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Interaction between a bisphosphonate, tiludronate and nanocrystalline apatite: in vitro viability and proliferation of HOP and HBMSC cells

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Abstract
Nanocrystalline apatites (NCA) are the inorganic components of mineralized tissues and they have been recently proposed as biomaterials for drug delivery systems. Bisphosphonates (BPs) are currently the reference drugs used to treat diseases involving bone disorders such as osteoporosis. Nevertheless, the interaction phenomena between BP molecules and apatite nanocrystals of bone are not well understood. Therefore, the adsorption characteristics have been examined and cellular activity of tiludronate molecules on NCA as models of bone mineral has been investigated. Adsorption experiments of tiludronate onto NCA were carried out and revealed a Langmuir-type adsorption isotherm. The uptake of tiludronate molecules is associated with a release of phosphate ions, indicating that the main reaction is an ion exchange process involving surface anions. The results evidence the strong affinity of BP molecules for the apatitic surface. The interactions of NCA–tiludronate associations with human osteoprogenitor cells and human bone marrow stromal cells do not reveal any cytotoxicity and evidence the activity of adsorbed tiludronate molecules. Moreover, an evolution of the physico-chemical characteristics of the apatitic substrate during biological study was observed, highlighting the existence of dynamic interactions. This work contributes to clarifying the reaction mechanisms between BPs and biomimetic apatites.

1. Introduction

Although calcium phosphate stoichiometric hydroxyapatite Ca₁₀(OH)₂PO₄ is frequently considered as a model compound for biological mineralization, the most relevant model concerning bone minerals is certainly that of non-stoichiometric calcium phosphate nanocrystalline apatites (NCAs) exhibiting the general formula (Montel et al 1981)

\[ \text{Ca}_{10-x} (\text{PO}_4)_{6-x} (\text{HPO}_4)^{2-} \text{ or } \text{CO}_3^{2-})_x (\text{OH})_{2-x} \quad \text{with} \quad 0 \leq x \leq 2. \] (1)

These nanocrystals exhibit a special feature as shown in previous investigations using spectroscopic techniques (Rey et al 2007a). Recently, precipitated nanocrystals possessed a prominent structured hydrated layer on their surfaces, which is gradually converted into more stable apatite domains during ageing. This hydrated layer contains mineral ions in ‘non-apatitic’ environments which are easy to exchange and substitute reversibly by other ions in aqueous solutions. The adsorption properties of nanocrystals depend on the hydrated layer composition and the extent of this layer relates to the maturation stage of the nanocrystals. Biomimetic NCA are
used as bioactive biominerals for orthopaedic applications (Rey et al. 2007b) and have been recently proposed as biomaterials for drug delivery systems (Autefage et al. 2009, Palazzo et al. 2007).

Bone is a dynamic tissue which undergoes a continuous rebuilding process during life as a result of the coupled actions of bone cells: osteoclasts promote resorption of old tissue while osteoblasts initiate new tissue formation (Eriksen et al. 2007, Brandi and Collin-Osldoby 2006). However the equilibrium between bone resorption and formation differs in different phases of life and is strongly affected by bone diseases, such as osteoporosis, Paget’s disease and hypercalcemia (Meunier 2005, Rodan and Fleisch 1996). Bisphosphonates (BPs) are currently the most prescribed drugs for the treatment of diseases involving bone turn-over disorders (Leu et al. 2006, Russell et al. 2008). BPs were first studied over 30 years ago as substitutes for inorganic pyrophosphate; they are characterized by a non-hydrolysable P-C-P structure with two phosphonic acid groups bonded to the same carbon. Their mechanisms of action are due to the conjunction of physicochemical effects and biological activity (like pyrophosphate, BPs present a high affinity to adsorb onto apatite crystals, and have been shown to prevent mineral dissolution. In addition, they inhibit osteoclast activity and bone resorption (Russell and Rogers 1999, Leu et al. 2006).

