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# Adsorption and Release of BMP-2 on Nanocrystalline Apatite-Coated and Uncoated Hydroxyapatite/ $\beta$ -Tricalcium Phosphate Porous Ceramics

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**Abstract:** The association of bone morphogenetic proteins (BMPs) with calcium phosphate bioceramics is known to confer them osteoinductive properties. The aim of this study was to evaluate the surface properties, especially regarding recombinant human BMP-2 (rhBMP-2) adsorption and release, of commercial sintered biphasic calcium phosphate ceramics after coating with biomimetic nanocrystalline apatite. The raw and coated ceramics exhibited similar macroporous structures but different nanometer-sized pores contents. Both types of ceramics showed Langmuir-type adsorption isotherms of rhBMP-2. The coating noticeably increased the rate of adsorption and the total amount of growth factor taken up, but the maximum coverage per surface area unit as well as the affinity constant appeared lower for coated ceramics compared with raw ceramic surfaces. The limited advantage gained by coating the ceramics can be assigned to a lower accessibility of the surface adsorption sites compared with the raw ceramics. The quantity of rhBMP-2 spontaneously released in cell culture medium during the first weeks was lower for coated samples than for uncoated ceramics and represented a minor fraction of the total adsorbed amount. In conclusion, the nanocrystalline apatite coating was found to favor the adsorption of rhBMP-2 while providing a mean to fine tune the release of the growth factor.

**Keywords:** bone morphogenetic protein 2 (BMP-2); bioceramics; biphasic calcium phosphate (BCP); nanocrystalline apatite; protein adsorption and release

## INTRODUCTION

One of the most efficient ways to improve the bone forming ability of biomaterials is their association with bone morphogenetic proteins (BMPs). Such growth factors, BMP-2 and BMP-7, the two most efficient variants of BMP growth factors available, have been associated with different types of biomaterials for orthopedic applications (collagen, calcium phosphate (Ca-P) cements, polymers,

ceramics).<sup>1</sup> Although calcium phosphates are among the most frequently used biomaterials for bone reconstruction, little is known about their association with BMP.<sup>2,3</sup> Regarding porous ceramics, the growth factors are generally associated with the implants by simple impregnation and drying and the type of bonding with the substrate and the release rate are often undetermined.<sup>4-7</sup> This lack of knowledge could explain divergences in the reported effect of such associations. Most studies testify to an improvement of bone formation with an acceleration of repair<sup>4-7</sup>; however, a few studies have mentioned a resorptive process of bone attributed to a stimulation of osteoclast activity.<sup>8,9</sup> Recently, a few studies have been published on the adsorption characteristics of BMP-2 on apatitic calcium phos-

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phate.<sup>2,3</sup> In a first study,<sup>2</sup> the adsorption isotherms have been drawn and the adsorption parameters determined. The data show a strong affinity of BMP-2 for apatite surfaces, enhanced in the presence of calcium ions as in the case of the adsorption of other proteins.<sup>10–12</sup>

The second work on this topic<sup>3</sup> is based on the use of theoretical modeling of the interactions of surfaces (in this case, the (001) apatite plane) with functional groups of BMP-2. The results indicate a preferential interaction between carboxylic functional groups from the protein and calcium ions from the apatitic surface.

These experiments however do not give information on the behavior of sintered bioceramics and do not evoke the crucial question of the release of the growth factor in biological medium. Biphasic ceramics composed of hydroxyapatite (HA) and  $\beta$ -tricalcium phosphate ( $\beta$ -TCP) have been commercialized for more than 15 years as bone substitute materials. They have been shown to combine an excellent biointegration with a bioresorption capable of being modulated according to the  $\beta$ -TCP content.<sup>13</sup> Because of high sintering temperatures, these ceramics generally exhibit a very low surface reactivity. Although natural osteoinductive properties have been suggested for these compounds,<sup>14,15</sup> they occur erratically and do not seem to be related to identified surface characteristics.<sup>15</sup> However, microporosity has been suggested to be an important factor likely to favor the binding of circulating growth factor on the material surface.<sup>16,17</sup>

This contribution aims at investigating the potential increase of the surface reactivity of sintered ceramics obtained by coating their surface with highly reactive biomimetic nanocrystalline apatites.<sup>18</sup> A very thin layer, a few micrometers thick, should not alter the porous structure of the ceramic and could enhance its adsorption capabilities with regard to growth factors such as rhBMP-2. This first report has particularly focused on comparing the adsorption properties of rhBMP-2 on sintered biphasic ceramics before and after coating with nanocrystalline apatite, while keeping similar macroporous structures and different nanometer-sized pore contents. The release of rhBMP-2 was evaluated in a biological fluid model, namely Dulbecco's modified Eagle's medium, in anticipation of subsequent studies on *in vitro* and *in vivo* behavior of rhBMP-2-containing bioceramics.

## MATERIALS AND METHODS

### Materials

Biphasic calcium phosphate (BCP) ceramics (hydroxyapatite/ $\beta$ -tricalcium phosphate, 65/35) were produced by Teknimed S.A. (l'Union, France). These highly porous BCP blocks (65% porosity) were crushed and sieved to obtain randomly shaped 1–1.4 mm diameter granules (12/10 powder, CERAFORM).

These granules were coated, when needed, with a nanocrystalline apatite layer, a few micrometers thick, using a process being patented (Teknimed, l'Union, France). This

layer represented  $1.6\% \pm 0.1\%$  of the samples weight. The nanocrystalline apatite deposit was found to settle on both the external surface of the granules and the inner pore surface, shown by scanning electron microscopy (SEM) observations (Figure 1).

