Long-term cell-mediated protein release from calcium phosphate ceramics

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Abstract: Efficient delivery of growth factors from carrier biomaterials depends critically on the release kinetics of the proteins that constitute the carrier. Immobilizing growth factors to calcium phosphate ceramics has been attempted by direct adsorption and usually resulted in a rapid and passive release of the superficially adherent proteins. The insufficient retention of growth factors limited their bioavailability and their efficacy in the treatment of bone regeneration. In this study, a coprecipitation technique of proteins and calcium phosphate was employed to modify the delivery of proteins from biphasic calcium phosphate (BCP) ceramics. To this end, tritium-labeled bovine serum albumin ([^{3}H]BSA) was utilized as a model protein to analyze the coprecipitation efficacy and the release kinetics of the protein from the carrier material. Conventional adsorption of [^{3}H]BSA resulted in a rapid and passive release of the protein from BCP ceramics, whereas the coprecipitation technique effectively prevented the burst release of [^{3}H]BSA. Further analysis of the *in vitro* kinetics demonstrated a sustained, cell-mediated release of coprecipitated [^{3}H]BSA from BCP ceramics induced by resorbing osteoclasts. The coprecipitation technique described herein, achieved a physiologic-like protein release, by incorporating [^{3}H]BSA into its respective carriers, rendering it a promising tool in growth factor delivery for bone healing. © 2009 Wiley Periodicals, Inc. J Biomed Mater Res 92A: 463–474, 2010

Key words: biomaterials; calcium phosphate ceramics; protein coprecipitation; protein delivery; cell-mediated release

INTRODUCTION

Skeletal trauma, arthroplasty revision, and bone tumor resection often require the implantation of bone grafts. Autologous cortical or cancellous bone grafts remain widely accepted as the standard of care in bone graft selection due to their unequaled success rates. There are critical limitations, however, associated with the graft harvest such as donor site morbidity, prolonged surgery time, and limited supply.[1–4] Allograft bone is readily available from bone banks but includes the added risks of disease transmission and immunogenically induced graft rejection.[2,4–7] Bone substitute materials provide a promising alternative to skeletal reconstructions with autogenic or allogenic bone grafts. Calcium phosphate-based materials, such as hydroxyapatite and tricalcium phosphate, are known for their excellent biocompatibility and their osteoconductive properties. Since these biomaterials are without intrinsic osteoinductive activity, attempts have been made to render these materials osteoinductive through the addition of osteogenic growth factors.[8–10] Because of the complex regulation and action of growth factors during bone healing, the quantitative and temporal framework of their delivery is critical for the successful osseointegration and replacement of bone substitute materials. So far, the immobilization of growth factors with calcium phosphate ceramics has mostly been accomplished by adsorption of proteins onto

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the surface of the materials.[9,11] This superficial deposition, however, leads to a burst release of the proteins upon exposure to a physiological environment.[12–14] The osteoinductive effects of these agents are thus restricted temporally and spatially, resulting in poor bioavailability and limited osteogenic potential. Researchers have tried to overcome this problem by increasing the concentrations of adsorbed growth factors. However, high concentrations of BMP-2 were shown to induce excessive bone formation and heterotopic ossification. Furthermore, high concentrations of BMP-2 were also shown to lead to a stimulation of osteoclastic bone resorption.[15–17]

Techniques providing the possibility to modulate the delivery of proteins from ceramic biomaterials may greatly enhance the efficacy of growth factor application for bone regeneration. By decreasing the amount of growth factor required to support bone regeneration, the risk of adverse effects, including neoplastic induction, may also be reduced. Various techniques to modify the release of therapeutic agents from biomedical materials including calcium phosphate-based materials, polymers, and metal implants have been described previously.[18–24] By incorporating agents into polymeric materials or binding of proteins to titanium implants following chemical modification of the metal surface, investigators were able to improve protein retention and to achieve slow release kinetics.[20,25–32] Recently, Liu et al.[13,33–35] have successfully used a coprecipitation technique of proteins together with calcium phosphate to immobilize bovine serum albumin (BSA) and BMP-2 on titanium implants by incorporating the proteins into a layer of crystalline calcium phosphate. The incorporation of the proteins significantly decreased the passive release of BSA when compared with a superficial deposition of the protein. BMP-2 coprecipitated onto the titanium implants in the presence of calcium phosphate retained its biological activity *in vitro* and *in vivo* after being released from its carrier.[34,35] So far, the coprecipitation of proteins onto biomedical materials has been applied primarily to titanium implants. The adaptability of this technique to calcium phosphate-based materials has not been investigated in detail. Although coating techniques meeting the requirements of sufficient protein retention and decreased burst release have been developed, investigations of the release kinetics have, with very few exceptions, focused on passive, noncell-mediated mechanisms of growth factor delivery.[18,19,25,28,31,36,37] Little is known about the extent to which cells influence the liberation of proteins from biomaterials and whether the release of proteins can be modulated by cell-mediated mechanisms.

It was hypothesized as follows: (a) that the burst release of proteins from ceramic biomaterials found with conventional protein adsorption can be minimized by applying the coprecipitation of proteins to calcium phosphate ceramics and (b) that protein incorporation may accomplish a sustained release of proteins mediated by the resorption activity of bone marrow cells differentiated to monocytes/macrophages and osteoclasts. Tritium-labeled bovine serum albumin ([^{3}H]BSA) served as a model protein to analyze the protein uptake onto the ceramics as well as the passive and cell-mediated release of the protein from the carrier material.

MATERIALS AND METHODS

Chemicals

Fetal bovine serum (FBS), minimal essential alpha medium (α-MEM), and Penicillin G/Streptomycin were from Gibco (Basle, Switzerland). Receptor activator of nuclear factor kappa-B ligand (RANKL) was from Peprotech (London, UK) and colony-stimulating factor-1 (CSF-1) was from Chiron Corporation (Emeryville, CA). Bovine serum albumin (BSA) was from Sigma Aldrich (Basel, Switzerland). The XTT cell proliferation kit and $1.25(OH)_2D_3$ were from Roche Diagnostics (Rotkreuz, Switzerland). The total protein quantification assay was from Bio-Rad (Bio-Rad Protein Assay, Bio-Rad, Reinach, Switzerland). The staining kit for tartrate resistant acid phosphate (TRAP) was from Sigma Aldrich ((Basle, Switzerland). Hoechst 33342 nucleic acid stain was from Molecular Probes (Basle, Switzerland). Sodium boro-[3H]-hydride, Ultima Gold™ scintillation fluid, and scintillation vials were from Perkin Elmer (Schwerzenbach, Switzerland). Osmium acid solution, Piperazine-1,4-bis(2-ethane-sulfonic acid) (PIPES), and glutardialdehyde solution were from Merck (Dietikon, Switzerland).

Bone substitute materials

Custom made porous biphasic calcium phosphate (BCP) ceramics (pore size 150–200 μm, porosity 75 ± 5%) composed of 80% hydroxyapatite (HA) and 20% β-tricalcium phosphate (β-TCP) were purchased from Progentix BV (MB Bilthoven, The Netherlands). Ceramic cylinders with a diameter of 14-mm were prepared and discs with a thickness of 0.5-mm were cut with a diamond saw. The BCP discs were cleaned with ethanol and UV sterilized (4 h) directly before use.

Preparation of [^{3}H]BSA

Fifty milligrams of bovine serum albumin **(BSA)** was labeled with tritium ([^{3}H]) by the reductive methylation procedure of Tack et al.[38] The reaction involves a brief exposure of the protein to formaldehyde and sodium boro-[^{3}H]-hydride (Cat. No. NET023H100MC, Perkin Elmer, Switzerland), which specifically labels the α-amino groups

of NH_2-terminal residues and ε-amino groups of lysyl residues. The reaction mix was loaded onto a Sephadex G-25 PD-10 column (GE Healthcare, Otelfingen Switzerland) to separate labeled protein from the nonincorporated free label. The protein concentration within the purified preparation was determined using a total protein quantification assay.

