Hydroxyapatite as a Carrier for Bone Morphogenetic Protein

Ramin Rohanizadeh*
Kimberly Chung

Bone morphogenetic proteins (BMPs) can induce the formation of new bone in numerous orthopedic and dental applications in which loss of bone is the main issue. The combination of BMP with a biomaterial that can carry and deliver proteins has been demonstrated to maximize the therapeutic effects of BMPs. However, no ideal candidate with optimal characteristics as a carrier has emerged for clinical use of BMPs. Hydroxyapatite (HA) is a potential BMP carrier with its osteoconductive properties and desirable characteristics as a bone graft biomaterial. In this study, 3 different methods to load BMP into HA materials were characterized and compared based on the BMP uptake and release profile. BMP was loaded into HA in 3 ways: (1) incorporation of BMP during HA precipitation, (2) HA immersion in BMP solution, and (3) BMP incorporation during dicalcium phosphate dihydrate (DCPD) conversion to HA. The size of HA crystals decreased when BMP was loaded during HA precipitation and HA immersion in BMP solution; however, it did not change when BMP was loaded during DCPD-to-HA conversion. The highest BMP uptake was achieved using the immersion method followed by HA precipitation, and the lowest via DCPD conversion. It is interesting to note that BMP loading during HA precipitation resulted in sustained and prolonged BMP release compared with the 2 other BMP loading methods. In conclusion, BMP incorporation during HA precipitation revealed itself to be the best loading method.

**Key Words:** hydroxyapatite, bone morphogenetic protein, drug delivery, carrier

**INTRODUCTION**

Although bone is one of the few regenerative tissues in the human body, its regenerative ability is limited. Some bone defects undergo incomplete fracture healing (nonunion fractures), or the defect size is beyond the body’s healing capacity (critical size defects). In such cases, further intervention is required, typically of a surgical nature, to replace the damaged bone with a bone graft material. Osteoinductive materials such as bone growth factors are able to lead and induce bone formation, even in nonskeletal sites (eg, muscle). Biomaterials used in orthopedic applications, such as ceramics, metals, and polymers, are only osteoconductive in nature and generally are not able to induce bone formation. The combination of an osteoconductive biomaterial with osteoinductive molecules overcomes the limitations of synthetic bone graft biomaterials. One such hybrid material involves incorporating bone morphogenetic proteins (BMPs) into a biodegradable carrier. Once this hybrid material is implanted, it will accelerate bone formation and regeneration in surrounding defective bone tissues.

BMPs are part of the transforming growth factor-β cytokine family. BMPs have a range of properties that make them suitable for bone tissue engineering. They are capable of inducing bone formation in both skeletal and non-skeletal sites and have been shown to be effective in the treatment of bone defects in various clinical applications.
of functions, including fetal organ development and postnatal kidney, neuronal, and bone development.\(^5\) Of greatest interest are its osteoinductive abilities, which allow BMP signaling to induce postnatal bone formation in nonskeletal sites.\(^6,7\) Of the many isoforms, BMP-2 and -7 show the greatest in vivo potential for osteogenesis.\(^8,9\) They recruit undifferentiated mesenchymal stem cells to the defective site that differentiate into osteoblasts, which form new bone;\(^6,7\) this is known as intramembranous bone formation.\(^10\) Another bone formation pathway, endochondral formation, occurs when chondroblasts (cartilage cells) are created from stem cells, modulating the bone shape; they are then calcified and replaced by bone.\(^10\) Both pathways can be induced by BMPs and are highly dependent on the properties of the BMP carrier and the isoform of BMP.\(^10,11\) The main interest behind using BMP in dental and orthopedic applications is its potential for reconstructing critical size bone defects and nonunion fractures.\(^3\) To date, areas for which BMP have been studied include spinal fusion, long bone trauma, ligament reconstruction, and orthopedic, craniomaxillofacial, and periodontal disease.\(^3,12\) Several BMP delivery systems are available, ranging from the gene therapy approach to local implantation and systemic administration.\(^3\) However, most research has focused on localized delivery systems, particularly in search of the ideal BMP carrier.

