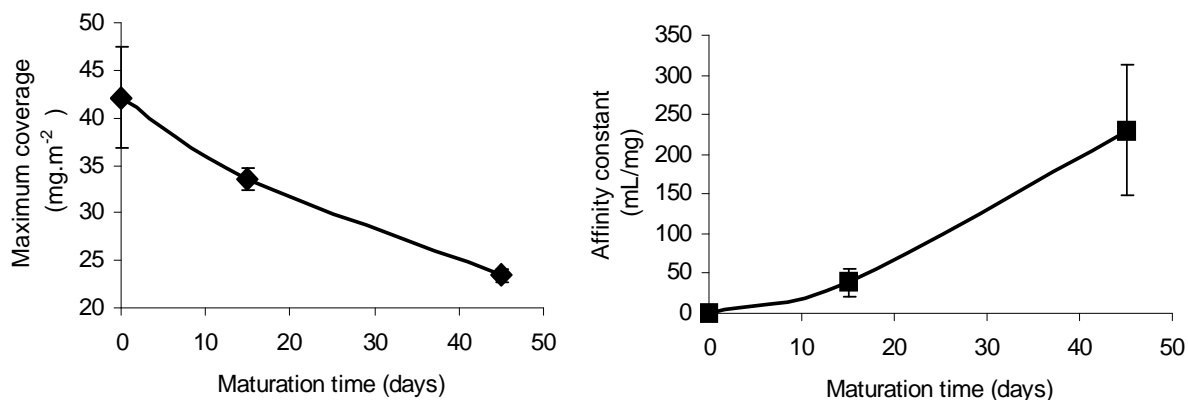


Figure 4: variation of the adsorption characteristics of BSA on nanocrystalline apatites with the maturation time²⁰.

-Heparin

Heparin is a calcium complexing polysaccharide involved in the control of blood clot formation which has been proposed as carrier of growth factors. The adsorption of heparin on well crystallized stoichiometric apatite was investigated in detail. Like BSA and small molecules the adsorption equilibrium is reached relatively rapidly in about 20 minutes. The adsorption isotherms are Langmuirian (Figure 5).

The analyses of the mineral ions in solution show a complex variation (Figure 6). A constant increase of the concentration of phosphate ions as the amount of heparin in solution increases is observed. Concerning the calcium concentration, a decrease occurs at low heparin content followed by an increase when the adsorption limit is reached. This behavior seems rather different from that of risedronate although the phosphate releases seems to increase with the amount adsorbed, the calcium content with a U shaped variation appears rather strange. However, like for risedronate, the addition of calcium ions in the absorption solution was found to increase the maximum amount adsorbed whereas it decreased in the presence of phosphate ions. Additional studies by solid state NMR on apatite

nanocrystals showed that the adsorption of heparin was associated with a decrease of the surface HPO_4^{2-} ions.

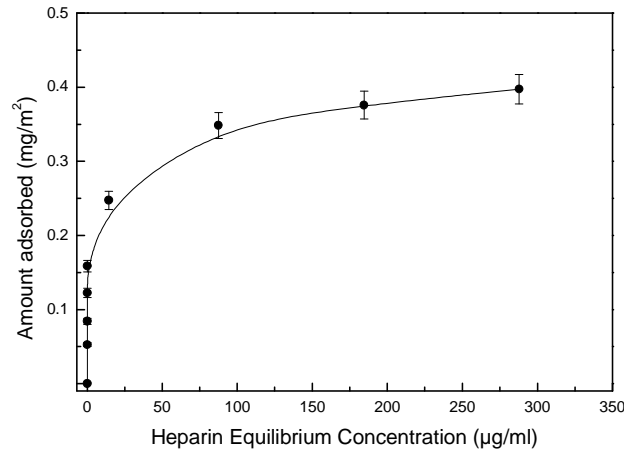


Figure 5 : adsorption isotherm of heparin on well crystallized nearly stoichiometric apatite. (Ca/P ratio 1.62, Specific surface area : $82 \text{ m}^2 \cdot \text{g}^{-1}$, pH=7.4 , T=25°C)