Immobilization of [^{3}H]BSA onto BCP ceramics

BCP ceramics were coated with [^{3}H]BSA either by adsorption or by protein coprecipitation. [^{3}H]BSA was superficially adsorbed to the surface of the materials by immersion of BCP discs in an aqueous protein solution (total volume of 2.5 mL) for 48 h at 37°C in 24-well plates using a custom made holder (coating 1: Adsorption). To coprecipitate proteins and calcium phosphate onto BCP ceramics, the materials were first immersed for 24 h at 37°C in five-time concentrated simulated body fluid (Na^+ 733.5 m*M*, Cl^- 720.0 m*M*, HPO_4^{2-} 5.0 m*M*, SO_4^{2-} 2.5 m*M*) under high-nucleation conditions in the presence of Mg^{2+} (7.5 m*M*) to inhibit crystal growth. An amorphous layer of calcium phosphate was thereby created which served as a seeding substratum for the subsequent crystalline calcium phosphate layer. The crystalline calcium phosphate layer was generated by incubation of the BCP discs in a supersaturated solution of calcium phosphate (Na^+ 140.0 m*M*, Ca^{2+} 4.0 m*M*, Cl^- 144.0 m*M*, HPO_4^{2-} 2.0 m*M*) for 48 h at 37°C in the presence of [^{3}H]BSA. [^{3}H]BSA and calcium phosphate were thereby coprecipitated onto the BCP ceramics (coating 2: Coprecipitation). The coprecipitation of [^{3}H]BSA was performed in volumes of 2.5 and 12 mL supersaturated calcium phosphate solution, respectively. In a third coating procedure, the coprecipitation technique was at first performed without addition of [^{3}H]BSA to create a protein-free layer of crystalline calcium phosphate. Subsequently, [^{3}H]BSA was adsorbed to the ceramics which had been precipitated with calcium phosphate beforehand (coating 3: CaP precipitation/adsorption). Volumes of 2.5 and 12 mL of supersaturated calcium phosphate solution and aqueous protein solution were used. With all coating techniques, [^{3}H]BSA was added to the coating solutions to a final concentration of 0.1, 1, and 10 μg/mL coating solution. For the first 60 min of the coating procedures, a vacuum was applied to remove air trapped within the porous structure of the BCP discs.

Cell cultures

Bone marrow cells (BMC) were harvested from femurs and tibias of 6-week-old, male *ddy* mice. After equilibration of the BCP discs in α-MEM supplemented with 10% FBS (not heat inactivated) and 1% Pen/Strep (100 U/mL and 100 μg/mL, respectively), 1×10^6 cells were seeded on each BCP disc. The cells were cultured in α-MEM containing 10% FBS and 1% Pen/Strep with CSF-1 (30 ng/mL) for differentiation toward the monocyte/macrophage lineage and with CSF-1 (30 ng/mL) and RANKL (50 ng/mL) for differentiation toward the osteoclast lineage. The media was changed in 3-day intervals. From day 6 of culture, the media were acidified by adding 15 meq/L H^+ according to Arnett et al.[39–41] to activate osteoclasts and to reduce the precipitation of calcium ions from the media onto the ceramics. To inhibit the activity of osteoclasts, calcitonin was added to the cultures on day 10 at a final concentration of $1 \times 10^{-10} M$.

TRAP and Hoechst staining of murine BMC

To visualize the BMCs grown on the ceramics, nuclei were stained using Hoechst 33342. Staining for tartrate resistant acid phosphatase (TRAP) was performed to identify osteoclast lineage cells. For this purpose, cell seeded ceramics were washed with PBS and subsequently fixed with 4% buffered paraformaldehyde for 10 min. Afterwards, the samples were washed with demineralized water and incubated with 0.5 mL TRAP staining solution for 5 min. The samples were rinsed again with demineralized water and incubated with Hoechst 33342 nuclear stain (concentration 1 mg/mL, Fluka Switzerland) for 10 min. Finally, the ceramics were washed with PBS and demineralized water and dried overnight at 45°C.

Scanning electron microscopy analysis

Scanning electron microscopy (SEM) was performed to analyze the surface morphology of the BCP ceramics using a Philips SEM unit (XL 30 FEG, Philips, The Netherlands). Dry samples, either with or without cells, were glued onto microscope holders with conductive carbon cement (Leit-C by Göcke, Baltec, Baltzers, Liechtenstein). For conductivity, a thin layer of 10-nm gold was deposited using a Baltec SCD 004 unit (Baltec, Baltzers, Liechtenstein). For imaging, the following microscope parameters were used as follows: voltage: 10 kV, working distance: ~10 mm, and electron source: secondary electrons.

Cell fixation and critical point drying

To analyze the behavior of the cells on the ceramics by SEM, cells were fixed as described by Baxter et al.[42] In brief, the ceramics with the cells were washed with 0.1*M* PIPES buffer pH 7.4 and fixed with 2.5% glutaraldehyde. After additional washings with PIPES buffer, the samples were immersed in 0.5% osmiumtetroxide and subsequently dehydrated through a graded series of ethanol. Thereafter, the samples were critical point dried with a CPD 30 critical point drier (Baltec, Baltzers, Liechtenstein).

Quantification of [^{3}H]BSA uptake

The uptake of [^{3}H]BSA onto the BCP discs was quantified by liquid scintillation counting. [^{3}H]BSA coated BCP discs were dissolved in 2 mL of 1*M* hydrochloric acid (HCl). Aliquots of 350-μL were then mixed with 1400 μL distilled water and 17.5 mL Ultima Gold™ scintillation fluid. Scintillation was measured with a liquid scintillation counter (TriCarb 2200CA, Packard, Downers Grove, IL).

Analysis of the release of [^{3}H]BSA from BCP ceramics

The passive and the cell-mediated release of [^{3}H]BSA from the BCP discs was monitored over a period of 19 days. BCP discs coated with [^{3}H]BSA were equilibrated in α-MEM culture media for 24 h. Afterwards, the discs were incubated in α-MEM culture media for another 18 days to investigate the spontaneous release of [^{3}H]BSA. To induce the cell-mediated release of [^{3}H]BSA, 1×10^6 murine BMC were seeded onto the BCP discs and cultured for 18 days in the presence of CSF-1 alone or in the presence of CSF-1 and RANKL as described above. The culture medium was changed in 3 day intervals and processed for subsequent analysis.

To quantify the spontaneous and the cell-mediated release of [^{3}H]BSA, 500 μL of the collected culture supernatants were added to 3 mL Ultima Gold™ scintillation fluid and [^{3}H] was measured with a liquid scintillation counter. At the end of the release experiments, the residual radioactivity of the BCP discs was determined by dissolving the materials in hydrochloric acid and subsequent liquid scintillation counting as described above. The percentage of [^{3}H]BSA released from the BCP discs was calculated according to the formula [dpm of the released fraction/(dpm of the released fraction + dpm of the residual radioactivity of the BCP discs) × 100].

Statistics

All numerical data are presented as mean values together with the standard deviation. The data were statistically evaluated by ANOVA using SigmaStat® software for Windows (Version 3.01, Systat Software, San Jose, CA). Pairwise multiple comparisons were made using the Tukey test. Differences were considered to be statistically significant if the p value was less than 0.05.