The classic BP treatment is the systemic way of oral administration or intravenous injection. However, systemic use of BPs can result in undesirable side effects (Coleman et al. 2011) such as fever (Mönkkönen et al. 1998), gastrointestinal disturbances such as ulcers (Elliott et al. 1997) or osteonecrosis of the jaw, especially observed with intravenous preparations (Kos and Luczak 2009). Moreover a low bioavailability is commonly observed for oral administration (Hoffman et al. 2001). On this basis, the development of strategies for local administration of BPs at bone sites exhibiting a risk of fracture (vertebra, hip, wrist, etc) becomes even more interesting. In the literature, BPs have been associated with calcium phosphate ceramics (Denissen et al. 1994, Josse et al. 2005), cements (Panzavolta et al. 2009) or hydroxyapatite (McLeod et al. 2006, Boanini et al. 2007, Errasisti et al. 2010, Nancollas et al. 2006) and rarely adsorbed onto NCAs (Errasisti et al. 2009, 2010, Palazzo et al. 2007), though they mimic bone mineral more precisely than stoichiometric hydroxyapatite generally used. The aim of this work was to clarify the reaction mechanisms between a BP molecule, tiludronate and a biomimetic apatite.

Moreover, with the main action of BPs being their inhibitory effects on osteoclasts (Fleisch 2000, Russell et al. 2008), very few studies have indeed examined the direct action of adsorbed BPs on osteoblasts (Panzavolta et al. 2009, Boanini et al. 2007). Although the activities of these cells are strongly linked (Fleisch 2000), suggesting indirect effects, the direct interaction between BPs and osteoblasts cells, or more generally osteoblast precursors, has never been appraised using the nanocrystalline apatitic model, and must be therefore explored. In this work, we investigated more precisely the interaction of NCA/tiludronate associations on pre-osteoblastic cells.

![Figure 1. Molecular structure of tiludronate.](image)

2. Materials and methods

2.1. Materials

A NCA powder close to mature bone mineral composition was synthesized by the double decomposition technique at ambient temperature and physiological pH (Rey et al. 1989, 1995). NCA was prepared by pouring a calcium solution (Ca(NO3)2.4H2O, C = 69.6 g L–1, in 750 mL) into a carbonate phosphate solution (NH4)2HPO4, C = 80 g L–1, and NaHCO3, C = 60 g L–1, in 1500 mL). The precipitate obtained was left to mature for one month at room temperature. Subsequently, NCA was quickly vacuum-filtered, washed with deionised water and freeze-dried. The powder was then sieved (<125 μm) and stored in a freezer.

Powder of tiludronate (di-sodium(4-chlorophenyl)thio)methylene-bis-phosphonate) hemihydrate (figure 1) was a gift from Sanofi Aventis (France).

2.2. Adsorption

2.2.1. Sample preparation. In a polyethylene tube, 50 mg of poorly crystalline apatite powders were dispersed in 5 mL of an adsorption medium, an aqueous solution of tiludronate at different concentrations: 0, 0.5, 1, 1.5, 2 and 2.5 g L–1 in KCl (1 mM) (Errasisti et al. 2009, Al-Kattan et al. 2010). Then pH was adjusted at physiological pH by addition of KOH and HCl solutions. The suspensions obtained were sonicated for 5 minutes and incubated for 24 h at physiological temperature (37 °C) without stirring to reach an adsorption equilibrium, then centrifuged for 20 min (at 5000 rpm). The supernatants were filtered onto Millipore (0.2 μm) before chemical analyses. The powder was washed once with 5 mL of deionized water and freeze-dried, and stored in a freezer. It has been checked that a second washing with the same amount of water removed only a negligible amount of tiludronate.