The carrier for BMP should enhance the activity of the protein by maintaining a certain BMP concentration in the defective area for a sufficient time to allow new bone formation.\(^3,6,12–14\) Good affinity should exist between BMP and its carrier to maintain sustained and prolonged BMP release. The carrier should be easily sterilized and biodegradable and should have no immunogenicity.\(^12,14\) An ideal carrier not only is a mechanism for drug delivery but preferably should support bone growth by having an appropriate porous structure for cell infiltration and ingrowth. Finally, the overall manipulation process that includes loading BMP onto a carrier and its release must retain the biological activity of BMP.\(^6,11,12\) A range of BMP carriers of an organic and an inorganic nature have been investigated.\(^2,13\) These carriers have showed varying levels of success, but the major components of human bone—collagen and hydroxyapatite—are preferred BMP carriers.\(^2\)

Hydroxyapatite (HA), \(\text{Ca}_{10}(\text{PO}_4)_{6}(\text{OH})_2\), has been used as a carrier for antibiotics, analgesics, and anticancer agents for the skeletal system.\(^15,16\) It is a preferred biomaterial in orthopedic and dental applications because of its chemically similar structure to the inorganic component of bone and other hard tissues.\(^2,17\) Although more soluble phases of calcium phosphate exist (eg, octacalcium phosphate, dicalcium phosphate dihydrate), HA has shown the greatest potential in bone tissue engineering.\(^3\) Synthetic HA may be created through several techniques and in a range of forms and shapes, such as powder, blocks, discs, and granules.\(^12\) HA is capable of directly bonding to bone, and emerging evidence suggests osteoinductivity with HA materials of certain pore sizes and characteristics.\(^18,19\)

BMP adsorption onto HA can enhance interfacial strength and contact between the HA implant and surrounding bone, thereby promoting greater bone regeneration around the implant than is seen with HA alone.\(^3,17\) On the other hand, incorporating BMP into an HA porous carrier, even in small doses, may increase the strength of the porous carrier.\(^20,21\) BMPs adsorb on HA crystals via binding between functional groups COO\(^-\), OH, and NH\(^2\) of BMP and the calcium site of HA.\(^22\) One BMP molecule may adsorb on HA, or up to 3 molecules may adsorb cooperatively at once.\(^22\) It has been shown that the right side of BMP-2 is most prone to adsorption, with maximal adsorption occurring in acidic conditions.\(^23\) Acidic
conditions result in higher BMP/HA affinity because below the isoelectric point of BMP (pH 7.9), BMP is positively charged and HA has a negative surface charge.

BMP adsorption is thought to be limited to the macropores of HA, but there is a tendency for a rapid initial burst release of BMP once a BMP/HA composite is implanted in the body. Transient high BMP concentrations in the bone defect can lead to adverse effects by activating osteoclast cells, which in turn resorb bone and promote inhibitory proteins of BMP, thus limiting the osteogenic ability of the implant. Most studies have loaded BMP to HA via coating of the surface of HA materials by BMP. This can be achieved by immersion of HA materials in a BMP solution because it can be done under physiologic conditions, reducing the possibility of BMP denaturation. In contrast, incorporating BMP into HA materials results in BMP adsorption onto both macropores and micro-pores of materials with predominant attachment to calcium sites. Hence, BMP incorporation into HA is thought to prolong the retention profile because BMP should be released as HA is dissolved. Liu et al recently demonstrated that incorporation of BMP into biomimetic calcium phosphate revealed osteoinductive properties in rats and was fivefold more efficacious in osteoinductivity compared with implants surface coated with BMP.

Many animal studies have been conducted on HA/BMP hybrid materials. These trials used HA materials immersed for a time in a BMP solution and convincingly demonstrated HA as a suitable carrier for BMP. Optimization of HA synthesis for BMP incorporation into HA powder

Recombinant human bone morphogenetic protein-2 produced by Escherichia coli was purchased from GenScript (Piscataway, NJ) as a sterile lyophilized powder that was reconstituted in 5 mM acetic acid. Sodium hydroxide, acetic acid, calcium chloride, sodium phosphate monobasic monohydrate, and sodium phosphate dibasic anhydrous were supplied by MP Biomedicals (Solon, Ohio). Water used throughout the project was purified by reverse osmosis (Milli-Q, Millipore, Billerica, Mass).