RESULTS

Uptake of [^{3}H]BSA by BCP ceramics

The uptake of [^{3}H]BSA by BCP ceramics was quantified after dissolution of the ceramics in 2 mL 1*M* hydrochloric acid. The coating efficacy was calculated as the percentage of the protein immobilized onto the ceramics in relation to the amount of protein within the coating solution. At a coating concentration of 0.1 μg/mL [^{3}H]BSA the coating efficacy was similar irrespective of whether [^{3}H]BSA was adsorbed or coprecipitated onto BCP ceramics [Adsorption (volume 2.5 mL): 14.1 ± 5.2%, Coprecipitation (volume 2.5 mL): 14.5 ± 2.7%, Coprecipitation (volume 12 mL): 17.0 ± 3.4%]. At a concentration of 1 μg/mL and 10 μg [^{3}H]BSA per mL coating solution, there was a statistically significant difference found in coating efficacy between adsorption and coprecipitation with 2.5 mL and 12 mL coating volume [Adsorption (volume 2.5 mL, 1 μg [^{3}H]BSA/mL): 26.3 ± 1.5%, Coprecipitation (volume 2.5 mL, 1 μg [^{3}H]BSA/mL): 21.4 ± 4.6%, Coprecipitation (volume 12 mL, 1 μg [^{3}H]BSA/mL): 20.7 ± 3.2%, Adsorption (volume 2.5 mL, 10 μg [^{3}H]BSA/mL): 31.8 ± 3.1%, Coprecipitation (volume 2.5 mL, 10 μg [^{3}H]BSA/mL): 23.2 ± 4.0%, Coprecipitation (volume 12 mL, 10 μg [^{3}H]BSA/mL): 24.4 ± 5.8%; $p < 0.05$ Adsorption (volume 2.5 mL) *versus* Coprecipitation (volume 2.5 mL and 12 mL)]. As shown in Table I, adsorption of [^{3}H]BSA subsequent to CaP precipitation of the ceramics (CaP precipitation/adsorption) at a concentration of 1 μg [^{3}H]BSA per mL coating showed similar coating efficacy as the coprecipitation of [^{3}H]BSA. The coating efficacy of CaP precipitation/adsorption of [^{3}H]BSA was significantly lower when compared with direct adsorption of [^{3}H]BSA onto BCP ceramics.

TABLE I
Uptake of [^{3}H]BSA onto Biphasic Calcium Phosphate Ceramics

Coating Technique	V (mL)	Uptake [^{3}H]-BSA (%)
Adsorption	2.5	26.3 ± 1.5$^{*\#}$
Adsorption	12.0	28.0 ± 2.4$^{*\#}$
Coprecipitation	2.5	21.4 ± 4.6
Coprecipitation	12.0	20.7 ± 3.2
CaP precipitation/adsorption	2.5	20.9 ± 1.8
CaP precipitation/adsorption	12.0	22.5 ± 1.4

The table depicts the value of protein uptake using [^{3}H]BSA at concentration of 1 μg per mL coating solution. Mean values ± standared deviation ($n = 6$ for each group), $^*p < 0.05$ *versus* Coprecipitation and same volume of coating solution, $^\#p < 0.05$ *versus* CaP precipitation/adsorption and same volume of coating solution.

Passive and cell-mediated release of [^{3}H]BSA from BCP ceramics

The passive and the cell-mediated release of [^{3}H]BSA from BCP ceramics was monitored over 19 days. Within 19 days, 65–73% of [^{3}H]BSA adsorbed to the ceramics was passively released into the medium. Coprecipitation of [^{3}H]BSA onto the material significantly decreased the passive release to 13–21% ($p < 0.001$ vs. Adsorption) of the total protein bound to the ceramics. Coprecipitation improved the retention of [^{3}H]BSA from passive release independent of whether the protein was coprecipitated to BCP ceramics at concentrations of 0.1, 1, or 10 μg/mL coating solution (Table II). With adsorption and coprecipitation of [^{3}H]BSA, the passive release proceeded almost completely within the first 4 days of incubation (Fig. 1). Adsorption of BCP ceramics with [^{3}H]BSA at a concentration of 1 μg/mL (volume

TABLE II
Cumulative Passive and Cell-Mediated Release of [^{3}H]-BSA from BCP ceramics

Coating technique	*V* (mL)	Cumulative Release (% of Protein Uptake)		
		Incubation a-MEM	BMC +CSF-1, RANKL	BMC +CSF-1, +RANKL
Adsorption	2.5	71.1 ± 5.3	80.5 ± 8.2	87.7 ± 6.9*
Adsorption	12.0	84.4 ± 2.6	93.0 ± 3.2*	91.6 ± 3.2*
Coprecipitation	2.5	15.5 ± 2.5	17.4 ± 2.8	29.3 ± 5.3$^{\#¥}$
Coprecipitation	12.0	12.5 ± 2.0	14.7 ± 3.0	37.8 ± 6.1$^{\#¥}$
CaP precipitation/adsorption	2.5	82.1 ± 9.3	87.4 ± 3.2	91.9 ± 3.1*
CaP precipitation/adsorption	12.0	81.8 ± 0.9	82.0 ± 2.0	92.3 ± 4.2^{*+}

The cumulative release of [^{3}H]-BSA (concentration 1μg per mL coating solution) was determined during 19 days with respects to the passive release (incubation of ceramics in α-MEM culture media) and cell-mediated release by murine bone marrow cells differentiated toward monocytes/macrophages (BMC +CSF-1, −RANKL) and osteoclasts (BMC +CSF-1, +RANKL). Mean values ± standard deviation (n = 6 for each group), *p < 0.05 *versus* α -MEM of the same group, $^+$p < 0.05 *versus* BMC, +CSF-1,−RANKL of the same group, $^\#$p < 0.001 *versus* a-MEM of the group, $^¥$p < 0.001 *versus* BMC, +CSF-1, −RANKL of the same group.

2.5 mL) resulted in a release of 63.0 ± 6.0% of the protein within 4 days when compared with 71.7 ± 5.3% within 19 days of incubation. The analysis of the release kinetics after coprecipitation of [^{3}H]BSA at a concentration of 1 μg/mL (volume 2.5 mL) showed a release of 12.8 ± 2.6% of the protein within 4 days and of 15.5 ± 2.5% release within 19 days. CaP precipitation with subsequent adsorption of [^{3}H]BSA (CaP prec./Ads.) did not modify the release kinetics of the protein when compared with adsorption of [^{3}H]BSA directly onto the ceramics. With the CaP precipitation/adsorption of [^{3}H]BSA at a concentration of 1 μg/mL (volume 2.5 mL) 65.5 ± 6.0% and 82.1 ± 9.3% of the protein were release within 4 days and 19 days, respectively. With all coating techniques, the volume of the coating solution did not influence the release kinetics during passive release. Adsorption, coprecipitation, and CaP precipitation/adsorption showed similar release kinetics independent of whether the respective technique was used with a coating volume of 2.5 or 12 mL (Fig. 1; Table II).

To assess the cell-mediated release of [^{3}H]BSA, BMCs were grown on the ceramics and differentiated to monocytes/macrophages and osteoclasts, respectively. The kinetics of the cell-mediated release of adsorbed [^{3}H]BSA was identical irrespective whether BMCs were differentiated to monocytes/macrophages or to osteoclasts [Fig. 2(A,B); Table II]. Furthermore, the kinetics of the release was independent of the presence of cells in ceramics adsorbed with [^{3}H]BSA. If [^{3}H]BSA was adsorbed to BCP ceramics subsequent to CaP precipitation (CaP prec./Ads.), there was no modification of the release of [^{3}H]BSA by murine BMCs observed independent of whether the cells were differentiated toward monocytes/macrophages or osteoclasts [Fig. 2(C,D); Table II].