2.2.2. Characterization techniques. The phosphate content of synthesized apatite sample was determined by spectrometry of the phosphovanadomolybdic acid complex (Charlot 1974). The calcium content was determined by complexometry with ethylenediaminetertraacetic acid (EDTA). The specific surface area of the sample was evaluated using the Brunauer–Emmett–Teller method (nitrogen adsorption) on a Quantachrome Instruments Monosorb Nova 1000. The average size of NCA aggregates d (0.5) was obtained using a laser granulometer (Malvern Mastersizer 2000). The amount of carbonate ions of the apatite powder was measured by coulometry.
techniques (UIC, Inc. CM 5014 coulometer with CM 5130 acidification unit). The solid phase was identified by x-ray diffraction (XRD) (Seifert XRD-3000TT diffractometer, CuKα radiation with λ = 1.5406 Å).

The characterization of the powders after adsorption was performed by different spectroscopic methods such as Fourier-transform infrared (FTIR), solid-state NMR and Raman microspectroscopy. FTIR experiments were performed on a Thermo Nicolet 5700 FTIR spectrometer using the KBr pellet method (2 mg sample/300 mg KBr); spectra were recorded in the 4000–400 cm⁻¹ range, at 64 scans accumulation and 4 cm⁻¹ resolution. Solid-state NMR experiments were recorded on a Bruker Avance 400 spectrometer equipped with a 4 mm probe. Samples were spun at 8 kHz at the magic angle using ZrO₂ rotors. For ³¹P MAS single-pulse experiments, small flip angles (~30°) were used with recycle delays of 20 s and with high-power proton decoupling conditions. ³¹P chemical shifts were referenced to an external 85% H₃PO₄ sample. Raman microspectroscopy analysis was carried out using a Horiba, Jobin Yvon Labram HR800 confocal microspectrometer, equipped with a helium–neon laser (λ = 632.82 nm), over a wavenumber range of 350–1750 cm⁻¹.

The tiludronate concentration in the solution was determined by UV absorption spectroscopy at 265 nm (single beam UV/Vis Hitachi U-1100 spectrophotometer). The amount of calcium and phosphate ions released upon adsorption was measured respectively by inductively coupled plasma atomic emission spectroscopy (Horiba, Jobin Yvon, 2004, Ultima 2, ICP-AES) and by spectrometry of the phosphovanadomolydbic acid complex.

2.3. Biological analyses

2.3.1. Sample preparation. For the substrates used in cell culture experiments, a large quantity of NCA with tiludronate was prepared by dispersing 0.5 g of apatite powder in 50 mL of adsorption medium (2 g L⁻¹ of tiludronate, 1 mM KCl) using experimental process previously described. The equilibrium concentration was about 1.57 mM. Several powder lots were mixed to obtain a homogenous sample. Subsequently, the powders were pressed by uniaxial pressing to form pellets (diameter 1 cm, weight 50 mg, at 190 MPa at room temperature) of NCA with and without BPs.

2.3.2. Isolation and culture of HBMSCs and HOPs. HBMSCs were obtained according to Vilamitjana-Amedee et al. (1993), with some modifications. Human bone marrow was aspirated during hip prosthesis surgery from the iliac crest of healthy donors aged between 20 and 50 years, in accordance with the French ethics committee. Cells were separated and counted. Sequential passages through syringes fitted with 16-, 18- or 21-gauge needles into a single suspension were the technique used to separate the cells. The suspension thus obtained was split into two samples, centrifuged for 15 min at 800 rpm and only one was resuspended in the Iscove Modified Dulbecco’s Medium (IMDM, Gibco, Invitrogen) supplemented with 10% (v/v) foetal calf serum (FCS, PAA) and 10⁻⁸ M dexamethasone (Sigma, St. Louis, MO, USA) in order to induce osteoblastic differentiation of adherent cells (Vilamitjana-Amedee et al 1993). These cells, called human osteoprogenitor cells (HOPs), express cbfa1/Runx2, ALP and type I collagen. Subculturing was carried out using 0.2% (w/v) trypsin and 5 mM EDTA.