### Characterization of the Nanocrystalline Apatite

Although many applications and materials containing nanocrystalline apatite are tested and produced nowadays,<sup>19</sup> they are generally not thoroughly characterized from a physicochemical point of view, despite the demonstration that adsorption properties<sup>20,21</sup> and other properties such as dissolution<sup>22</sup> depend strongly on the type of nanocrystals considered. The composition of the nanocrystalline apatite used to coat the granules in this work was determined by chemical analysis: calcium was titrated by complexation with ethylene diaminetetraacetic acid (EDTA)<sup>23</sup>; phosphate ions were quantified by spectrophotometry using a phosphovanadomolybdic complex<sup>23</sup>; the carbonate content was measured by coulometry (UIC.Inc CM5014). The nanocrystals were also characterized by X-ray diffraction (XRD, Inel CPS 120 diffractometer) using a Co anticathode ( $\lambda = 1.78892 \text{ \AA}$ ) and Fourier transform infrared (FTIR) spectroscopy (Nicolet 5700), by transmission, using the KBr pellet method. Decomposition of  $v_3\text{CO}_3$  and  $v_4\text{PO}_4$  was performed from FTIR spectra using GRAMS curve-fitting software.<sup>24</sup> Crystal morphology was observed by high-resolution transmission electron microscopy (TEM) at 480 and 3200 kV using a Jeol Jem 2100F microscope.

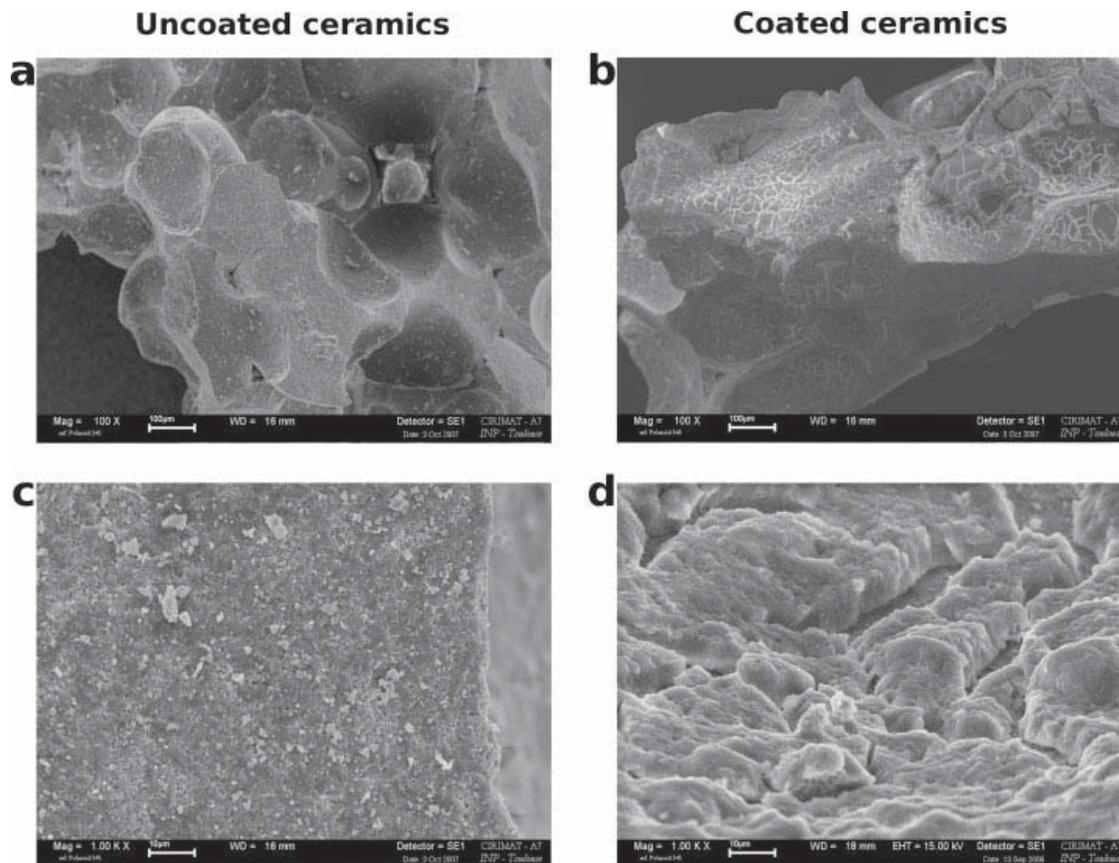
### Characterization of the Granules

Morphological characterization of the coated and uncoated granules was carried out by SEM using a Leo 435 VP microscope. The specific surface area was measured by the Brunauer-Emmett-Teller (BET) method using nitrogen adsorption (Quantachrome Nova 1000). The porosity of coated and uncoated granules was analyzed using a mercury intrusion porosimeter (Micromeritics Autopore III) between 8 and 60,000 psi. Considering the complex shapes of the granules, the adhesion strength of the coating could not be determined by usual methods (ISO 2409 standards).