In BCP ceramics coprecipitated with [^{3}H]BSA, BMCs were able to induce a cell-mediated release of [^{3}H]BSA if differentiated toward osteoclasts. [Fig. 2(E,F); Table II]. From day 7, the cell-mediated release of coprecipitated [^{3}H]BSA by osteoclasts gave rise to a sustained liberation of 2.6 ± 0.5% to 7.3 ± 2.0% of the protein bound to the materials per

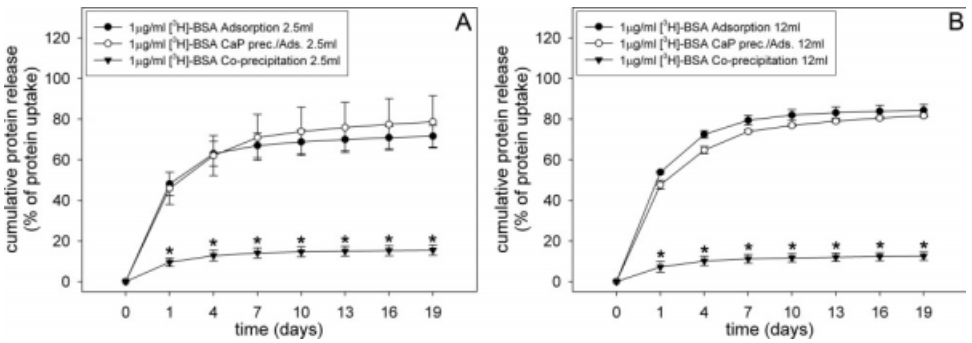

Figure 1. The graphs show the passive release kinetics of [^{3}H]-BSA coated onto BCP discs at a concentration of 1 μg/mL using coating volumes of 2.5 mL (A) and 12 mL (B) during the course of 19 days of incubation. Mean values are represented ± standard deviation (n = 6 for each group), *p < 0.001 *versus* adsorption and *versus* CaP precipitation/adsorption.

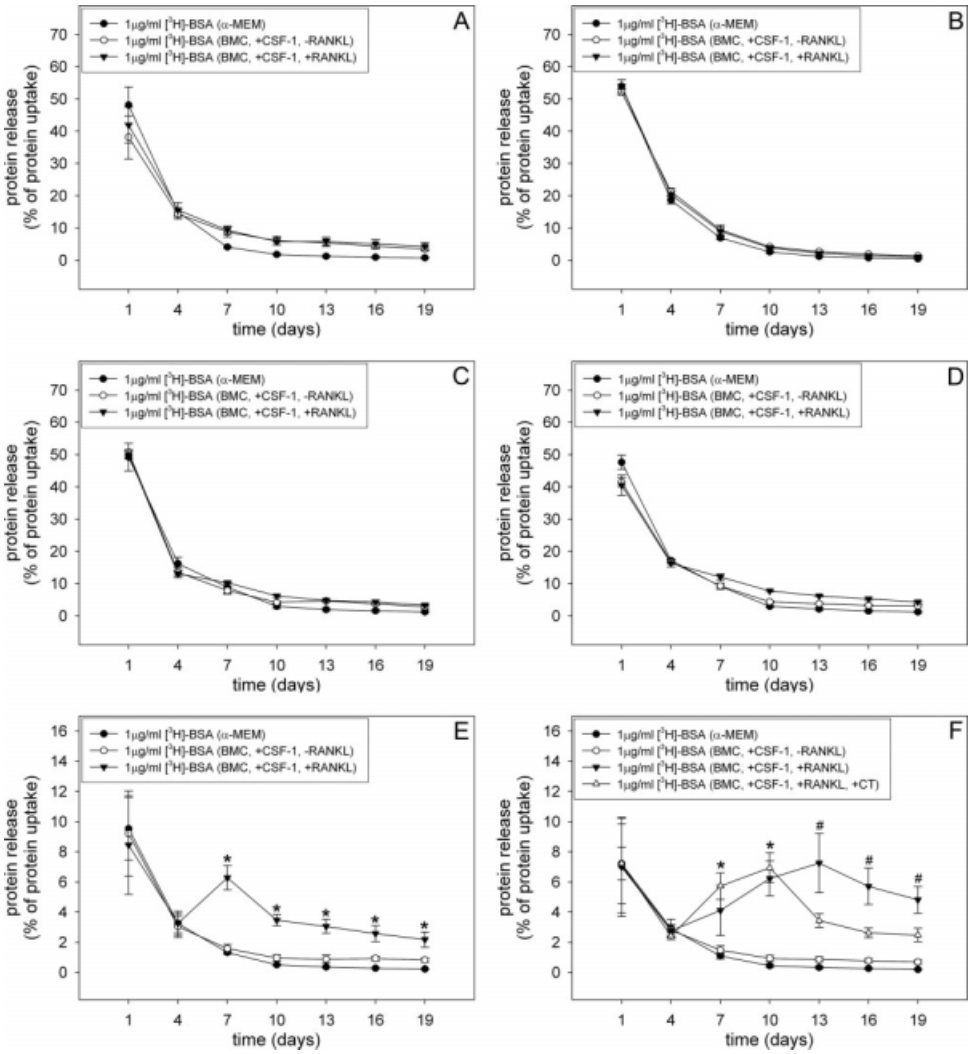

Figure 2. The spontaneous release after incubation of ceramics in α-MEM culture media and the cell-mediated release in the presence of murine bone marrow cells were investigated over 19 days. Graphs show the release of [^{3}H]BSA loaded to the ceramics at a concentration of 1 µg/mL. Adsorption (A, B): Release of [^{3}H]BSA adsorbed to the surface of the ceramics with a volume of 2.5 mL (A) and 12 mL (B). CaP precipitation/adsorption (C, D): Release of [^{3}H]BSA from ceramics that first underwent precipitation of calcium phosphate and were subsequently adsorbed with [^{3}H]BSA at a volume of 2.5 mL (C) and 12 mL (D). Coprecipitation (E, F): Release of [^{3}H]BSA coprecipitated to the ceramics with 2.5 mL (E) and 12 mL (F) calcium phosphate solution. Mean values are represented ± SD (n = 6 for each group), *$p \leq 0.001$ *versus* 1 µg/mL [^{3}H]BSA (α-MEM) and *versus* 1 µg/mL [^{3}H]BSA (BMC, +CSF-1, −RANKL); #$p \leq 0.001$ *versus* 1 µg/mL [^{3}H]BSA (α-MEM), *versus* 1 µg/mL [^{3}H]BSA (BMC, +CSF-1, −RANKL), and *versus* 1 µg/mL [^{3}H]BSA (BMC, +CSF-1, +RANKL, +CT).

3 days period. The addition of calcitonin to BMCs differentiated toward osteoclasts on day 10 of culture efficiently inhibited the cell-mediated release of [^{3}H]BSA. In contrast to osteoclasts, BMCs differentiated to monocytes/macrophages did not induce a cell-mediated release of coprecipitated [^{3}H]BSA. There was no modification of the passive release kinetics by monocytes/macrophages found

Proliferation and differentiation of BMCs grown on BCP ceramics

The proliferation and differentiation of BMCs on the ceramics was visualized using Hoechst and TRAP staining on day 19 of the cell culture. As shown in Figure 3(A,B), BMCs treated with CSF-1 but without RANKL proliferated on the surface of BCP ceramics but were TRAP negative. BMCs treated with CSF-1 and RANKL differentiated into TRAP-positive multinucleated cells [Fig. 3(C–F)]. Hoechst/TRAP staining was similar for ceramics adsorbed and coprecipitated with [^{3}H]BSA (not shown).