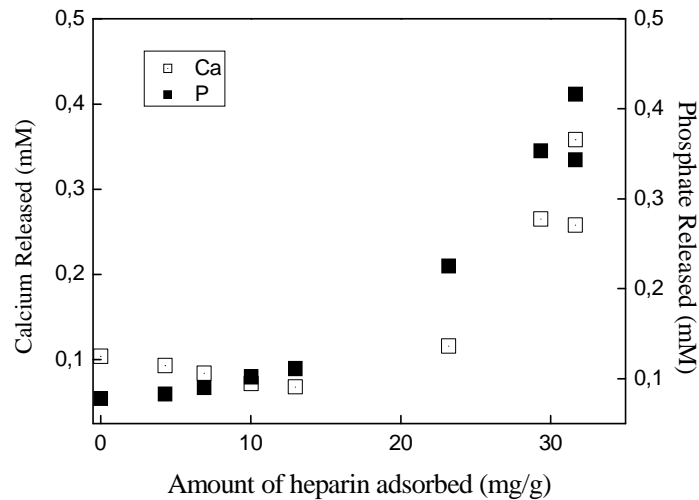


Figure 6: variation of the calcium and phosphate concentrations in solution with heparin adsorption.

-rhBMP 2

BMP 2 is one of the most efficient growth factor involved in bone reconstruction. Only a very few studies have been made on the adsorption of this molecule on apatites. Boix et al. have produced a rather complete study on the adsorption of BMP on well crystallized apatites³⁰. This molecule seems to follow the general characteristics of the adsorption of other molecules with a Langmuir type isotherm. Like for many other molecules for which the attachment to the apatite surface is determined by anionic carboxylate functions, the adsorption limit varies considerably with the mineral ions present in the solution. The increase of the calcium concentration in the adsorbate solution increases the adsorption limit whereas that of phosphate shows a reverse effect. This growth factor can also be attached to nanocrystalline coatings³¹. The adsorption is also Langmuirian, and most of the growth factor molecules are irreversibly bound and they are not spontaneously released in a cell culture media (Figure 7). This characteristic has also been observed for other growth factors like FGF 2 or VEGF^{32,33}.

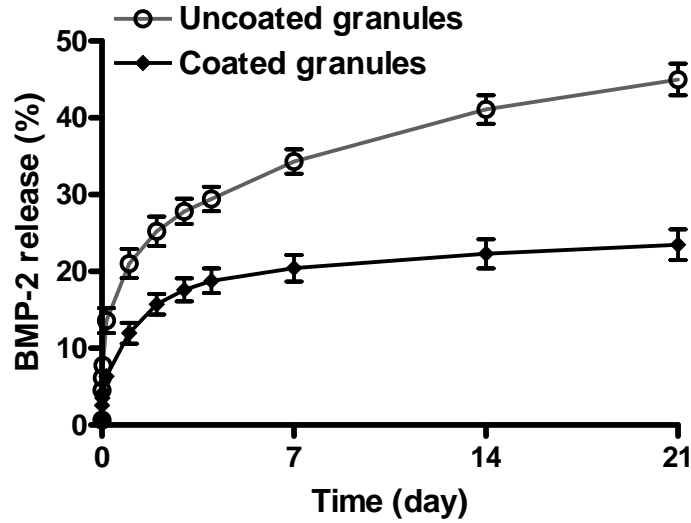


Figure 7: Release of rhBMP 2 in DMEM complete culture medium with 10% fetal calf serum from adsorbed rhBMP-2 on nanocrystalline coated and uncoated Biphasic Calcium Phosphate³¹.

DISCUSSION

These few examples illustrate the variability of the adsorption processes on apatites but common features have been observed.

Adsorption isotherms

Generally the adsorption equilibrium of molecules strongly interacting with an apatite surface is well depicted by a Langmuir isotherm (C_e equilibrium concentration of the adsorbate and Q amount adsorbed):

$$Q = NK_L C_e / (1 + K_L C_e)$$

characterized by two adsorption parameters, the affinity constant (K_L) and the maximum coverage (N). The empirical Freundlich isotherms:

$$Q = K_F C_e^{1/n}$$

have been found generally in the adsorption of molecules weakly bound to the apatite surfaces, and rather far from the saturation limit. It has been found for example for serine in the example given here and also for different other ions or molecules like glycine and acetate ions for example.