### rhBMP-2 Labeling

The recombinant human BMP-2 (rhBMP-2), expressed in *E. coli*, was supplied by Würzburg University (Pr. W. Sebald).

rhBMP-2 was labeled by  $^{125}\text{I}$  using a modified chloramine T method previously described.<sup>25</sup> Briefly, 10  $\mu\text{g}$  of rhBMP-2 was dissolved in 50  $\mu\text{L}$  of a 0.25M phosphate buffer pH 7.5 and mixed with 10  $\mu\text{L}$  of  $\text{Na}^{125}\text{I}$  (1 mCi). The reaction was initiated by adding 5  $\mu\text{L}$  of a chloramine T solution (0.1 mg/mL of phosphate buffer). After an incubation period of 2 min at room temperature, a second 5  $\mu\text{L}$  aliquot of chloramine T solution was added, and after 1.5



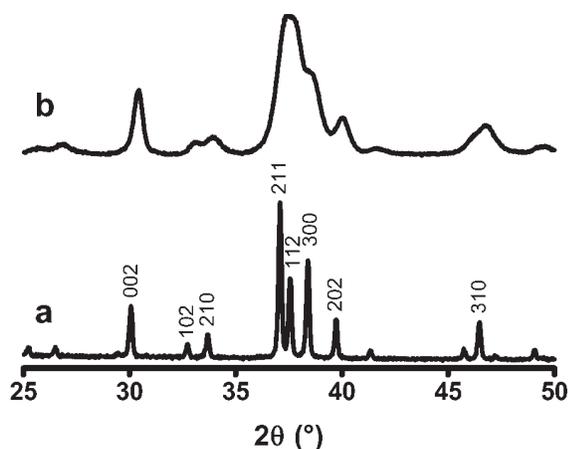
**Figure 1.** SEM micrographs of uncoated [magnification  $\times 100$  (a) and  $\times 1000$  (c)] and coated granules [magnification  $\times 100$  (b) and 1000 (d)]. The 2- to 5- $\mu\text{m}$ -thick coating settles on the porous surface.

min a last 5  $\mu\text{L}$  aliquot was added and left for 1 min. The reaction was stopped with 20  $\mu\text{L}$  of 50  $\text{mM}$  L-tyrosine, 200  $\mu\text{L}$  of potassium iodide (60  $\text{mM}$ ), and 200  $\mu\text{L}$  of ultrapure urea (1.2  $\text{mg}/\text{mL}$  in acetic acid 1 $M$ ).  $^{125}\text{I}$ -rhBMP-2 separation was carried out using a previously equilibrated chromatography Sephadex G25 column. Labeled proteins were eluted with a 4  $\text{mM}$  HCl, 75  $\text{mM}$  sodium chloride, and 0.1% bovine serum albumin (BSA) solution.

#### rhBMP-2 Adsorption on BCP Granules

**Adsorption Isotherms.** The adsorption protocol used in these experiments was based on preliminary results showing an increase of the maximum adsorbed amount of several proteins, including BMP, in the presence of calcium ions.<sup>2,10–12</sup> The solutions used for the adsorption experiments were prepared just before their incubation with granules. They were obtained by diluting a rhBMP-2 solution with a buffer solution (NaCl 1 $M$ , Tris 25  $\text{mM}$ , and  $\text{CaCl}_2$  10  $\text{mM}$ , pH 7.4). The  $^{125}\text{I}$ -rhBMP-2 was then added to the solution to reach a  $^{125}\text{I}$ -labeled-to-unlabeled protein ratio of 1:2000. The adsorption experiments were performed at 37°C in batches containing 20  $\text{mg}$  of coated or uncoated ceramic granules immersed into 40  $\mu\text{L}$  of rhBMP-2 solution. After 24 h, the supernatant was removed and the free

radiolabeled molecules in solution were counted. The amount of rhBMP-2 adsorbed was determined from the quantity of  $^{125}\text{I}$ -rhBMP-2 remaining in solutions. One of the main problems using BMP-2 solutions at physiologic pH is precipitation. The concentrations of rhBMP-2 varied from 1000 to 150  $\mu\text{g}/\text{mL}$  (39.9–6  $\mu\text{M}$ ). In this range, no precipitate was observed in the solutions. To confirm that the decrease of the amount of BMP-2 in solution was solely due to adsorption and not to precipitation from an unstable solution, a second control of the amount adsorbed was performed on the ceramic itself: the granules were washed three times with 1  $\text{mL}$  of a solution saturated with respect to  $\beta$ -TCP (the most soluble phase of the BCP ceramics) to eliminate nonadsorbed  $^{125}\text{I}$ -rhBMP-2 (including possibly precipitated BMP-2) without dissolving the granules and the coating and dried at ambient temperature for 72 h. These granules were then dissolved in perchloric acid 6 $N$  and the  $^{125}\text{I}$ -rhBMP-2 present in the solutions was counted. Both methods gave very close results, thus confirming the bonding of BMP-2 on the ceramic surfaces and the absence of bias related to a possible BMP-2 precipitation. We will report only the data obtained by analysis of the supernatant solutions carried out in duplicate. Results were expressed as mean  $\pm$  standard deviation (SD).



**Figure 2.** X-ray diffraction pattern of hydroxyapatite (a) and apatite nanocrystals used for coating the granules (b).

**Adsorption Kinetics.** The adsorption experiments were performed at 37°C in batches containing 30 mg of coated or uncoated ceramics immersed into 60 μL of rhBMP-2 solution at 300 μg/mL (adsorption solution). After 15 min and up to 24 h, the supernatant was removed and the free radiolabeled molecules in solution were counted. Each assay was carried out in triplicate. Results were expressed as mean ± standard error of the mean (SEM).

**rhBMP-2 Release.** After removal of the radiolabeled adsorption solution, the granules were washed and dried as described earlier. Two samples were dissolved in perchloric acid, and the <sup>125</sup>I-rhBMP-2 contained in the dissolution solutions was counted to determine the initial amount of adsorbed rhBMP-2. Three other samples were used for the release experiment. The desorption kinetics were monitored in 1 mL of culture medium (Dulbecco's modified Eagle's medium) complemented with 10% of fetal bovine serum and 1% of penicillin and streptomycin at 37°C. The medium was removed and replaced by a fresh one after 2 min and at irregular intervals (12 times) over a period of 21 days. The radiolabeled molecules released in solution were counted. The loss of radioactivity because of the half-time life of <sup>125</sup>I was considered in the determination of the amount of protein released. Assays were carried out in triplicate. Results were expressed as mean ± SEM. It shall be noticed that the amount of BMP released was always much lower than the solubility limit of nonglycosylated rhBMP-2 in physiologic conditions.<sup>26</sup>

**Statistical Analysis.** The experiments performed in triplicate were compared using the Student's *t*-test. A probability value (*p*-value) of less than 0.05 was considered significant.