Scanning electron microscopy analysis

Scanning electron microscopy demonstrated that the adsorption of [^{3}H]BSA to the materials did not

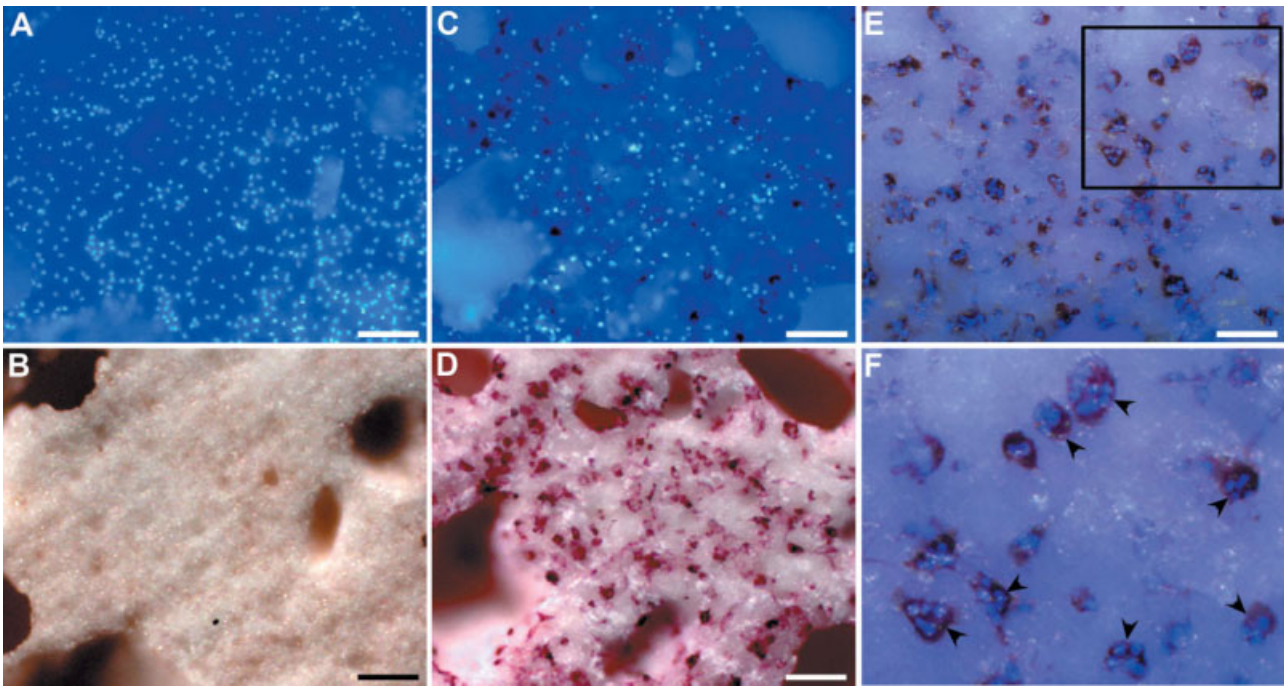

Figure 3. After 19 days of culture of BMC on BCP ceramics, cells were visualized using a nuclear stain (Hoechst 33342). To assess the differentiation of BMC to osteoclast lineage cells, TRAP staining was performed. Staining of nuclei showed that BMCs cultured in the presence of CSF-1 but without RANKL (A) and BMC treated with CSF-1 and RANKL (C) were able to proliferate on BCP ceramics (A, C: fluorescence illumination, BMCs: bright blue, ceramic material: dark blue). BMCs treated with CSF-1 only did not show positive staining for TRAP and were therefore not visualized on the ceramics if fluorescence illumination was not applied (B). BMCs cultured in the presence of CSF-1 and RANKL differentiated toward TRAP-positive (red) osteoclasts (D). Multichannel imaging approved the presence of multinucleated TRAP positive osteoclast derived from BMCs treated with CSF-1 and RANKL (marked by arrowheads) [E, F (detailed view)]. Scale bars represent 100 μm.

change the surface morphology of the materials. By SEM, no differences in the structure of the original ceramics and ceramics adsorbed with [^{3}H]BSA were detectable [Fig. 4(A–D)]. The surface morphology of BCP ceramics subjected to the coprecipitation protocol, however, was markedly modified. The immersion of the material in five-times concentrated simulated body fluid created an amorphous layer of calcium phosphate on the surface [Fig. 4(E,F)]. Subsequent coprecipitation of [^{3}H]BSA by immersion of the materials in a supersaturated solution of calcium phosphate together with the protein resulted in the superimposition of a crystalline layer of calcium phosphate [Fig. 4(G,H)]. Ceramics that underwent precipitation of calcium phosphate without addition of [^{3}H]BSA first to create a protein free-layer of crystalline calcium phosphate and were subsequently adsorbed with the protein showed similar surface morphologies when compared with ceramics coprecipitated with [^{3}H]BSA [Fig. 4(I,J)].

SEM analysis following the culture of murine BMCs on the ceramics showed that BMCs differentiated toward monocytes/macrophages were not able to resorb the crystalline or amorphous layer of calcium phosphate created by the coprecipitation procedure [Fig. 5(A–C)]. BMCs cultured with CSF-1 and RANKL developed into active osteoclasts, able to resorb the calcium phosphate structure of the materials [Fig. 5(D–F)].

DISCUSSION

The limitations associated with autogenic or allogenic bone grafts have lead investigators to search for alternative solutions in the treatment of bone defects. Recent approaches include the use of bone substitutes combined with osteogenic growth factors such as members of the BMP family. Others have utilized regional delivery of mesenchymal stem cells (MSC) to promote bone healing. MSCs offer the promising potential to augment healing of critically sized defects.[43] Their application, however, is complex, requiring cells to be harvested from a secondary site, expanded *in vitro*, and seeded onto the bone substitute material directly prior to implantation. Ready to use biomaterial-growth factor constructs may provide a solution to some of the difficulties