To maintain undifferentiated HBMSCs in a non-oriented lineage, the pellet was resuspended with an alpha minimal essential medium (αMEM), (Invitrogen), supplemented with 10% (v/v) FCS. Cells were plated into 75 cm² cell culture flasks (Falcon) at a density of 5 × 10⁵ cells cm⁻² and incubated in a humidified atmosphere of 95% air, 5% CO₂ at 37 °C. Subculturing was performed as previously described.

2.3.3. Cell viability and proliferation assays. Cell viability was determined with the live/dead viability/cytotoxicity kit (molecular probes), according to the manufacturer’s protocol. Cell proliferation assays were carried out according to Mosmann’s technique (Mosmann 1983). All assays were replicated four times for each condition tested. The positive control used for cell proliferation was tissue culture polystyrene (TCPs) of plastic culture dishes. The pellets of materials were put on an agarose layer (2% (v/v)), prepared in 0.1M phosphate buffered saline (PBS at pH 7.4), to prevent cell attachment to plastic dishes. Then cells were seeded and incubated overnight at 37 °C in the corresponding medium of each cell type. Thereafter, pellets were seeded with a density of 30 × 10⁵ cells per cm², cultured in the IMDM for HOPs or in αMEM for HBMSCs supplemented with 10% (v/v) FCS and incubated at 37 °C in a humidified atmosphere. After 1, 3, 7, 15 and 21 days, the cell metabolic activity was measured by the MTT assay in order to quantify the cell growth. The MTT assay is an observation technique based on the ability of mitochondrial enzyme of viable cells to metabolize a water-soluble tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide into an insoluble formazan salt. The MTT solution was incubated at 37 °C for 3 h, removed and insoluble formazan crystals formed were dissolved in dimethylsulfoxide. Then, 100 μL of this solution was aspirated and poured into another 96-well plate to measure the absorbance at 540 nm. This technique was allowed to quantify directly the cell proliferation because the intensity of the staining solution obtained is proportional to cell proliferation. The scanning electron microscope (SEM) examination was performed using a Hitachi S-2500 SEM with a LaB₆ filament on samples gold-coated beforehand, in order to observe the morphology of the cells seeded onto the surface of the materials and the formation of a cell layer.

3. Results and discussions

3.1. Powder characterization

The solids obtained exhibited XRD patterns (figure 2) characteristic of poorly crystalline apatitic calcium phosphates (Bichert et al 2009) and no additional crystalline solid phase was observed after tiludronate adsorption. Chemical analysis of NCA gave a CO₃²⁻ concentration of 5.1 ± 0.4% wt
Figure 2. XRD diagrams for nanocrystalline apatite (NCA) powders before and after adsorption: (a) NCA and (b) NCA+BP (equilibrium concentration at about 1.57 mM).

Figure 3. FTIR spectra for NCA powders before and after adsorption: (a) BP, (b) NCA and (c) NCA+BP (equilibrium concentration at about 1.57 mM).

(C/P ratio of 0.17) and Ca/P ratio of 1.61 characteristic of a calcium-deficient apatite (CDA). The specific surface area was evaluated to be 156 ± 9 m² g⁻¹ and the average size of NCA aggregates d (0.5) was 31 µm. The nanocrystal size was determined by XRD using Scherrer’s equation (Scherrer 1918): average length about 16.7 nm and average width thickness about 4.3 nm.

FTIR spectra of apatitic powders synthesized before adsorption (figure 3) showed characteristic vibration bands of poorly crystalline apatite: especially apatitic PO₄ bands at 469 (v2), 562–603 (v4), 962 (v1) and 1000–1104 cm⁻¹ (v3) (Rey et al 2007a; Eichert et al 2009) and the apatitic HPO₄²⁻ band (550 cm⁻¹). Other vibration bands reveal the presence of non-apatitic environments of PO₄³⁻ (617 and 634 cm⁻¹) and HPO₄²⁻ (530 cm⁻¹) ions in the surface hydrated layer (Bóhis et al 2000). This NCA model presents bands at 870, 1420 and 1470 cm⁻¹ due to CO₃ vibrations characteristic of non-apatitic carbonates and type B carbonate species. The original tiludronate molecule presents several lines, particularly those associated with aromatic ring chains at 1480, 1500, 1580 and 1620 cm⁻¹, C–H and C–S vibrations at 1390 and 820 cm⁻¹ and 710 cm⁻¹, respectively (Bonney et al 1994). After adsorption, the spectrum of NCA displayed additional lines and shoulder characteristic of tiludronate molecules at 1480 and 820 cm⁻¹, especially supporting the presence of a tiludronate on the precipitate.