Optimization of HA synthesis for BMP incorporation into HA powder

A 10 mL solution of 2 M calcium chloride and a 5 mL solution of 1.2 M sodium phosphate dibasic anhydrous were prepared to obtain a calcium (Ca)/phosphate (P) ratio of 1.67, equal to that of stoichiometric HA. The calcium chloride solution was slowly added to the phosphate solution while stirring using a magnetic stirrer. The pH of the solution was adjusted to 8, and the solution was heated to one of the following
temperatures: 37°C, 40°C, 43°C, 45°C, 47°C, and 50°C. During heating, the solution was covered to prevent evaporation. Stirring continued for 2 hours to mature the precipitated HA crystals. The mixture was subsequently filtered quickly using a filter-funnel and flask vacuum; this was followed by rinsing once with 25 mL of warmed (50°C) water. The filter paper and the powder were then removed from the funnel and were placed immediately in an oven at 40°C to be dried overnight. The HA fabrication process was repeated at a pH of 7.5 over the same temperature range and conditions. The synthesis condition that resulted in precipitation of crystalline HA and that had the lowest risk of causing BMP denaturation (temperature and pH close to 37°C and 7.4, respectively, ie, physiologic conditions) was chosen for BMP incorporation.

Preparation of DCPD and optimization of DCPD-to-HA conversion for BMP incorporation into HA powder

Two equimolar (0.8 M) 15 mL solutions of calcium chloride and sodium phosphate dibasic anhydrous were prepared. Calcium chloride was added to the phosphate solution at room temperature, and the pH was adjusted to 5. The solution was covered and was left to be stirred at room temperature for 2 hours before filtering and rinsing with 25 mL of room temperature water. The DCPD powder was dried overnight at room temperature.

Once dried, 1 g of the DCPD powder was placed in 8.25 mL of 0.1 M sodium hydroxide (dissolved as a buffered solution). The pH of the solution was adjusted to 8, and hydrolytic conversion was allowed to proceed at the following temperatures: 37°C, 40°C, 43°C, 45°C, 47°C, and 50°C in an oven with shaker. After 24 hours, the solution was removed, filtered, and dried at 40°C to retain the powder. The entire process was repeated at a pH of 8.5 and 9 at the same temperatures. The reaction was also investigated at 50°C and at 60°C at pH 9, 9.5, 10, and 11. A 0.2 M solution of sodium hydroxide at 70°C was also trialed. Similarly, the DCPD-to-HA conversion condition with the least risk of denaturing BMP was selected for BMP incorporation.

Methods to load BMP into HA powder

- **Incorporation during HA precipitation:** Based on the results of optimization conditions for HA synthesis (Table 1), 37°C and pH 8 were the selected parameters for this method. BMP-2 solution was added to 5 mL unheated calcium chloride solution, which then was slowly poured into the phosphate solution (as described earlier) to obtain a concentration of 120 µg/mL of BMP in the 15 mL solution used for HA preparation. The weight ratio between obtained HA and the BMP available in the solution was approximately 1000:1; the same ratio was used across all other BMP loading methods.

- **Immersion of HA powder in a BMP solution:** The second method of loading BMP into HA involved immersing HA powder in a solution containing BMP. A total of 700 mg of preformed HA was immersed at 37°C in a 5.8 mL solution containing 120 µg/mL BMP-2, yielding the same HA/BMP weight ratio and BMP concentration in solution as

<table>
<thead>
<tr>
<th>pH</th>
<th>Temperature, °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>37 DCPD + HA</td>
</tr>
<tr>
<td></td>
<td>40 HA DCPD + HA</td>
</tr>
<tr>
<td></td>
<td>43 DCPD + HA</td>
</tr>
<tr>
<td></td>
<td>45 HA DCPD + HA</td>
</tr>
<tr>
<td></td>
<td>47 HA DCPD + HA</td>
</tr>
<tr>
<td></td>
<td>50 HA DCPD + HA</td>
</tr>
</tbody>
</table>

*DCPD refers to dicalcium phosphate dehydrate; HA, hydroxyapatite.*
the previous method. After 24 hours, the solution was filtered and was rinsed with 25 mL warmed water (50°C). The powder was allowed to dry overnight at 37°C.