In a recent study on adsorption Rill et al. tested different adsorption models for the analysis of adsorption data of phosphonate-containing molecules on apatites³⁴. The best fits were given by a Langmuir-Freundlich isotherm:

$$Q = N(K_{LF} C_e)^n / (1 + (K_{LF} C_e)^n)$$

and a Tóth isotherm:

$$Q = [N(K_T C_e)^n / (1 + (K_T C_e)^n)]^{1/n}$$

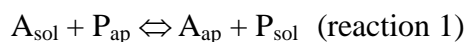
which are taking into account the existence of a distribution of adsorption energies on the apatite

surfaces. However for molecules of interest the simple Langmuir's isotherms are satisfactory. A major characteristic of adsorption on apatites is the absence of reversibility, often noticed, which is totally contradictory with the Langmuir theory of adsorption.

The adsorption process

Historically two types of adsorption sites have been recognized, on apatite calcium surface sites and phosphate surface sites respectively. Negatively charged molecules (or moieties) were considered to bind to calcium sites and positively charged ones to phosphate sites. The adsorption of negative molecules seems particularly important and it is involved in most chromatographic use of apatites and also in most studies involving molecules of biological interest. The zeta potential of apatites has been considered to play a major role in the adsorption process and most adsorption behaviors were, and still are, related to this surface parameter although the correlation between this global physical parameter describing the surface charge of apatite particles and chemical interactions with specific ions at an atomic level is not straightforward. It shall be considered that the global charge of a particle does not give any information on the local distribution of cations and anions at a mineral surface and in fact zeta potential does not appear as a decisive parameter: for example the adsorption of positive molecules on positive apatite surface and inversely negative molecules on negative apatite surface has been described^{35, 36}. Similarly the isoelectric point of proteins, by itself, do not give specific information on the ionization state of the different functions of a molecules and they should be considered with caution for the prevision of an adsorption behavior related to a chemical interaction.

In several of the examples given in this report the adsorption of negative molecular ions has been related to a release of phosphate ions evidencing a chemical ion exchange reaction with the apatite surface. This process has been described in different instances, as for example in the adsorption of polyacids³⁷, citrate ions³⁸, zoledronate²⁵, or serum proteins on apatites¹⁶. In the case of risedronate it has been found that one molecule adsorbed removed one phosphate ion out of the apatite surface. This chemical process seems rather general and supports a chemical description of the adsorption reaction rather than a physical one based on global electrostatic interactions. The adsorption could then be represented, for example, as a chemical equilibrium as follows (in a case of a one to one exchange):



with A the adsorbate in a given ionic state either in solution (sol) or on the apatite surface (ap), P phosphate group in a given ionic state either in solution or on the apatite surface. Other ion exchange possibilities have been discussed by Misra in the case of citrate adsorption³⁸. These descriptions seem consistent with the use of apatites in chromatography where negatively charged molecules are desorbed using phosphate solutions (and positively charged ones using calcium solutions).

The peculiar behavior of heparin deserves some additional explanations. This molecule can give soluble complexes with calcium ions. In the presence of apatite, calcium ions are complexed by heparine and apatite dissolves to reach a solubility equilibrium. During the first raising part of the adsorption isotherm these complexes are bound to the apatite surface and essentially phosphate ions are released. As the surface saturation occurs, the addition of heparin still results in Ca-complex formation but the complex cannot attach to the saturated surface and remains in solution, resulting in an increase of both calcium and phosphate concentrations in the solution. This behavior could also occur in the adsorption of citrate anions on apatites or more generally in the adsorption of calcium-complexing molecules.

In addition to complexation other processes can disturb the adsorption equilibrium and have to be taken into account: the possible precipitation of calcium (or phosphate) salts, the dissolution equilibrium of the apatite and the surface hydrolysis reactions of apatite.

Insoluble calcium salts may sometimes form and precipitate with the adsorbate molecules, depending on their respective concentrations. Then the apatite phase is progressively destroyed as the new phase forms leading to a release of phosphate in the solution until a new equilibrium is reached. This event does not generally give a Langmuir type isotherm with a typical saturation domain. On the contrary, the addition of adsorbate solutions aliquots results in precipitation of calcium salt of the adsorbate and more destruction of the apatite. In some cases however this process could result in a Langmuir-like adsorption behavior with an apparent saturation domain if there is a coating of the apatite surface by the new phase preventing further reaction. The observation of the solid phase by X-ray diffraction or electron microscopy allows generally the detection of new phases formation.