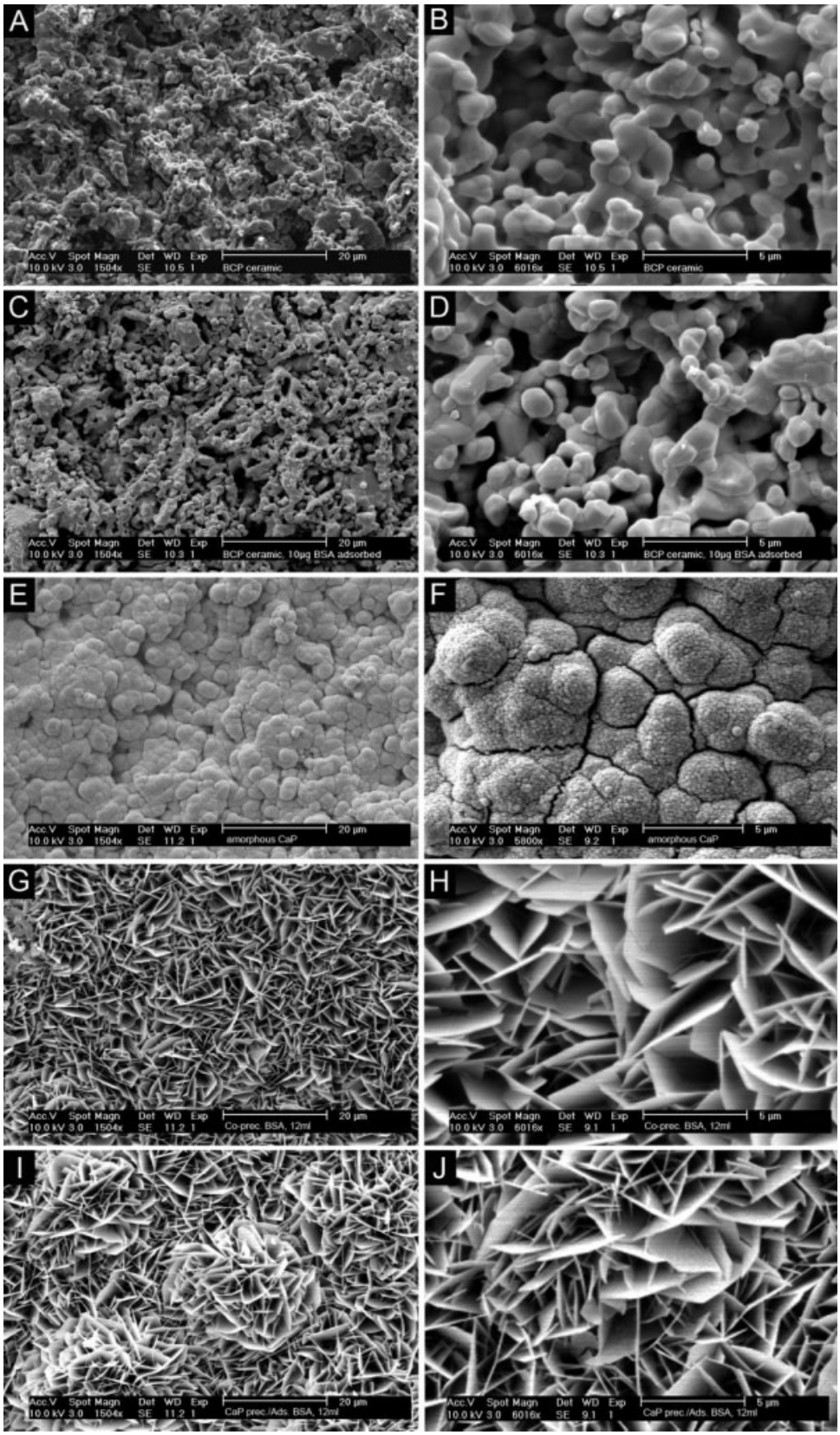

Figure 4. Scanning electron microscopy (SEM) was performed to analyze the surface morphology of BCP ceramics before and after the coating procedures. Untreated BCP ceramic (A, B). Adsorption of [^{3}H]BSA to the ceramics did not change the surface structure of the material (C, D). Immersion of BCP ceramics within five-times concentrated simulated body fluid created an amorphous layer of calcium phosphate (E, F). Subsequent incubation of ceramics in a supersaturated solution of calcium phosphate in the presence of [^{3}H]BSA created a crystalline layer of calcium phosphate on the surface of the materials (G, H; coprecipitation of [^{3}H]BSA). Protein free calcium phosphate precipitation and subsequent adsorption of [^{3}H]BSA (CaP precipitation/adsorption of [^{3}H]BSA) resulted in the formation of a layer of crystalline calcium phosphate similar to that found with the coprecipitation technique (I, J).

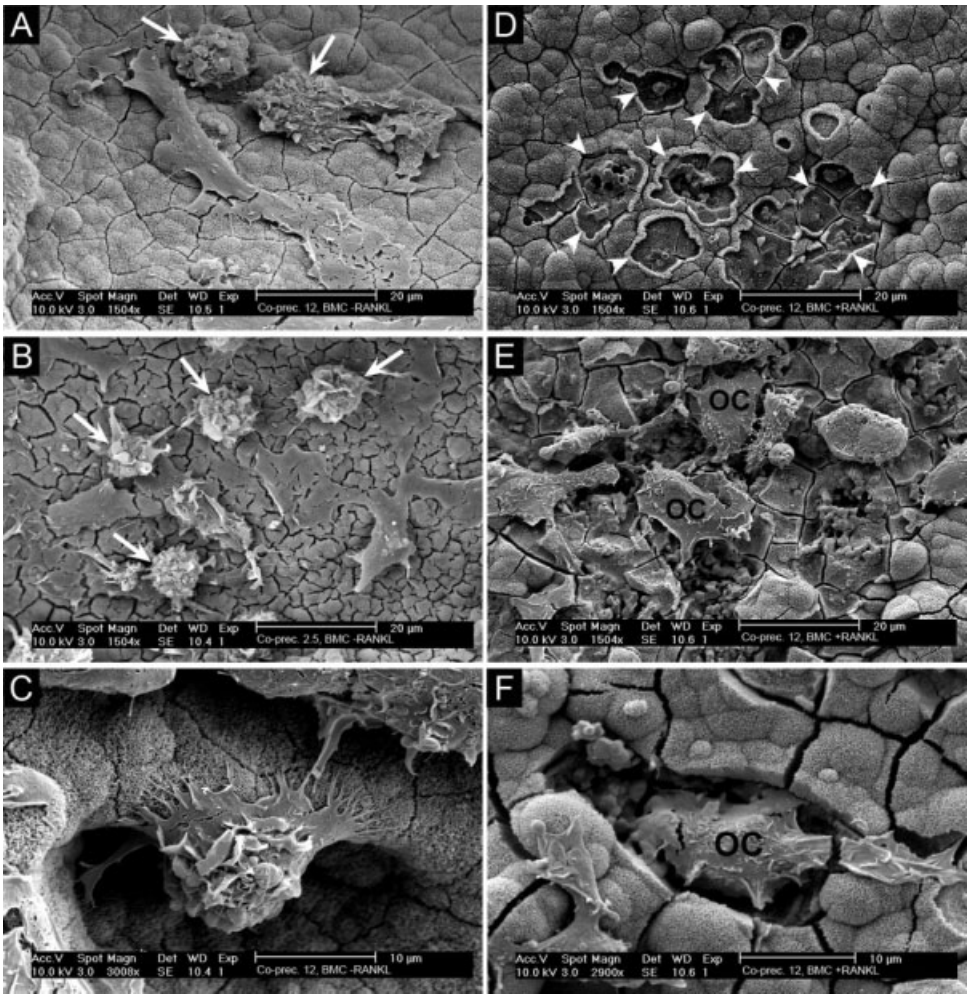

Figure 5. Scanning electron microscopy was performed after 19 days of culture of murine bone marrow cells on BCP ceramics coprecipitated with [^{3}H]BSA in the presence of calcium phosphate. (A–C) BMCs which grown on the ceramics and differentiated toward monocytes/macrophages were not able to resorb the amorphous phase of the crystalline layer of calcium phosphate created by the coprecipitation procedure (monocytes/macrophages marked by arrows in A and B). (D–F) BMCs cultured in the presence of CSF-1 and RANKL developed into active resorbing osteoclasts capable to dissolve the calcium phosphate layers. (D) Osteoclastic resorption of the calcium phosphate layers with typical resorption lacunae (marked by arrowheads). (E, F) Osteoclasts (OC) resorbing the calcium phosphate structure of the ceramics.

associated with stem cell use and may be advantageous to MSCs in clinical settings.

Efficient delivery of growth factors from carrier biomaterials depends critically on the release kinetics of the proteins that constitute the carrier. Woo et al.[44] have previously shown that a sustained availability of low concentrations of BMP-2 induced a significantly faster and broader repair of osseous defects in rat calvaria when compared with an immediate release model. In this study, [^{3}H]BSA was coprecipitated together with calcium phosphate onto BCP ceramics,[33–35,45,46] in attempt to achieve a sustained delivery of the protein from BCP ceramics. The coprecipitation technique was compared with a conventional protein adsorption procedure.[9,11] In accordance with previous findings,[12–14] adsorption of [^{3}H]BSA to BCP ceramics resulted in a rapid passive release of a large proportion of the protein bound to the material. On average, 62% of [^{3}H]BSA was released passively within the first 4 days. By applying the coprecipitation technique, ~80% of [^{3}H]BSA was retained from passive release during 19 days of investigation and the burst release within the first 4 days was significantly reduced to 13–16%. The residual burst release found with the coprecipitation technique was in the range of previously described coating techniques exhibiting slow release kinetics.[19,31,36,37] Kim and Valentini[36] immobilized rhBMP-2 onto hyaluronic acid-based carrier materials and observed a slow, passive release of approximately one third of the growth factor during 30 days. In accordance with this study, ~16% of immobilized rhBMP-2 was passively released within the first 4 days.