## RESULTS

### Characterization of the Nanocrystals

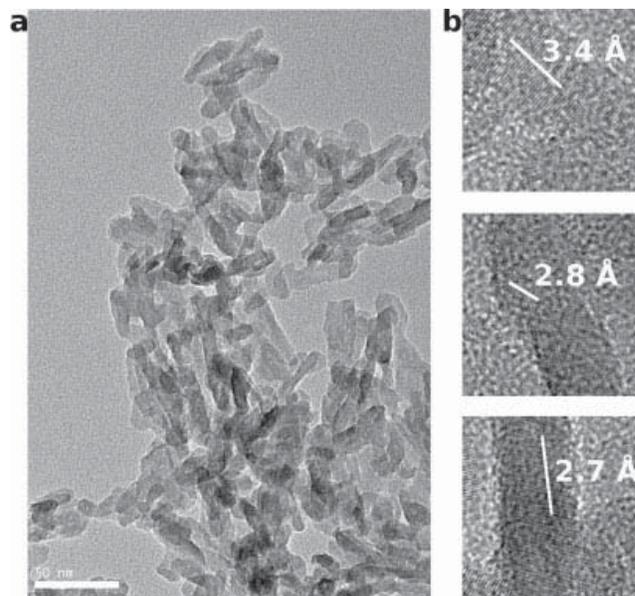
The XRD pattern of the nanocrystals used for coating the samples (Figure 2) was characteristic of a poor crystalline

apatite with broad diffraction peaks. The average crystal size was estimated using Scherrer's formula. The nanocrystals appeared elongated along the *c*-axis of the hexagonal structure ( $245 \pm 5$  Å average length determined from the (002) peak at  $2\theta = 30.4^\circ$ ). The average width thickness was  $102 \pm 3$  Å (from the (310) peak at  $2\theta = 46.6^\circ$ ).

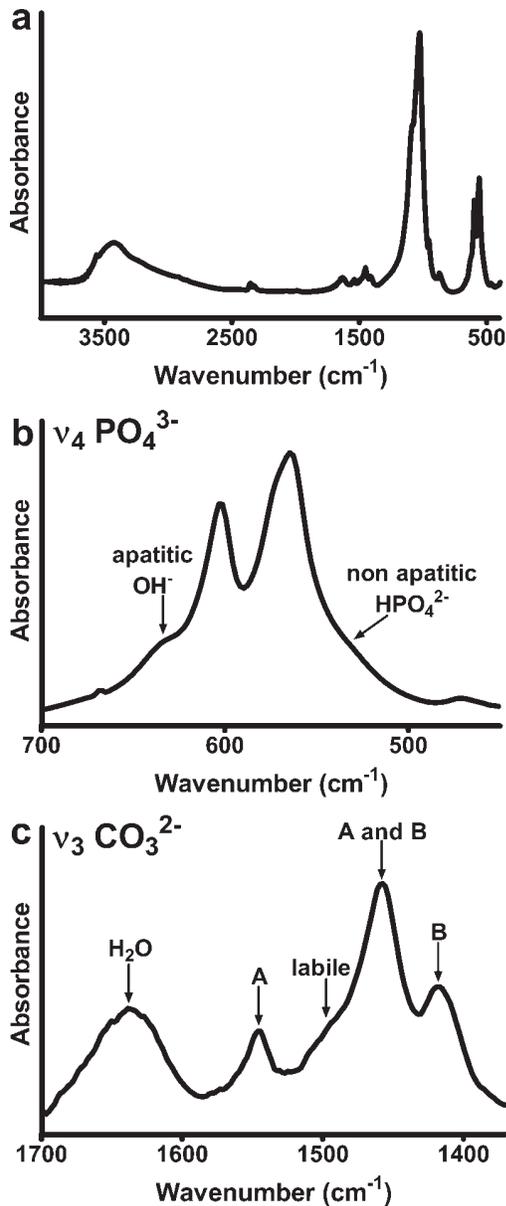
High-resolution TEM observations (Figure 3) of the coating confirmed the nanocrystalline nature of the apatite. The nanocrystals appeared as irregularly shaped platelets often agglomerated. The different *d*-spacings between interference fringes corresponded to the apatite structure: 3.4 Å assigned to the (002) lattice plane, 2.7 Å to (300) plane, and 2.8 Å to (211) lattice plane.