The dissolution equilibrium of apatite has also to be considered. Generally apatite dissolution is congruent when the apatite/solution ratio is small enough to neglect surface reactions. However there are some conceptual divergencies on the solubility product of hydroxyapatite most often related to inhomogeneity of composition and non-stoichiometry. In the case of nanocrystalline apatites the dissolution behavior is more complex than for stoichiometric apatites and it can probably be represented by a metastable equilibrium solubility (MES)³⁹ showing a dependency of the apparent equilibrium concentrations with the amount dissolved, although the dissolution still remains congruent. The amount of calcium and phosphate in the equilibrium solution depends also, in these cases, on the maturation stage of the nanocrystals. The result of dissolution equilibria is the existence of calcium and phosphate ions in the solution which may interfere with the adsorption equilibrium and especially with the maximum amount adsorbed and the affinity constant.

Additionally in cases where the amount of apatite with a high specific surface area is elevated in the solution, a hydrolysis of the phosphate ions on the surface may occur associated with a release of calcium in the solution. This phenomenon leads to an apparent non-congruent dissolution equilibrium which appears mainly related to a surface equilibration reaction and then depends on the solid/solution ratio. As adsorption studies are often made with rather large solid/solution ratios, this phenomenon leads to calcium and phosphate ions in solution in a ratio different from that in the solid, which may also interfere with the adsorption parameters. To avoid these anomalies a pre-equilibration of the apatite with a solution at the same pH and ionic strength than the adsorption solution can be realized, before putting the apatite in contact with the adsorption solution.

The description of adsorption as an ion exchange reaction permits to explain several characteristics of the adsorption of charged molecules on apatites and especially the apparent irreversibility of the adsorption process.

Irreversibility of the adsorption

Langmuir adsorption is generally an equilibrium and thus a reversible process. The most convenient way to determine the reversibility of adsorption is to dilute the solution in equilibrium with the apatite surface. Then the concentration of the adsorbate is reduced and a new equilibrium shall occur resulting in the release of molecules from the solid surface. The observation of a total or partial irreversibility of adsorption on apatites upon solution dilution is a common observation which has received several explanations such as a kinetic limitation due to multiple bonds of macromolecules, necessitating a rupture of all of them to release the molecule, change in configuration and surface properties. These possibilities may of course exist, but irreversibility is also observed for simple molecules such as phosphoserine or bisphosphonate for which none of these explanations seem satisfactory. Considering the ion exchange equilibrium reaction, it is apparent that the adsorption process is not only related to the concentration of the molecule in solution but also to that of the mineral ion displaced. From reaction 1 we can write:

$$K_{ex} = P_{sol} \cdot Q / (C_e(N-Q))$$

with K_{ex} , the equilibrium constant of the exchange reaction, Q the amount adsorbed, C_e the equilibrium adsorbate concentration, P_{sol} the phosphate concentration in solution, N the total number of adsorption sites (i.e. the maximum of molecules adsorbed). This equation can be transformed into a Langmuir type isotherm equation:

$$Q = NK_{ex}(C_e/P_{sol})/(1 + K_{ex}(C_e/P_{sol}))$$

The equilibrium concentration of the adsorbate (C_e) in the traditional Langmuir equilibrium is simply replaced by the ratio C_e/P_{sol} in the exchange reaction equilibrium. This model fits well with data points obtained in our studies, in addition it explains simply the apparent irreversibility on diluting the equilibrated solution. As dilution involves a reduction of both concentrations (C_e and P_{sol}) in equal proportion, the conditions of the equilibrium are roughly unchanged (if the very weak dissolution of apatite to re-establish the solubility equilibrium and hence the corresponding faint increase of calcium and phosphate in solution are neglected). The recently reported observation that the reversibility of the adsorption of phosphonate complexes³⁴ on apatite on dilution was depending on the equilibrium concentration of the adsorbate in solution can be explained with the same arguments. In these experiments the authors were testing the reversibility at two points of the isotherm corresponding to low and high adsorbed amount. The dilution was performed with a solution saturated with respect to the apatite to avoid any dissolution of the adsorbent. Assuming an exchange reaction of a complex molecule with a phosphate ion, the dilution with a saturated solution was correlatively bringing a relative increase of phosphate ions in solution more important for the solution equilibrated with a low amount adsorbed, and thus with the lowest amount of exchanged phosphate, than for the solution at a higher equilibrium concentration and a higher amount of phosphate in solution. Thus the reverse reaction appeared slightly stronger, in relative amounts, at low adsorbate equilibrium concentration. Similarly washing an apatite sample with adsorbed molecules resulting of ion exchange does not create any reverse exchange possibility and no desorption should be expected, except for faint release effects due to the dissolution of the apatite.