Various investigations have described coating techniques which exhibited slow release kinetics of proteins from implantable biomedical materials *in vitro* by entrapping agents into carrier materials, such as polymers and compounds of calcium phosphates and gelatin, or by binding proteins to titanium implants following chemical modification of the metal surface.[18–20,25,26,29–32,37] In these studies, investigations of release kinetics were performed by incubating protein coated biomaterials within in physiological solutions such as cell culture media or simulated body fluid, thus addressing the passive, noncell-mediated release of the proteins from the carrier only. *In vivo*, however, inflammatory cells such as monocytes/macrophages and osteoclasts may interact with the biomaterial and accelerate the release of proteins; thereby, compromising the slow release kinetic patterns found in an acellular environment. It is reasonable to assume that cell-mediated protein liberation significantly influences the temporal bioavailability of growth factors within a biomaterial implantation site. This suggestion is supported by a recent *in vitro* study by Lee et al.[28] who found a significant increase of the release of bovine serum albumin from polymer scaffolds in the presence of rat vascular smooth muscle cells when compared with the passive release of the protein into DMEM culture media. We, therefore, analyzed the release of [^{3}H]BSA from BCP ceramics mediated by monocytes/macrophages and osteoclasts derived from bone marrow mesenchymal cells *in vitro*.

In BCP, ceramics adsorbed with [^{3}H]BSA the release kinetics were not modified by the presence of monocytes/macrophages or osteoclasts, showing that the release of the protein was induced exclusively by passive mechanisms. Superficial adsorption does not provide the possibility to modulate the liberation of proteins by means of cell-mediated release. When [^{3}H]BSA was coprecipitated to BCP ceramics, BMC derived osteoclasts markedly influenced the liberation of [^{3}H]BSA inducing a sustained, cell-mediated protein release. The cell-mediated release was exclusively found with BMC's differentiated toward osteoclasts. BMC's differentiated toward monocytes/macrophages did not modify the release kinetics of [^{3}H]BSA. Additionally, SEM analyses of BCP ceramics coprecipitated with [^{3}H]BSA showed that osteoclasts, but not monocytes/macrophages were able to resorb the crystalline calcium phosphate structure of the coating. These results indicate that the cell-mediated release of [^{3}H]BSA was accomplished by osteoclastic resorption of the crystalline calcium phosphate layer in which the protein was incorporated, thereby liberating the protein from the carrier material.

SEM was also employed to investigate the morphology of the ceramic carrier materials and revealed that the surface microarchitecture was markedly modified by the coprecipitation technique when compared with the adsorption of [^{3}H]BSA. To distinguish whether the amelioration of protein retention and the modification of the protein release kinetics was due to the incorporation [^{3}H]BSA in the crystalline layer of CaP or merely due to the modification of the surface morphology, we performed a third coating technique. With this procedure, the surface structure was first modified by creating a protein free layer of crystalline calcium phosphate. Subsequently, [^{3}H]BSA was superficially adsorbed onto the modified surface. The release kinetics of [^{3}H]BSA using this technique mimicked the kinetics found in conventional adsorption and lacked a detectable cell-mediated release. These results demonstrate that the improvement of release kinetics applying the coprecipitation technique occurred, primarily, through the incorporation of proteins into the three-dimensional structure of the crystalline CaP layer and was not significantly affected by modifications of the surface morphology. Imbedding proteins into three-dimensional calcium phosphate based scaffolds may enhance bone regeneration by simultaneously achieving two goals: providing long-term bioavailability of osteogenic agents by cell-mediated release and providing highly osteoconductive matrices for bone formation.

A potential drawback of the coprecipitation technique may be the diminished coating efficacy under certain coating conditions, which was found to be decreased by a factor of 1.2–1.35 in comparison with conventional protein adsorption. A lower coating efficacy would implicate a need to employ larger amounts of a growth factor to finally immobilize equal amounts of the protein on its carrier. Considering the high costs of recombinantly produced growth factors, this decrease in coating efficacy may result in an increase in manufacturing costs of biomaterial-growth factor constructs. On the other hand, coprecipitation of [^{3}H]BSA greatly enhanced protein retention by reducing the burst release of the protein by a factor of 4–4.9. Therefore, the decreased coating efficacy of protein coprecipitation is easily outweighted by the greatly ameliorated efficacy of protein delivery of this procedure.

In conclusion, coprecipitation significantly improved the release kinetics of [^{3}H]BSA from calcium phosphate ceramics. By incorporating [^{3}H]BSA into the three-dimensional structure of calcium phosphate ceramics, we were able to sufficiently retain [^{3}H]BSA on the biomaterials, minimize the burst release of the protein, and achieve a sustained, cell-mediated release induced by resorbing osteoclasts. Because of the physiologic-like release mode, the coprecipitation technique looks to be a promising tool in optimizing growth factor delivery for bone

healing. Additionally, a long-term release of low concentrations of osteogenic growth factors may reduce the risks of excessive bone formation, soft tissue ossification, and neoplastic induction which are attributed to their applications.