The Raman spectroscopic study complemented FTIR data. The Raman spectrum of tiludronate (figure 4, table 1) exhibited four main regions: the first one between 550 and 800 cm⁻¹ characteristic of ν(C–Cl) bands, the second one containing the ν(C–S) aliphatic bands in the 630–790 cm⁻¹ region, the third one at 1072 and 1097 cm⁻¹ corresponding to the two phosphonic acid groups and the last one is assigned to the aromatic ring chain at 1010, 1470, 1565 and 1600 cm⁻¹ (Bonner et al 1994, Wood 1930, Wood and Collins 1932). The Raman spectrum of powders obtained after adsorption (figure 5) showed the characteristic bands of the apatitic structure (Penel et al 1998), especially PO₄ bands at 469 (v2), 562–603 (v4), 962 (v1).

Table 1. Raman bands observed for tiludronate.

<table>
<thead>
<tr>
<th>Domain, Assignments</th>
<th>Raman (cm⁻¹)</th>
</tr>
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<tbody>
<tr>
<td>ν(C–Cl)</td>
<td>550–800</td>
</tr>
<tr>
<td>ν(C–S) aliphatic</td>
<td>630–790</td>
</tr>
<tr>
<td>ν(C–S) aromatic</td>
<td>1080–1100</td>
</tr>
<tr>
<td>ν(PO₄³⁻)/ν(PO₄²⁻)</td>
<td>1072, 1097</td>
</tr>
<tr>
<td>ν(C–C) aromatic ring vibrations</td>
<td>1010, 1470, 1565, 1600</td>
</tr>
</tbody>
</table>
and 1000–1104 cm⁻¹ (v3) (Rey et al. 2007a, Eichert et al. 2009) and CO₃²⁻ bands at 1070 cm⁻¹. Additional lines at 746 and 1575 cm⁻¹ corresponding to C–S and aromatic ring chain vibrations of tiludronate can be assigned to tiludronate. Unfortunately, in FTIR and Raman spectra other lines of tiludronate, especially those of phosphonate groups, probably involved in the tiludronate/NCA association, are hidden by those of phosphate groups of NCA. In order to obtain more information on such interactions a solid-state NMR study was carried out.

The ³¹P NMR spectra of tiludronate, NCA and tiludronate/NCA association are shown in figure 6. The spectrum of tiludronate shows two main lines at 15 and 19.3 ppm attributed to the ³¹P nuclei of the two phosphate groups of BPs, chemically non-equivalent (Errassifi et al. 2009). The spectrum of NCA is characterized by a single asymmetric broad line at 3.3 ppm due to PO₄³⁻ and HPO₄²⁻ species (Eichert et al. 2004). The spectrum of tiludronate/NCA association presents, in addition to the phosphate line of NCA, a weaker broad line at 15.7 ppm at about a chemical shift of phosphonate groups of tiludronate. Such broad lines have already been observed in a previous study by Mukherjee et al. (2008) concerning the adsorption of several BPs bound to bone. The broadness of this line very different from that of tiludronate Na₂PO₃ suggests a lack of crystalline structure and seems in agreement with adsorbed BPs (Grossmann et al. 2000).