An equivalent, though slightly different, description of the exchange reaction can be proposed taking into account the solubility equilibrium of the apatite:

$$(Ca)^R(P) = K_{sp,pp}$$

where $K_{sp,pp}$ is the solubility product of the apatite corresponding to one phosphate and R the Ca/P ratio of the apatite, assuming a congruent dissolution (the pH is considered constant, for example a physiological pH, and the OH contribution to the solubility is therefore also constant and included in the solubility product).

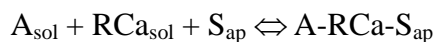
Then the adsorption isotherm may also be written as

$$Q = NK_{ex}(C_e(Ca)^R/K_{sp})/(1 + K_{ex}(C_e(Ca)^R/K_{sp}))$$

or:

$$Q = NK_b (C_e.(Ca)^R)/(1 + K_b C_e.(Ca)^R)$$

where $K_b = K_{ex}/K_{sp,pp}$. The adsorption process can then be described as mobilizing calcium ions from the solution to serve as a binding agent of the molecule with the mineral, instead of a phosphate exchange. The corresponding chemical equation can be written as:



where S_{ap} represents a binding site on the apatite. These raw equations shall be adapted to each case, and there can be for example one binding calcium per molecule, which could be described in the equivalent ion exchange process as a replacement of a fraction of phosphate ion. These descriptions (ion exchange with phosphate or calcium binding) are totally equivalent from a chemical point of view although they shall be adapted to take into account the number of ions involved and their charges.

The adsorption process does not differ strongly between well crystallized apatites and nanocrystalline apatites. An interesting aspect is the variation of the adsorption parameters with the characteristics of the nanocrystals

Adsorption on apatite nanocrystals and ageing

An interesting problem, regarding nanocrystalline apatites, is the possible change of surface properties with the maturation state of the nanocrystals.

The available data on adsorption properties of apatite nanocrystals are very scarce, but those obtained with several molecules such as bovine serum albumin, phosphoserine, and risedronate suggest a general behavior and the findings reported in figure 4, in the case of albumin, were also observed in other cases: the number of adsorption sites decreases when the maturation time of the nanocrystals increases but the affinity constant increases.

The variation of the affinity has been related to a decrease of the surface energy as the hydrated layer becomes progressively transformed into apatite. Although it is very difficult to determine the surface energy of nanocrystals, several measurements on Ca-P materials have shown that hydrated Ca-P salts always had a much lower surface energy than anhydrous ones⁴⁰. Thus, the hydrated layer, which most probably reduces the surface energy of the nanocrystals in aqueous media, also weakens, to some extent, the interfacial energy released by protein adsorption. Another explanation could be that ions in the hydrated layer become less exchangeable as the maturation time increases, which is effectively observed²⁹.

The decrease of the maximum amount of adsorbed molecules with maturation is more difficult to explain. It does not seem related to a decrease of the specific surface area of the crystals and the rate of coverage does not support the formation of multilayers of proteins on immature crystals. A different conformation could be suggested in the case of albumin. However the small phosphoserine exhibits the same behavior. The adsorption process by ion displacement suggests that the amount of protein incorporation could be related to the amount of labile mineral ions available in the hydrated layer and then to the extension of the hydrated layer. The adsorption process could then involve the whole hydrated layer and possibly its re-structuration and stabilization. As a support for this hypothesis it can be noted that the adsorption limit is well correlated to the development of the hydrated layer on nanocrystals as shown for example for the adsorption of phosphoserine (Figure 7). It has also been shown that the development of the hydrated layer was related to the ion exchange capability and a parallel seems to exist between ions exchange and adsorption.

Another common point to adsorptions on apatites is the variability of the adsorbed maximum amount with several physical chemical parameters.

Maximum amount adsorbed

The maximum amount adsorbed is supposed to correspond to the saturation of the surface adsorption sites. The question regarding the variations of this quantity is generally answered by reference to the surface charge model and its alterations. For example an increase of the pH produces a decrease of the surface charge and of the adsorption capabilities of negatively charged molecules.