As shown by chemical and FTIR analyses (Figure 4), these nanocrystals were composed of a carbonated, calcium-deficient apatite phase. The atomic Ca/P molar ratio was  $1.51 \pm 0.01$ ; the carbonate content reached  $1.50\% \pm 0.02\%$ . The study of the IR bands characteristic of carbonate species  $\nu_2$  ( $850\text{--}900\text{ cm}^{-1}$ ) and  $\nu_3$  ( $1350\text{--}1600\text{ cm}^{-1}$ ) suggested type A (carbonate substituted for OH) and type B (carbonate substituted for phosphate) carbonate incorporation in the apatite structure, with a very few carbonate ions on nonapatitic sites at the crystal surface.<sup>27</sup> The curve fitting of the bands characteristic of carbonate species  $\nu_3\text{CO}_3$  indicated that the ratio "labile  $\text{CO}_3/\text{total CO}_3$ " reached 0.30. The spectra also revealed the presence of  $\text{OH}^-$  and  $\text{HPO}_4^{2-}$  ions [Figure 4(b)]. These results indicate the presence of a very thin hydrated layer at the surface of the apatite crystals, as already discussed elsewhere.<sup>18</sup>

**Granules Characterization.** The specific surface area of uncoated granules was found to be rather low ( $0.44 \pm 0.09\text{ m}^2/\text{g}$ ), as usually observed for sintered ceramic materials,



**Figure 3.** TEM micrographs of the nanocrystals (a) and amplifications of the interference fringes (b).



**Figure 4.** FTIR spectrum of the nanocrystalline apatite (a) and expansions of  $\nu_4$   $\text{PO}_4^{3-}$  domain (b) and  $\nu_3$   $\text{CO}_3^{2-}$  domain (c).

whereas that of coated samples reached  $2.03 \pm 0.09 \text{ m}^2/\text{g}$ . Thus, the nanocrystalline coating led to a 4.7-fold increase in the specific surface area of the samples. This significant increase seems consistent with the number of nanocrystals deposited, considering their specific surface area close to  $84 \pm 10 \text{ m}^2/\text{g}$ , and suggests that most of the initial surface of the nanocrystal agglomerates continues to be accessible for nitrogen molecule adsorption.

The porosity data [Figure 5(a)] of uncoated samples revealed three types of pores: macropores (with diameters greater than  $100 \mu\text{m}$ ) and microsized pores ( $10 \mu\text{m}$  and  $100 \text{ nm}$ ) related to sintering defects. The coated samples exhibited the same porosity characteristics, but an additional type of (nanosized) pore was also evidenced, characterized

by a rather narrow size distribution (ranging from 9 to 21 nm, with a maximum at 14.3 nm). Thus, it can be noted in particular that the micron-thick coating did not alter the starting porosity of the sintered ceramic, including the microsized pores. The additional pore-type observed on the coated samples can be assigned to internanocrystal spaces. SEM observations [Figure 5(b)] confirmed the existence of different levels of porosity on the raw samples. On the coated samples, the micron-thick layer appeared to cover the macropore wall, although some discontinuities and cracks were observed.

**rhBMP-2 Adsorption.** The adsorption rates and adsorption isotherms are represented in Figures 6 and 7 for coated and uncoated samples, respectively.

As shown in Figure 6, the coating significantly increased the amount of growth factor uptake. Thus, after 24 h, coated granules adsorbed  $517 \pm 9 \mu\text{g}$  of rhBMP-2 per gram of granules, whereas uncoated ceramics adsorbed  $397 \pm 19 \mu\text{g/g}$  ( $p < 0.005$ ). The initial rate of adsorption appeared also faster for coated granules than for uncoated ones:  $444 \mu\text{g}/(\text{g}\cdot\text{h})$  versus  $185 \mu\text{g}/(\text{g}\cdot\text{h})$  and the adsorption limit was reached much faster.

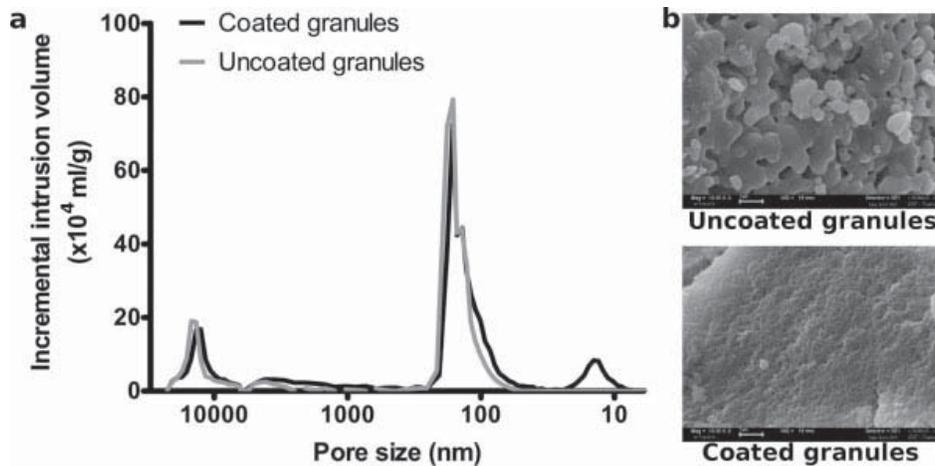
The adsorption isotherm exhibited a characteristic Langmuir shape, as often observed in the adsorption of various proteins on apatites<sup>2,20,28</sup> (Figure 7). The isotherms were correctly described by the linearized expression of the Langmuir isotherm:

$$C_{\text{eq}}/Q = C_{\text{eq}}/N + 1/(N \cdot K)$$

where  $C_{\text{eq}}$  and  $Q$  indicate the concentration at equilibrium and the amount of adsorbed protein, respectively, either per surface unit or per gram of granules depending on data description. The parameters  $K$  and  $N$ , expressed as  $K \pm \Delta K$  and  $N \pm \Delta N$ , represent the affinity constant of the adsorption equilibrium and the maximum adsorbed amount, respectively. The affinity constant of the coated granules seemed slightly lower than that of the uncoated ones ( $0.007 \pm 0.002 \text{ mL}/\mu\text{g}$  vs.  $0.011 \pm 0.006 \text{ mL}/\mu\text{g}$ ). The theoretical maximum quantity of rhBMP-2 absorbed was higher for coated ceramics ( $1608 \pm 170 \mu\text{g/g}$ ) than for uncoated ones ( $758 \pm 68 \mu\text{g/g}$ ). However, considering the maximum amount adsorbed per surface area unit, it appeared lower for coated samples ( $0.032 \pm 0.003 \mu\text{mol}/\text{m}^2$ ) than for raw samples ( $0.069 \pm 0.006 \mu\text{mol}/\text{m}^2$ ). In addition, the total amount of growth factor uptake from the same rhBMP-2 solution at  $300 \mu\text{g}/\text{mL}$  (recovery yield) appeared higher for coated samples ( $87\% \pm 3\%$  vs.  $66\% \pm 4\%$ ).