3.2. Analytes of the solution

The evolution of the amount of tiludronate adsorbed from dilute solutions (0–6.7 mM) as a function of its remaining concentration in the solution is plotted in figure 7. The adsorption isotherms obtained are Langmuirian in shape in agreement with a previous study on another BP, risedronate (Errassifi et al. 2010). The adsorption plateau was reached at relatively low equilibrium concentrations (about 1 mM) corresponding to a tiludronate load of 11 wt%, highlighting the high affinity between tiludronate molecules and the NCA surface. Similar findings have been reported for the adsorption on NCA of biological macromolecules such as albumin, heparin, growth factors and risedronate molecules (Errassifi et al. 2010). The affinity constant (K) and the amount adsorbed at saturation (N) were determined using a Langmuir linear regression (Langmuir 1918) as shown in figure 7:

\[
\frac{C_{eq}}{Q_{ads}} = \frac{C_{eq}}{N} + \frac{1}{(K \times N)}
\]

with \(C_{eq}\) (mmol L⁻¹) being the equilibrium concentration in the solution of tiludronate, \(Q_{ads}\) (μmol m⁻²) the amount adsorbed per unit area, \(N\) (μmol m⁻²) the amount adsorbed at saturation, \(K\) (L mmol⁻¹) the affinity constant of tiludronate for the NCA surface.

The values of K and N were respectively 10.14 ± 0.02 L mmol⁻¹ and 2.17 ± 0.01 μmol m⁻². These values are close to the values previously obtained for the adsorption of risedronate molecules using similar experimental conditions (Errassifi 2011, Al-Kattan et al. 2010). In addition, these studies have shown that higher amounts of BP adsorbed at saturation were obtained on NCA compared to well-crystallized stoichiometric hydroxyapatite. This phenomenon has been attributed to the presence of a surface hydrated layer on NCA offering more flexibility for molecule substitutions or incorporations than a patterned rigid mineral surface, like in a well-crystallized apatite. The affinity constant has been found to vary strongly depending on the nature of the adsorbing molecules (Nancollas et al. 2006) and NCA surface characteristics (Ouizat et al. 1999). The values obtained here confirm the high affinity of tiludronate for the NCA surface.

The calcium ion concentration in the solution was very low and weakly altered by the adsorption reaction (data not shown). It has been assumed to be mainly related to apatite dissolution equilibrium (Errassifi et al. 2010, Al-Kattan et al. 2010). A correlation exists between phosphate released in the solution and tiludronate adsorbed by the NCA surface (figure 8). In fact, the uptake of tiludronate is proportional to the amount of phosphate ions released in the solution (slope of 1.3 ± 0.1). Thereby, the adsorption of tiludronate onto NCA seems to be a chemisorption process corresponding to
an ion exchange between $\text{PO}_3$ anionic ends of tiludronate molecules in the solution and phosphate species from the NCA surface and/or carbonates. This phosphate exchange has already been observed for risedronate adsorbed onto NCA and hydroxyapatite by Errassifi et al (2009) and for zoledronate bound to $\beta$TCP and CDA by Roussiere et al (2008). For example, the study of the adsorption of risedronate molecules onto hydroxyapatite showed an exchange process with close to the one-to-one substitution process (Al-Kattan et al 2010, Errassifi 2011). The exchange ratio appears different here and could depend on the composition of the hydrated layer, on the speciation of BP ions at the surface and, probably, on the surface zeta potential variations. Other explanations could be proposed for the release of phosphate ions and Josse et al (2005), for example, have shown that for zoledronate interaction with calcium phosphate compounds, at high BP concentrations, crystalline calcium zoledronate needles were formed corresponding to a dissolution–precipitation mechanism. However, in our case, no foreign phase formation was detected. In addition, it shall be noted that in the case of dissolution–precipitation, a Langmuir isotherm would not have been obtained (Errassifi et al 2010).

3.3. Cell viability and differentiation

Live/dead assays performed on cellularized materials revealed that cells kept their viability over the time of cell culture (figure 9). This is corroborated by SEM examination (figure 10). These results highlight the absence of cytotoxicity of NCA, whatever the cell types. Cells kept their viability with time of culture onto these functionalized materials. Moreover, the morphology of the cells on pellets containing tiludronate evidences the absence of cytotoxicity of the drug at the concentration used. Cells remain spread on the materials and exhibit filipodia. These qualitative data were confirmed by the quantitative analysis and cell proliferation study.