References

1. Finkemeier CG. Bone-grafting and bone-graft substitutes. J Bone Joint Surg Am 2002;84A:454–464.
2. Gazdag AR, Lane JM, Glaser D, Forster RA. Alternatives to autogenous bone graft: Efficacy and indications. J Am Acad Orthop Surg 1995;3:1–8.
3. Coventry MB, Tapper EM. Pelvic instability: A consequence of removing iliac bone for grafting. J Bone Joint Surg Am 1972;54:83–101.
4. Damien CJ, Parsons JR. Bone graft and bone graft substitutes: A review of current technology and applications. J Appl Biomater 1991;2:187–208.
5. Buck BE, Malinin TI, Brown MD. Bone transplantation and human immunodeficiency virus. An estimate of risk of acquired immunodeficiency syndrome (AIDS). Clin Orthop Relat Res 1989;240:129–136.
6. Stevenson S, Shaffer JW, Goldberg VM. The humoral response to vascular and nonvascular allografts of bone. Clin Orthop Relat Res 1996;326:86–95.
7. Stevenson S, Emery SE, Goldberg VM. Factors affecting bone graft incorporation. Clin Orthop Relat Res 1996;324:66–74.
8. Liu Y, Huse RO, de Groot K, Buser D, Hunziker EB. Delivery mode and efficacy of BMP-2 in association with implants. J Dent Res 2007;86:84–89.
9. Alam MI, Asahina I, Ohmamiuda K, Takahashi K, Yokota S, Enomoto S. Evaluation of ceramics composed of different hydroxyapatite to tricalcium phosphate ratios as carriers for rhBMP-2. Biomaterials 2001;22:1643–1651.
10. Uludag H, D'Augusta D, Palmer R, Timony G, Wozney J. Characterization of rhBMP-2 pharmacokinetics implanted with biomaterial carriers in the rat ectopic model. J Biomed Mater Res 1999;46:193–202.
11. Aebli N, Stich H, Schawalder P, Theis JC, Krebs J. Effects of bone morphogenetic protein-2 and hyaluronic acid on the osseointegration of hydroxyapatite-coated implants: An experimental study in sheep. J Biomed Mater Res A 2005;73: 295–302.
12. Lasserre A, Bajpai PK. Ceramic drug-delivery devices. Crit Rev Ther Drug Carrier Syst 1998;15:1–56.
13. Liu Y, Hunziker EB, Randall NX, de Groot K, Layrolle P. Proteins incorporated into biomimetically prepared calcium phosphate coatings modulate their mechanical strength and dissolution rate. Biomaterials 2003;24:65–70.
14. Schmidmaier G, Wildemann B, Bail H, Lucke M, Stemberger A, Flyvbjerg A, Raschke M. Local liberation of IGF-I and TGF-beta 1 from a biodegradable poly(d,l-lactide) coating of implants accelerates fracture healing. Chirurg 2000;71:1016–1022.
15. Kanatani M, Sugimoto T, Kaji H, Kobayashi T, Nishiyama K, Fukase M, Kumegawa M, Chihara K. Stimulatory effect of bone morphogenetic protein-2 on osteoclast-like cell formation and bone-resorbing activity. J Bone Miner Res 1995;10: 1681–1690.
16. Kaneko H, Arakawa T, Mano H, Kaneda T, Ogasawara A, Nakagawa M, Toyama Y, Yabe Y, Kumegawa M, Hakeda Y. Direct stimulation of osteoclastic bone resorption by bone morphogenetic protein (BMP)-2 and expression of BMP receptors in mature osteoclasts. Bone 2000;27:479–486.
17. Valentin-Opran A, Wozney J, Csimma C, Lilly L, Riedel GE. Clinical evaluation of recombinant human bone morphogenetic protein-2. Clin Orthop Relat Res 2002;395:110–120.
18. Takahashi Y, Yamamoto M, Tabata Y. Enhanced osteoinduction by controlled release of bone morphogenetic protein-2 from biodegradable sponge composed of gelatin and beta-tricalcium phosphate. Biomaterials 2005;26:4856–4865.
19. Chen PR, Chen MH, Lin FH, Su WY. Release characteristics and bioactivity of gelatin-tricalcium phosphate membranes covalently immobilized with nerve growth factors. Biomaterials 2005;26:6579–6587.
20. Adden N, Gamble LJ, Castner DG, Hoffmann A, Gross G, Menzel H. Phosphonic acid monolayers for binding of bioactive molecules to titanium surfaces. Langmuir 2006;22:8197–8204.
21. Determan MD, Cox JP, Mallapragada SK. Drug release from pH-responsive thermogelling pentablock copolymers. J Biomed Mater Res A 2007;81:326–333.
22. Gao H, Wang YN, Fan YG, Ma JB. Conjugates of poly(D,L-lactide-*co*-glycolide) on amino cyclodextrins and their nanoparticles as protein delivery system. J Biomed Mater Res A 2007; 80:111–122.
23. Defail AJ, Edington HD, Matthews S, Lee WC, Marra KG. Controlled release of bioactive doxorubicin from microspheres embedded within gelatin scaffolds. J Biomed Mater Res A 2006;79:954–962.
24. Levy Y, Zilberman M. Novel bioresorbabale composite fiber structures loaded with proteins for tissue regeneration applications: Microstructure and protein release. J Biomed Mater Res A 2006;79:779–787.
25. Desire L, Mysiakine E, Bonnafous D, Couvreur P, Sagodira S, Breton P, Fattal E. Sustained delivery of growth factors from methylidene malonate 2.1.2-based polymers. Biomaterials 2006;27:2609–2620.
26. Ennett AB, Kaigler D, Mooney DJ. Temporally regulated delivery of VEGF in vitro and in vivo. J Biomed Mater Res A 2006;79:176–184.
27. Kempen DH, Lu L, Kim C, Zhu X, Dhert WJ, Currier BL, Yaszemski MJ. Controlled drug release from a novel injectable biodegradable microsphere/scaffold composite based on poly(propylene fumarate). J Biomed Mater Res A 2006;77: 103–111.
28. Lee M, Chen TT, Iruela-Arispe ML, Wu BM, Dunn JC. Modulation of protein delivery from modular polymer scaffolds. Biomaterials 2007;28:1862–1870.
29. Prabu P, Dharmaraj N, Aryal S, Lee BM, Ramesh V, Kim HY. Preparation and drug release activity of scaffolds containing collagen and poly(caprolactone). J Biomed Mater Res A 2006;79:153–158.
30. Puleo DA, Kissling RA, Sheu MS. A technique to immobilize bioactive proteins, including bone morphogenetic protein-4 (BMP-4), on titanium alloy. Biomaterials 2002;23:2079–2087.
31. Qiu B, Stefanos S, Ma J, Lalloo A, Perry BA, Leibowitz MJ, Sinko PJ, Stein S. A hydrogel prepared by in situ cross-linking of a thiol-containing poly(ethylene glycol)-based copolymer: A new biomaterial for protein drug delivery. Biomaterials 2003;24:11–18.
32. Yoshinari M, Hayakawa T, Matsuzaka K, Inoue T, Oda Y, Shimono M, Ide T, Tanaka T. Oxygen plasma surface modification enhances immobilization of simvastatin acid. Biomed Res 2006;27:29–36.
33. Liu Y, Layrolle P, de Bruijn J, van Blitterswijk C, de Groot K. Biomimetic coprecipitation of calcium phosphate and bovine serum albumin on titanium alloy. J Biomed Mater Res 2001;57:327–335.
34. Liu Y, Hunziker EB, Layrolle P, De Bruijn JD, de Groot K. Bone morphogenetic protein 2 incorporated into biomimetic coatings retains its biological activity. Tissue Eng 2004;10: 101–108.

35. Liu Y, de Groot K, Hunziker EB. BMP-2 liberated from biomimetic implant coatings induces and sustains direct ossification in an ectopic rat model. Bone 2005;36:745–757.
36. Kim HD, Valentini RF. Retention and activity of BMP-2 in hyaluronic acid-based scaffolds in vitro. J Biomed Mater Res 2002;59:573–584.
37. Rai B, Teoh SH, Hutmacher DW, Cao T, Ho KH. Novel PCL-based honeycomb scaffolds as drug delivery systems for rhBMP-2. Biomaterials 2005;26:3739–3748.
38. Tack BF, Dean J, Eilat D, Lorenz PE, Schechter AN. Tritium labeling of proteins to high specific radioactivity by reduction methylation. J Biol Chem 1980;18:8842–8847.
39. Arnett TR, Dempster DW. Effect of pH on bone resorption by rat osteoclasts in vitro. Endocrinology 1986;119:119–124.
40. Arnett TR, Boyde A, Jones SJ, Taylor ML. Effects of medium acidification by alteration of carbon dioxide or bicarbonate concentrations on the resorptive activity of rat osteoclasts. J Bone Miner Res 1994;9:375–379.
41. Arnett TR, Spowage M. Modulation of the resorptive activity of rat osteoclasts by small changes in extracellular pH near the physiological range. Bone 1996;18:277–279.
42. Baxter LC, Frauchiger V, Textor M, ap Gywn I, Richards RG. Fibroblast and osteoblast adhesion and morphology on calcium phosphate surfaces. Eur Cell Mater 2002;4:1–17.
43. Petite H, Viateau V, Bensaid W, Meunier A, de Pollak C, Bourguignon M, Oudina K, Sedel L, Guillemin G. Tissue-engineered bone regeneration. Nat Biotechnol 2000;18:959–963.
44. Woo BH, Fink BF, Page R, Schrier JA, Jo YW, Jiang G, DeLuca M, Vasconez HC, DeLuca PP. Enhancement of bone growth by sustained delivery of recombinant human bone morphogenetic protein-2 in a polymeric matrix. Pharm Res 2001;18:1747–1753.
45. Kokubo T, Kushitani H, Sakka S, Kitsugi T, Yamamuro T. Solutions able to reproduce in vivo surface-structure changes in bioactive glass-ceramic A-W. J Biomed Mater Res 1990;24:721–734.
46. Kokubo T. Bioactive glass ceramics: Properties and applications. Biomaterials 1991;12:155–163.