#### rhBMP-2 Release

The release of rhBMP-2 in Dulbecco's modified Eagle's medium complemented with calf serum was monitored as cumulated amounts (Figure 8). Similar release curves were observed exhibiting a sharp initial release, slowing down at



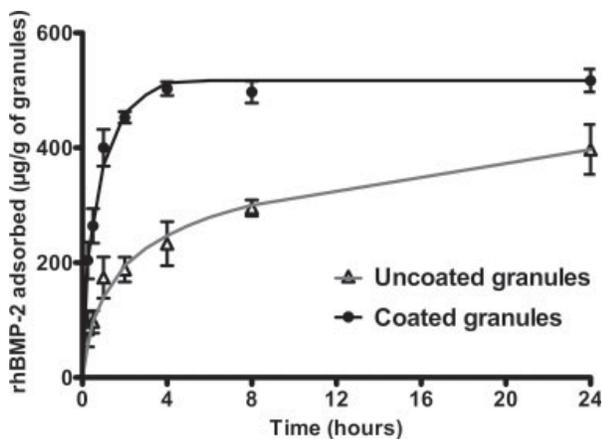
**Figure 5.** Microporosity of the uncoated and coated granules. Incremental microporous volume (a) and SEM micrographs (magnification  $\times 10,000$ ) (b). An additional porosity corresponding to internanocrystal spaces is observed on the coated samples.

48 h. The total amount released at 21 days was slightly but significantly ( $p < 0.005$ ) lower for coated granules than for controls ( $100 \pm 12 \mu\text{g/g}$  vs.  $139 \pm 10 \mu\text{g/g}$ ). The released quantities represented however a minor fraction of the total amount adsorbed for coated samples (23% at 21 days), and about one half (46%) of the adsorbed growth factor for raw ceramics (the initial amount of BMP-2 adsorbed was higher on coated granules compared with uncoated ones for the same concentration of the adsorption solutions).

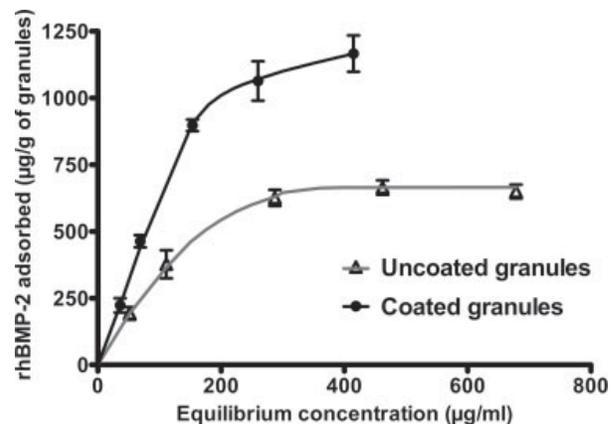
## DISCUSSION

The use of a ceramic coated with nanocrystalline apatite was expected to present several advantages over raw sintered ceramics. The first advantage in this study was indeed found to be an increase of the specific surface area and of the maximum adsorbable amount of growth factor in the porous ceramic.

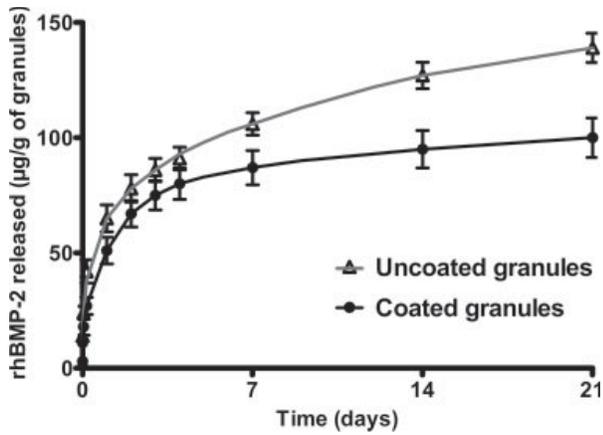
The second expected advantage was related to the improvement of the surface reactivity. Previous comparative studies on the adsorption properties of several molecules on well-crystallized apatites and nanocrystalline ones<sup>20,21</sup> had shown an increase of the maximum amount adsorbed per specific surface area and a decrease of the affinity constant on nanocrystalline apatites. These properties were attributed to the existence at the surface of the nanocrystals of a hydrated layer containing mobile ions with a higher reactivity than those present at the surface of well-crystallized apatite. This study indicated that this hydrated layer was also present in our case. The third advantage was expected to be due to the formation of nanosized pores allowing the gathering of proteins, which could enhance the biological activity of apatite coatings. Porosity has indeed been suggested to be of prime importance in bone healing: macroporosity was found to be necessary for cell invasion and body fluid circulation,<sup>29</sup> and many studies have shown that microporosity was also an important factor. In particular, Habibovic et al.<sup>16</sup> have



**Figure 6.** Adsorption of rhBMP-2 on uncoated and coated granules as a function of time.



**Figure 7.** Adsorption isotherm of rhBMP-2 on uncoated and coated granules.



**Figure 8.** Cumulative release of rhBMP-2 adsorbed on uncoated and coated granules after immersion for 24 h in a 300 µg/mL rhBMP-2 solution.

demonstrated that microporosity provides a microenvironment essential for osteoinduction, and Rouahi et al.<sup>17</sup> have shown the increased adsorption of serum proteins related to an increase of the microporosity of HA ceramics.