Similarly the increase of the calcium ions concentration in solution increases the surface charge and favors the adsorption of negatively charged molecules whereas the increase of the phosphate concentration has a reverse effect. As already mentioned, this global model does not consider that the most important adsorption processes on apatite involve strong local interactions with individual ions on the surface and not simply a global electrostatic interaction between a charged surface and a charged molecule.

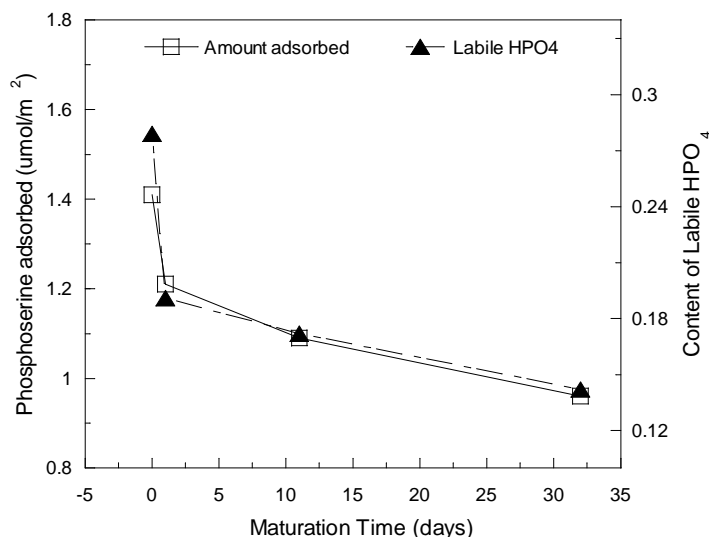


Figure 7: Correlation between the amount of phosphoserine adsorbed and the development of the hydrated layer as measured by the intensity of the surface HPO_4^{2-} determined by FTIR spectroscopy.

Considering the proposed ion exchange reactions, the chemical equilibriums involved cannot explain a variation of the adsorption limit: when the concentration of adsorbate increases to large values, all sites should be occupied. Such variations suppose that maximum number of sites which can be occupied (N) varies according to different factors. It is obvious that for most molecules, and this is especially true for macromolecules, the dimension is much larger than that of phosphate or calcium ion exchanged by specific molecular functions and a total replacement of all surface ions by these molecular functions is not possible. Thus there are always more possibilities of exchange and attachments than what is feasible due to steric constraints. The effect of phosphate and calcium ions in the adsorption solution are always similar regarding the adsorption limit of negatively charged molecules: phosphate ions decrease the maximum amount of adsorbed molecules compared to a solution containing only the adsorbate, and calcium ions increase it. These added mineral ions affect the adsorption limit in a wide range (from 1 to 6 for example in the cases of heparin). If we compare with the variations observed for nanocrystals at different maturation stages it can be suggested that the mineral ions in solution may affect the hydrated layer at the surface of the apatite crystals. The strength of attachment and surface mobility of the adsorbed molecules has also to be considered and could be involved in the reorganization of molecules on the surface.

Consequences for bone mineral

Two main roles have been assigned to the interactions of proteins with apatite crystals in biological systems: initiation of crystal nucleation and inhibition of crystal growth⁴¹. Several in vitro experiments have provided support for this notion. However the classification of proteins as initiators or inhibitors of mineral formation suffers from numerous exceptions. It has been shown that the role of proteins may depend on their state and configuration. Thus, the same protein may act as an inhibitor

when it is in solution and as an initiator when it is bound to a substrate. In addition most adsorbing proteins seem to show both aspects depending on their concentration¹⁰: at low concentrations they accelerate crystal formation whereas at high concentrations they act as inhibitors.

Most mineral binding proteins are rich in anionic groups. Phosphoproteins especially have been suggested to bind to calcium and to initiate the first nuclei for mineral formation⁴². Due to their lower mobility compared to that of mineral ions and the relatively fast rate of formation of nanocrystals, proteins can only play a role when they are already in situ. In addition to their role in the nucleation and growth of mineral crystals, proteins may also be used to control mineral maturation. Maturation of the nanocrystals considerably reduces their surface reactivity and their action in homeostasis. Remodeling appears, therefore, as a necessity for the organism to maintain the ion reservoir function of bone. The strong variation of the surface properties of the nanocrystals with maturation and especially the change in adsorption properties suggest that some proteins could act as signals between mineral crystals and cells to control the maturation state and possibly initiate the remodeling process. This hypothesis needs, however, to be verified. The variation of the adsorption properties of bone nanocrystals with time is rarely considered in a dynamical approach of the system; but, considering the very large specific surface area of bone crystals, it could be fundamental.