**Incorporation during DCPD-to-HA conversion:** None of the conditions tested to convert DCPD to HA was successful (Table 2); therefore based on previous works of Le-Geros,28 0.2 M sodium hydroxide solution at 70°C was used for this method. A total of 120 mg/mL of BMP was added to the 8.25 mL of 0.2 M sodium hydroxide solution (70°C). The HA/BMP weight ratio and the BMP concentration in solution were adjusted as in the previously mentioned methods. The conditions of DCPD-to-HA conversion were described earlier.

**BMP release profile**

The amount of BMP incorporated into the HA powder was determined by subtracting the BMP concentration remaining in the calcifying or immersion solution at the end of the loading process (before filtration) from the initial protein concentration of 120 μg/mL. A 1.2 mM solution of sodium phosphate monobasic monohydrate adjusted to pH 7.4 was the phosphate buffered solution (PBS) used for the in vitro BMP release assay. The rate of BMP release was determined by incubating 120 mg BMP-loaded HA in 1.2 mL of PBS in an Eppendorf tube at 37°C in a shaker oven. In all, 850 μL of the supernatant was withdrawn and frozen at 12 hours and at days 1, 3, 7, and 14. The removed supernatant was replaced with 850 μL of fresh PBS at each time point. The experiment was set up for all 3 BMP-loading methods using 5 samples per method and time point. As a control, HA without BMP was also incubated in PBS at 37°C with identical time points for supernatant withdrawal. All collected supernatant was stored at −20°C until the protein concentration was measured using 2 different protein assays.

The Lavapep total protein fluorescence assay (Fluorotechnics, Sydney, Australia) and the Quantikine (R&D Systems Inc, Minneapolis, Minn) BMP-2 enzyme-linked immunosorbent assay (ELISA) were used to determine total protein uptake into HA and its release profile in vitro. The microplate reader used for both protein kits was the POLARStar OPTIMA in conjunction with FLUOstar OPTIMA software (BMG Labtech, Ortenberg, Germany). Because calcium ions released from HA powder may interfere with the measurement of BMP concentration in the total protein assay, 2 g of HA (without BMP) was soaked in 16.7 mL of water for 14 days. The supernatant was removed and was used in making the BMP stock solution and in the serial dilutions to make standards for the total protein assay.

**Physicochemical properties of HA (before and after BMP loading)**

X-ray diffraction (XRD) (Siemens D5000 X-ray diffractometer, Siemens Healthcare Diagnostics, Deerfield, Ill) was used to determine the crystal
The parameters used were 40 kV and 30 mA, with a scan range between 10 and 40 degrees 2° and a step size of 0.02 degrees with 2 seconds per step. Scanning electron microscopy (SEM) determined the crystal morphology of the synthesized materials and the effects of BMP incorporation on HA crystal size and shape. The Zeiss Ultra Plus (Carl Zeiss, Göttingen, Germany) electron microscope with a working distance of 4.4 mm and 20 kV was used in this study.

**RESULTS**

**Optimization of HA synthesis for BMP loading**

XRD analysis of powders obtained from the calcium and phosphate precipitation reactions demonstrated that a mixture of DCPD and HA formed at pH 7.5 and at temperatures of 37°C and 40°C (Table 1). At temperatures above 43°C, only HA crystals were precipitated at pH 7.5. By increasing the pH to 8, across all tested temperatures, the materials obtained had only HA crystal structure. Table 1 summarizes the products obtained from precipitation in calcium and phosphate solutions. For the second loading method (DCPD-to-HA conversion), because the conditions shown in Table 2 did not elicit conversion of DCPD crystals to HA, the DCPD powder was soaked in 0.2 M NaOH (pH 13) at 70°C to be hydrolyzed to HA.

**Physicochemical properties of HA (before and after BMP loading)**

The typical XRD spectrum of HA precipitates obtained from solution at pH 8 and 37°C is shown in Figure 1A. Diffraction peaks in this spectrum are identified as those of HA. Major peaks at 2θ = 26 degrees, 32 degrees, and 34 degrees indicate HA lattice planes of (002), (211), and (300), respectively. Figure 1B and C shows the typical XRD spectra of DCPD and HA converted from DCPD, respectively. Figure 1B shows major peaks at 2θ = 11.7 degrees, 21 degrees, and
29 degrees, corresponding to DCPD lattice planes of (020), (121), and (112) respectively. Figure 1C identifies the typical HA peaks after DCPD-to-HA conversion; no DCPD peaks were observed after the conversion.