The rather slow adsorption process of rhBMP-2 observed here could be related to the diffusion rate of bulk proteins in the pores of the ceramic and/or indicate a rearrangement of the proteins on the surface. The computed modeling of the adsorption of BMP-2 on the (001) apatite surface has recently been reported.<sup>3</sup> Three types of interactions have been involved in the adsorption process: an electrostatic interaction of charged COO<sup>-</sup> groups from the protein with Ca<sup>2+</sup> ions on the apatite surface and water-bridged H-bonds between OH and NH<sub>2</sub> residues of rhBMP-2 and with PO<sub>4</sub><sup>3-</sup> ions on the surface. Such interaction models are still imperfect. In particular, they need to consider the alteration of the crystals' surface in aqueous media and the existence of a surface hydrated layer (either nascent in apatite nanocrystals or acquired on raw ceramic surfaces). In addition, the (001) apatite surface is not generally the most developed and active in apatites. Nevertheless, these interaction models are possibly also valid on other apatite crystal surfaces and possibly on other Ca-P compounds as they essentially involve electrostatic and H-bonding with Ca<sup>2+</sup> and PO<sub>4</sub><sup>3-</sup> ions, showing similar distribution and distances on selected crystallographic planes for calcium phosphate compounds.

The rhBMP-2 adsorption data obtained for the two ceramics can be compared with the results already published on the adsorption of BMP-2 on HA powder (Table I). In particular, the maximum amounts adsorbed are in the same range and seem very close. Therefore, the presence of β-TCP in the ceramic does not seem to affect the maximum BMP-2 coverage, which suggests that the adsorption process is not fundamentally different on β-TCP and HA. In addition, the comparison of our data with those of Boix et al.<sup>2</sup> indicates that the maximum coverage is relatively independent of the experimental procedure. The

presence of BSA in our rhBMP-2 solution, for example, does not seem to interfere with the amount of growth factor adsorbed. The nanocrystalline apatite-coated ceramics exhibit a lower maximum coverage per surface area unit than the raw ceramic. A nanocrystalline coating appears thus to be much less advantageous than expected. This limited gain could be related to a lower accessibility of the surface of nanocrystals in the coating, which is consistent with the existence of nanosized pores preventing an easy diffusion of the protein in the layer. The size of such pores (about 15 nm) appeared in fact only slightly larger than the protein dimensions (7 nm × 3.5 nm × 3 nm)<sup>30</sup> and pore occlusions may easily occur. In addition, the nanocrystals of the coating exhibit a low amount of nonapatitic species at their surface limiting protein adsorption.<sup>20,31</sup> Despite these observations, the uptake of rhBMP-2 appears to be related mostly to the specific surface area of the samples.

Thus, the main advantage of the nanocrystalline coating is the clear increase of the specific surface area and of the amount of BMP-2 taken up by the ceramic. The affinity constants obtained in this study appear much lower than those previously published on HA powder.<sup>2</sup> This parameter is however very sensitive to experimental conditions and substrate characteristics. Thus, it was shown to vary considerably (up to a factor 100) on nanocrystalline apatites depending on their maturation state.<sup>20</sup> The affinity constant appears slightly lower for the nanocrystalline apatite-coated ceramics when compared with the raw ceramic, corresponding with previously published data concerning the adsorption of different molecules such as albumin and phosphoserine on apatites with varying degrees of crystallinity.<sup>20,21</sup> Several interpretations have been given in the literature concerning the variation of the affinity constant, including a variation of the surface charge and/or of the surface energy. Considering however that the adsorption process is supposed to involve essentially interactions between calcium ions from the surface and carboxylic groups from the protein, the adsorption behavior cannot be solely described in terms of physical properties of the surface. From a practical point of view, our studies were performed with a rather high mineral surface/solution ratio to favor a high recovery yield of the growth factor from the solution (more than 85% in the case of the nanocrystalline-coated sample) for *in vitro* and *in vivo* studies. However, they do not represent optimized adsorption conditions.

**TABLE I.** Adsorption Parameters *N* and *K* of rhBMP-2 on Apatite

Samples	$N \pm \Delta N \times 10^8$ (mol/m <sup>2</sup> )	$K \pm \Delta K \times 10^{-6}$ (M <sup>-1</sup> )	Reference
Raw BCP ceramics	6.9 ± 0.6	0.28 ± 0.15	This work
Coated BCP ceramics	3.2 ± 0.3	0.17 ± 0.05	This work
HA powder	3.8 – 11.5 <sup>a</sup>	0.24 – 23 <sup>a</sup>	[2]

<sup>a</sup> Depending on adsorption conditions.

The release conditions of the growth factor adsorbed on apatite ceramics have rarely been investigated,<sup>31,32</sup> although they are believed to determine the biological activity of these associations. The importance of a sustained release for bone repair has recently been stressed by Seeherman and Wozney and Liu et al.<sup>1,8</sup> Generally, growth factors are incorporated within porous biomaterials by simple impregnation followed by drying and the release rate and binding properties are often unknown.<sup>4-7</sup> It is suspected that such associations do not allow the chemical bonding of the growth factor to the material. Precipitation and clustering of the growth factor molecules may occur in the material and the release is only determined by local dissolution and diffusion rules. For example, the release kinetics of the rhBMP-2 in fibrin and collagen sponges has been related to its solubility.<sup>33,34</sup> However, the release rate in the case of growth factors associated with biomaterials by impregnation and drying is often difficult to control, and the uncontrolled release of BMP has, in some instances, been related to an accelerated resorption of bone tissue and of the implant.<sup>8,9</sup>

Several modes of action of the adsorbed growth factors can be considered: (i) the spontaneous release of growth factors and their interactions with cell receptors; (ii) the cell-mediated release of growth factors; and (iii) the direct interaction of the adsorbed growth factors, exposing their binding domains to cell receptors.