The interactions between nanocrystals and the collagen matrix have not yet been elucidated at the atomic level. It seems probable that ions mobility in the hydrated layer could facilitate an intimate contact between the two main constituents of bone and could influence the mechanical properties of the tissue. The fact that the mechanical properties of bone depend strongly on its hydration state supports this hypothesis.

Considering the specific mode of interaction of drugs like bisphosphonates with apatites, the study of the adsorption process allows some findings to be explained. The absence of accumulation of the drug in bone even after years of use, for example, is related to the adsorption process and the exchange equilibrium: once equilibrium concentrations have been reached in bone tissue the loading cannot continue. This would not be the case if a dissolution re-precipitation process were involved. The release after interruption of the treatment depends on the phosphate concentration in bone fluids; as it is very low this release is very slow.

It seems important to consider the dynamic of bone tissue, not only in terms of bone cell behavior but also as a regulating system involving interfacial interactions with the mineral. Without taking this aspect into account the approach will necessarily be incomplete.

Consequences for biomaterials

Adsorption on apatites and other calcium phosphates may find several uses in biomaterials¹. Generally active molecules such as growth factors are associated with biomaterials by impregnation often followed by drying. However this easy, rapid and inexpensive method does not allow a precise control of the physical state of the active molecules which may appear partly crystallized partly adsorbed or partly precipitated as calcium or phosphate salt or more likely a combination of these possible states depending on the conditions of preparation and drying. Thus the release rate is difficult to predict it can be rapid and only determined by the diffusion properties of the molecule if no interactions with the biomaterial surface have been established or depend on solubility behavior or desorption conditions. These uncertainties depending on the conditions of impregnation can lead to anomalies. For example a local excess of released BMP may activate bone resorption instead of bone formation⁴³. Adsorption on the contrary leads to stable association and the molecules can only be displaced by mineral ions and/or soluble proteins with a stronger affinity for apatite surfaces but in a predictable manner. Generally, the spontaneous release is very low and the active molecules show a sustained release related to the degradation of the biomaterial. It is probable, although this has not been systematically investigated, that the release rate is related to the nature of the apatite samples and their maturation stage. Another interesting property of adsorbed growth factors is their availability for cells

as demonstrated in the case of VEGF. In vivo studies have confirmed the efficiency and reproducibility of adsorbed BMP-2 in osteoinduction.

Cell adhesion on an apatite surface appears to be generally poorer than on specially treated surfaces like those of cell culture dishes. Therefore, several attempts have been made to favor cell attachment by using cell-binding proteins or specific protein sequences, such as RGD (arginine, glycine, aspartic acid), involved in cell attachment. The results generally show an improvement of cell adhesion and differentiation. It has been suggested that the osteoconductive properties of apatite could be due to the ability of its surface to bind such proteins.

CONCLUSION

This non exhaustive review of some adsorption studies, essentially made in our research groups, in the last years relates the progressive shift of our conception of the adsorption process from an electrostatic model based on previous considerations largely developed, to a more focused ion exchange model. The possibilities of interactions of a molecule, especially a macromolecule, with a mineral apatite surface are of course more numerous than the simple ion exchange reactions proposed here, but our experience suggests that this is a fundamental basic reaction determining the strong adsorptive properties of many molecules and bio-macromolecules of interest and explaining some specific behaviors like the effect of phosphate and calcium ions on the adsorption equilibrium and the apparent irreversibility on dilution. The observation of such ions exchange interactions needs a precise evaluation of the amount adsorbed and of the mineral ion content of the solution which is rarely made in most adsorption studies. Among the competitive reactions which have to be taken into account we have identified the apatite dissolution equilibrium and the surface equilibration reactions of the mineral in solution which involves essentially a surface hydrolysis of phosphate species associated with a release of calcium ions. These different equilibriums can lead to different but totally equivalent descriptions of the adsorption phenomena.

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