Following BMP incorporation via (1) incorporation during HA precipitation, (2) HA immersion in BMP solution, and (3) incorporation during DCPD conversion to HA, the XRD spectra (Figure 2A through C) revealed that HA remained the only mineral compound present, with major peaks at 2θ = 26 degrees and 32 degrees. The XRD spectra showed a much higher level of noise after BMP incorporation (Figure 2A through C). No detectable alterations in the broadness of these peaks of the XRD spectra were observed following BMP loading.

SEM micrographs in Figure 3A through G show crystal morphology of different groups before and after BMP loading. The HA crystals synthesized from wet precipitation were nanosized and mostly had a plate-like shape (Figure 3A), whereas those formed from DCPD conversion were more needle-shaped and were larger, agglomerated, and more elongated (Figure 3C). The micrograph of DCPD (Figure 3B) shows large (micron-sized) plate-like crystals. The micrographs in Figure 3D through G reveal morphologic changes in HA crystals following BMP loading to HA powder. The BMP incorporated during HA precipitation reduced the size of the crystals but retained the HA plate-like shape (Figure 3A vs Figure 3D). After HA powder was immersed in the BMP solution, in most areas, HA crystals had a plate-like shape (Figure 3E); however, in some areas, crystals appeared to be very small and agglomerated compared with those before BMP loading (Figure 3F: magnified of boxed area in Figure 3E). BMP incorporated during DCPD-to-HA conversion resulted in little variation in the morphology of HA crystals compared with those formed without the presence of BMP (Figure 3C vs Figure 3G).

**Release profile of BMP**

The extent of BMP uptake into HA powder was measured using ELISA and total protein assays (summarized in Table 3). Based on the
Figure 3. Scanning electron micrograph (SEM) images of (A) hydroxyapatite (HA) precipitates at pH 8 and 37°C; (B) dicalcium phosphate dihydrate (DCPD) precipitates at pH 5 and at room temperature; (C) DCPD converted to HA at pH 13 and 70°C; (D) HA crystals after bone morphogenetic protein (BMP) incorporation during HA precipitation; (E) HA crystals after immersion in a BMP solution; (F) Figure 3E boxed area magnified (HA crystals); and (G) HA crystals after BMP incorporation during DCPD-to-HA conversion.
total protein assay, the highest percentage of BMP uptake into HA was achieved with the immersion technique followed by incorporation during HA precipitation, then DCPD-to-HA conversion. When measured by total protein assay, BMP uptake during the HA immersion method was significantly higher than that obtained using other loading methods (Table 3). The ELISA assay demonstrated significantly lower BMP uptake during HA precipitation and comparable percentage (around 96%) when BMP was loaded by immersion and the DCPD-to-HA conversion method. No significant differences were seen when the amount of BMP in solution was measured before and after the filtration process, indicating that BMP adsorption on filter materials and funnel was negligible.

Using the ELISA assay, the release profiles of BMP were determined for all 3 BMP-loading methods and are shown in Figure 4. The total amount of BMP released after 14 days was 1028 ± 181.6, 413.6 ± 183.5, and 619.3 ± 139.8 ng/mL, respectively, for HA precipitation, HA immersion, and DCPD-to-HA conversion loading methods. The percentage of BMP released (based on the percentage of BMP uptake) after 14 days was 22.9 ± 4.3, 0.43 ± 0.18, and 1.23 ± 0.28, respectively, for each of the methods previously mentioned (Figure 5). All 3 BMP-loading methods revealed a rapid increase in BMP release during the first 12 hours when compared with control. However, BMP released for samples prepared using the immersion method did not significantly increase after 12 hours, unlike the 2 other loading methods, which showed further BMP release after 12 hours. Loading BMP into HA powder during DCPD conversion showed a secondary burst of BMP release at the 7 day time point with minimal increase thereon, whereas for samples in which BMP was loaded during HA precipitation, the BMP

![Figure 4](image-url)  
**Figure 4.** Release profile of bone morphogenetic protein (BMP) from hydroxyapatite (HA) powders prepared using 3 BMP loading methods.