In the first case, the growth factor may act at a distance from the implant but the area of action cannot really be controlled and possible negative effects could be observed. In addition to an accelerated resorption of bone tissue and of the implant mentioned earlier,<sup>8,9</sup> the growth factors could potentiate unexpected cell differentiation in adjacent soft tissues. The data obtained in this work show that the spontaneous release of bound growth factors on calcium phosphate ceramics is rather low: after 21 days only 23% of the adsorbed rhBMP-2 on coated ceramics and 46% on raw ceramics are released.

It should be emphasized that the cell culture media are supersaturated with respect to the nanocrystalline coating and the most soluble phase of BCP ceramics (i.e.,  $\beta$ -TCP), meaning that spontaneous dissolution is not likely to occur.<sup>35</sup> In our case, the protein is chemically bound to the surface of the ceramic, and thus desorption appeared clearly as the limiting step of the release. The release or displacement of adsorbed growth factors are only possible when the binding domains on the mineral surface are accessible to competing adsorbing species, such as phosphate ions or other molecules. For example, phosphate ions generally displace anionic proteins from apatite in chromatography columns and the same phenomenon could also occur with adsorbed BMP-2 on Ca-P ceramics in cell culture media or in body fluids. Generally, however, the release by fast diffusing small phosphate ions is rather rapid and depends only on their concentration. In our case, the long-term release observed could be due to the displacement of growth factors by other molecules present in the media and exhibiting an affinity for the apatite

surface greater than that of BMP-2, and/or to a change of the surface characteristics.

In the second case, namely cell-controlled release, the growth factor release is under the control of the organism, and several examples have been given showing the superior efficiency of this mode of release.<sup>36</sup> However, such an interesting process can probably not be involved for adsorbed growth factors on ceramics, where both spontaneous release and cell-mediated release (related to a remodeling-like process on the ceramics) are simultaneously involved. The data suggest however that the amount released by spontaneous displacement of the adsorbed BMP-2 is low, especially in the case of coated samples. Thus, the release of most of the adsorbed growth factor would necessitate a destruction of the ceramic. Such processes occur *in vivo*, with bioabsorbable ceramics like BCP ceramics, because of the activity of osteoclast-like cells colonizing the implants surface shortly after implantation.<sup>1,37</sup> Thus, in our case, crystal dissolution and protein release would essentially be controlled by osteoclast cells. As osteoclast resorption is generally coupled with osteoblast reconstruction, such ceramics would increase the retention of BMP-2 at orthopedic treatment sites for a sufficient period of time to allow regenerative-tissue-forming cells to migrate up to the area of injury and to proliferate and differentiate, as stated by Seeherman and Wozney.<sup>1</sup> The local concentration of BMP appears as a crucial parameter delicate to tune, and a release of too much of BMP-2 could induce an exaggerated biodegradation of the ceramic and of adjacent bone tissues.<sup>8,9</sup> It seems, however, that the range of concentrations leading to adverse effects<sup>8,9</sup> corresponds to a massive burst release which will not occur with chemically bound growth factors. Bone reconstruction processes involving porous ceramics and controlled by cell activity are generally centripetal (i.e., progressing from the surface toward inside). Therefore, growth factors firmly bound to the implant surface would probably be active from the beginning to the end of the reconstruction.

A third possible pathway for growth factor activity involves a direct recognition of the bound growth factor by the cell receptors. Such reports of growth factor activity are scarce.<sup>38</sup> However, examples of bound enzymes showing equivalent or even enhanced activities have been published.<sup>39</sup> Yet, the adsorption generally prevents the protein reorientations and structural reconfiguration and would probably limit the recognition of bound growth factors by cells, and the direct activity of growth factors bound on ceramics has not yet been demonstrated.

It shall be noticed that the adsorption does not induce any alterations of the protein, and the preliminary cell culture experiments demonstrated that the rhBMP-2 previously adsorbed on the granules was active (results to be published).

## CONCLUSIONS

The coating of sintered BCP granules with highly reactive nanocrystalline apatite significantly improved rhBMP-2

adsorption. The amount of rhBMP-2 fixed from the solution and the rate of the adsorption process were noticeably increased on the coated ceramics when compared with the uncoated ones. These results were correlated with the increase in specific surface area, although not all of the surface of the nanocrystals was found to be active. It is suggested that the protein penetrates only partly in the nanocrystalline apatite layer because of the small size of nanosized pores arising from intercrystalline spaces. A preliminary association of the growth factor with the nanocrystals before coating could probably further increase the amount of rhBMP-2 attached to the ceramic surface as already shown by Liu et al.<sup>8</sup>

Most interestingly, only a low quantity of protein was spontaneously released in the cell culture medium during the first week, indicating a firm attachment on the surface and a long-term release without uncontrolled initial bursts sometimes observed in growth factor-enriched bioceramics. In an *in vivo* situation, it is inferred that protein release would essentially be controlled by the surface dissolution of the ceramic, involving osteoclast cells. Further experiments in an *in vivo* model are being carried out to evaluate the bone-healing performances of such coated biomaterials with adsorbed rhBMP-2.

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