Our results indicated that HOPs and HBMSCs exhibit similar kinetics of cell proliferation on both types of scaffolds (figure 11). Based on the MTT assay, tiludronate stimulated cellular proliferation whatever the nature of the cells (figure 11). The effect of tiludronate is evidenced on the two cell types throughout the culture time, from D1 to D21. Here, these results give evidence of tiludronate actions on undifferentiated cells, i.e. HBMSCs as was demonstrated for MG63, an osteoblastic cell line (Panzavolta et al 2009, Boamini et al 2008) and for human trabecular differentiated bone cells (Im et al 2004) with different BPs. In addition,
nanocrystals towards more crystalline stoichiometric structure was expected in the aqueous solution (Rey et al. 2007b).

The pellets were analysed after cell experiments by FTIR spectroscopy. In the ν₄ PO₄ region, five main bands were detected for poorly crystalline synthetic apatite samples (Eichert et al. 2009), as in bone mineral (Bohic 2000): a shoulder at 617 cm⁻¹ assigned to labile phosphate groups, three bands at 600, 575 and 560 cm⁻¹ due to phosphate ions in an apatitic environment and a shoulder at 533 cm⁻¹ to non-apatitic HPO₄ ion vibration, as explained previously. Curve fitting was done with the GRAMS/386 program (Galactic Industries, Salem, NH), fixing or varying parameters (position and width of apatitic and non-apatitic bands) to provide the best curve fit. The band shape was considered Lorentzian in all instances and the baseline was always linear. The relative band integrated intensities were defined as the ratio of peak area to total area of the phosphate bands. Figure 12 reports the relative band integrated intensity for labile PO₄ and HPO₄ ions. These values decreased for non-apatitic HPO₄ ions with cell test times from 0 to 21 days, whatever the type of cells. These results are in agreement with the observed evolution of NCA with maturation time in the solution (Eichert et al. 2009). Although such modifications of the surface composition probably have an effect on molecule adsorption and cell behaviour, this point has to be further investigated.

3.4. Characterization of the pellets after cells experiments

As supports used for osteoblastic tests were non-stoichiometric poorly crystallized apatites, a physico-chemical evolution of these data confirmed qualitative results obtained after 21 days (figures 9 and 10) showing spreading cells on NCA+BPs and the formation of a cell layer on these substrates.

Figure 12. Evolution of labile PO₄⁻² and HPO₄²⁻ contents with different time in culture medium: time 0 (D0); 3 days (D3) and 21 days (D21) with HOP (a) and HBMSC (b) cells for NCA, as determined by FTIR spectroscopy.
4. Conclusion

This work contributes to clarify the reaction mechanisms between tiludronate and biomimetic apatites. The adsorption characteristics were examined and the results obtained highlight the strong affinity of bisphosphonate (BP) molecules for the NCA surface. Spectroscopic techniques on solids such as the FTIR and Raman appear to be relevant methods to demonstrate molecular adsorption. Generally, adsorption studies of bone-bonding bioactive molecules or drugs are performed on well-crystallized hydroxyapatite and this work proposes models of bone nanocrystals with controlled physico-chemical characteristics and surface properties, allowing the screening of drugs in repeatable conditions closer to the biological situation. Whereas most papers investigate cell activity of BPs in the solution (Von Knoch et al. 2005, Im et al. 2004, Fromigue and Body 2002), our data clearly show that adsorbed molecules like tiludronate exhibit a biological effect, specifically a stimulation of cell proliferation of both cell types used in this work, HOPs and HBMSCs. Such findings appear important for the development and applications of drug delivery systems based on apatite nanocrystals and they have to be considered in bone mineral interactions with drugs. In further studies, the influence of these NCA characteristics (composition, crystallinity, etc) and thus the nature of the models used on the adsorption properties and cells response must be investigated.

Acknowledgments

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