![Figure 5](image-url)  
**Figure 5.** Percentage of bone morphogenetic protein (BMP) released from hydroxyapatite (HA) powders, based on the extent of BMP uptake for each respective BMP loading method.

**Table 3**  
Percentage of BMP uptake into HA measured by total protein and ELISA assays*

<table>
<thead>
<tr>
<th>BMP Loading Method</th>
<th>Incorporation During HA Precipitation</th>
<th>HA Immersion in BMP Solution</th>
<th>Incorporation During DCPD Conversion to HA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay</td>
<td>% BMP loaded</td>
<td>Total Protein</td>
<td>ELISA Total Protein</td>
</tr>
<tr>
<td>Total Protein</td>
<td>54 ± 3.5</td>
<td>94.6 ± 0.5t</td>
<td>96.2 ± 0.5</td>
</tr>
<tr>
<td>ELISA</td>
<td>72 ± 3.7</td>
<td>96.7 ± 0.5</td>
<td></td>
</tr>
</tbody>
</table>

*BMP refers to bone morphogenetic protein; DCPD, dicalcium phosphate dehydrate; ELISA, enzyme-linked immunosorbent assay; HA, hydroxyapatite.

†Significantly higher than the other 2 BMP loading methods (n = 5; ANOVA P < .001; post hoc Tukey pairwise).
release profile continued to increase over the 14 day period.

**DISCUSSION**

Precipitation of calcium phosphates from homogenous solutions requires the saturation point to be reached, followed by subsequent nucleation and crystal growth. HA crystals are obtained in human physiologic conditions, alkaline conditions, and temperatures above 37°C; HA crystallinity is increased by increasing the temperature in the wet precipitation method.\(^{29,30}\) Table 1 reflects these observations by showing that increasing the acidity and decreasing the temperature led to the precipitation of a mixture of DCPD and HA. However, at a pH of 8, only HA and no DCPD was formed. The XRD pattern (Figure 1A) showed that the HA obtained at a pH of 8 and at 37°C was poorly crystalline, demonstrated by broad peaks in the diffraction spectra. The significance of poorly crystalline HA is correlated with its increased solubility, which may facilitate BMP release once incorporated.\(^{29}\)

SEM micrograph (Figure 3A) showed that HA crystals were nano-sized (500 x 100 nm) plate shapes. The HA powder obtained had a tendency to agglomerate, which has been previously reported in wet precipitation of HA.\(^{31,32}\) Smaller HA crystals are desirable for their expected increased bioactivity property and their higher surface area for protein adsorption.\(^{32}\) In contrast to HA, DCPD precipitation from calcium and phosphate solutions is predominant at a pH less than 6.5 and at room temperature.\(^{33}\) Figure 1B shows highly crystalline DCPD precipitates as seen by sharp peaks in the XRD spectra when formed under acidic conditions (pH 5) and at room temperature. Figure 3B shows macrop-sized (up to 10 μm x 3 μm) plate-like crystals typical of DCPD crystal morphology. In alkaline conditions, DCPD undergoes hydrolytic transformation to HA. The following reaction details the hydrolysis of DCPD to HA:\(^{34}\)

\[
10 \text{CaHPO}_4 \cdot 2 \text{H}_2\text{O} \rightarrow \\
\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 + 18 \text{H}_2\text{O} + 12 \text{H}^+ + 4\text{PO}_4^{3-}
\]

A range of temperatures (60°C–140°C) and pH values (6–14) have been investigated to facilitate this hydrolysis reaction.\(^{35}\) It was seen that conditions at pH greater than 9 produced more elongated HA crystals. The condition selected in this study—0.2 M NaOH (pH 13) and 70°C—are of an extreme nature but were used here because the threshold for DCPD transformation was not determined (Table 2). At these conditions, the HA produced showed different crystal morphology with elongated rod particles (as previously reported) but with reduced width in comparison with HA crystals obtained through direct wet precipitation (Figure 3C vs Figure 3A).

BMP incorporation in HA has not been specifically investigated in terms of the physicochemical effects of BMP on HA. However, the presence of other bone matrix proteins, bovine serum albumin, and amylase has retarded HA crystal growth when present during precipitation of HA.\(^{35–38}\) SEM images in Figure 3D through G show smaller HA crystals upon BMP uptake compared with HA without BMP, supporting what has been previously found with other proteins. Incorporation of BMP during HA precipitation (Figure 3D) reveals uniform rod needle-like crystal morphology (approximately 200 × 10 nm) compared with HA crystals upon BMP uptake. The reduction in HA crystal size in BMP loaded samples might be due to binding of BMP to calcium sites in HA, thereby inhibiting further crystal growth.

Samples prepared via immersion of HA powder in BMP solution (Figure 3E) revealed less uniform crystal morphology after BMP uptake compared with those prepared by other loading methods. In this group, most crystals have a plate-like shape with crystals.
of similar size to those obtained via BMP incorporation during HA precipitation. Figure 3F (magnified boxed area in Figure 3E) shows an area of dissolution/reprecipitation of HA crystals during the 24 hour immersion period in the BMP solution. Dissolution of HA crystals results in a local increase in calcium and phosphate ions concentrations, leading to reprecipitation of very small HA crystals (50 x 100 nm) off the primary HA crystals. These secondary crystals showed different morphology and appeared smaller and more agglomerated than the primary HA crystals.

Unlike the other BMP-loading methods, BMP incorporated into HA powders during DCPD-to-HA conversion resulted in little change in crystal morphology (Figure 3G).

The XRD spectra from all the methods in the presence of BMP (Figure 2) show that the XRD spectra with a much higher level of noise compared with those prepared without BMP (Figure 1) indicate the presence of organic matter (such as BMP). The high level of noise in XRD spectra made it difficult to precisely determine the changes in peak broadening; therefore, for all 3 groups, no detectable changes in HA crystallinity were observed after BMP loading.

**BMP uptake**

The amount of BMP uptake into HA powders was determined via the protein assays seen in Table 3. However, assumptions underlined the calculated percentage of BMP uptake. Foremost is the BMP concentration in calcifying or immersion solutions, which was taken to imply that the remaining BMP was loaded into the HA powder. BMP adsorption to filter paper, by sampling before and after filtering, in each method was considered and revealed little adsorption of BMP onto filter materials. However, the washing step in every method may have reduced the extent of the actual amount of loaded BMP by removal of BMP, which was loosely attached or incorporated. Finally, BMP adsorption to the containers used for BMP loading was not accounted for.

The amount of BMP uptake into HA via the 3 methods—incorporation during HA precipitation, incorporation during DCPD-to-HA conversion, and immersion of HA in BMP solution—varied significantly based on the method of measuring protein in solution used (ELISA vs total protein assays). The total protein assay determines the state of BMP as total protein (both denatured and undenatured protein molecules), whereas the ELISA assay determines the amount of BMP in the biologically active conformation (undenatured molecules). Table 3 shows that the most extensive BMP loading occurred during HA immersion in the BMP solution, demonstrated by both ELISA and total protein assays. This may have happened as a result of the longer duration of BMP loading in the immersion method (24 hours) compared with the 2 hour interval for BMP incorporation during HA precipitation. Moreover, HA crystals immersed in the BMP solution experienced a secondary HA crystal growth, allowing for BMP adsorption onto the newly formed HA crystals, increasing the overall amount of BMP loaded. Based on the total protein assay, BMP uptake during HA precipitation was the next highest percentage. Unexpectedly, the DCPD-to-HA conversion loading method resulted in the lowest percentage, despite having a 24 hour loading interval. HA formed via hydrolysis of DCPD is known to be calcium deficient, which may reduce the availability of BMP binding sites to calcium, thereby reducing the extent of BMP loading during DCPD-to-HA. Moreover, the conversion reaction involves DCPD dissolution and reprecipitation of HA from calcium and phosphate ions. However, precipitation of new HA crystals does not take the allotted 24 hour period, thereby reducing the time BMP had to bind to calcium sites. More important, the HA precipitates obtained via DCPD conversion were largest in comparison with those obtained by the 2 other tested BMP loading methods.
Therefore, the decrease in total surface area of HA crystals for BMP adsorption may be another factor in reduced BMP uptake in the DCPD-to-HA conversion method.

It should be noted that the total protein assay did not differentiate between denatured and undenatured BMP molecules, whereas the ELISA assay detected BMP only in the biologically active conformation state. The ELISA assay is unable to measure denatured BMP molecules in the remaining calcifying or immersion solution; therefore a higher percentage of BMP was presumed to be loaded into HA when the ELISA assay was used, which is inaccurate. For this reason, across all 3 BMP-loading methods, the percentages of BMP uptake were elevated when measured by ELISA assay rather than the total protein assay (Table 3). Because the total protein assay accounted for denatured and active BMP, it gave a more accurate depiction of BMP uptake into HA powder.

The ELISA assay showed that BMP uptake for the DCPD-to-HA conversion method was significantly greater than the respective value obtained using the total protein assay. This may be explained by the conditions used to load BMP during DCPD-to-HA conversion. It has been reported that after 8 hours of heat treatment (70°C), the activity of BMP was significantly decreased.27 Conversion of DCPD powder to HA occurred at 70°C over 24 hours, increasing the percentage of denatured BMP molecules and subsequently reducing their detection in remaining solution via the ELISA assay. This contrasts with BMP loading during HA precipitation at 37°C and pH 8, wherein the secondary structure of BMP is better maintained, allowing more accurate BMP measurement in solution with ELISA and thereby BMP uptake in HA powder.

**BMP release profile**

Unlike the ELISA assay, which is capable of BMP-2 detection in the range of picogram, the total protein assay used in this study was unable to detect the very low concentration of BMP released from HA powder. BMP release from the samples prepared using HA precipitation and DCPD conversion was greater than in those prepared using HA immersion in BMP solution. BMP uptake during HA precipitation showed the best release profile, with the most BMP (22.9%) released in conjunction with a sustained and prolonged release profile. The low release of BMP (total of 0.43%) from samples prepared by immersion in BMP solution could be due to BMP detachment during washing caused by loose adsorption of BMP on HA crystals. BMP uptake during DCPD-to-HA conversion showed a biphasic release profile with 2 stages of burst release, within the first 12 hours and 7 days. For this group, the BMP release profile may indicate BMP initially released from the HA crystals located on the surface of HA agglomerates; after a week, BMP was released from crystals located in the inner regions of HA agglomerates. However, the release profiles for all loading methods showed an initial burst of BMP release within the first 12 hours. It should be noted that these release profiles reflect only the amount of undenatured protein (detected by ELISA assay); the actual amount of total released BMP (both denatured and undenatured) is probably higher.

Based on results obtained in this study, further optimization of BMP loading during the HA precipitation method should be considered. Enhanced understanding of BMP in HA adsorption would develop with variation of the stage in which BMP can be added during the HA precipitation process (eg, in the calcium solution vs in the phosphate solution vs during HA crystal maturation). Upon optimization, the biological osteogenic potential of these implants would have to be evaluated through in vivo trials. The addition of other bone augmenting drugs may be considered with HA as a potential carrier.
Overall, these findings show the suitability of HA as a BMP carrier. This study highlights the necessity of investigating the protein loading method because it directly affects many properties of the carrier and its ability to take up and release protein.

**CONCLUSION**

The potential for BMP in bone tissue engineering remains limited so long as its delivery is hampered. This study has demonstrated different methods of combining BMP, and that a single carrier, HA, can produce great variation in protein uptake and release, also affecting carrier physicochemical and morphologic properties. Based on parameters for HA synthesis closest to physiologic conditions, BMP incorporation reduced HA crystal size during HA precipitation (pH 8, 37°C) and the immersion method. However, protein loading during DCPD conversion to HA (pH 13, 70°C) showed no significant alteration in HA crystals.

The negligible amount of BMP released through the immersion method reflects the need for future studies to optimize methods of BMP loading, because this is the predominant BMP loading method used in past and current trials. BMP incorporation during HA precipitation produced the best release profile, despite not acquiring the highest amount of BMP loaded. Its BMP release profile could be described as slow, sustained, and prolonged, as desired in BMP clinical applications.

**ABBREVIATIONS**

BMP: bone morphogenetic protein  
DCPD: dicalcium phosphate dihydrate  
HA: hydroxyapatite  
ELISA: enzyme-linked immunosorbent assay  
PBS: phosphate buffered solution  
SEM: scanning electron microscopy  
XRD: X-ray diffraction

**ACKNOWLEDGMENT**

This work was supported by a research grant from American Academy of Implant Dentistry Research Foundation.

**REFERENCES**


