CHAPTER 7

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 co-precipitation 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 ([3H]BSA) was utilized as a model protein to analyze the co-precipitation efficacy and the release kinetics of the protein from the carrier material. Conventional adsorption of [3H]BSA resulted in a rapid and passive release of the protein from BCP ceramics, while the co-precipitation technique effectively prevented the burst release of [3H]BSA. Further analysis of the in vitro kinetics demonstrated a sustained, cell-mediated release of co-precipitated [3H]BSA from BCP ceramics induced by resorbing osteoclasts. The co-precipitation technique described herein, achieved a physiologic-like protein release, by incorporating [3H]BSA into its respective carriers, rendering it a promising tool in growth factor delivery for bone healing.

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.\textsuperscript{8-10} Due to 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 the surface of the materials.\textsuperscript{9,11} This superficial deposition, however, leads to a burst release of the proteins upon exposure to a physiological environment.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{20,25-32} Recently, Liu \textit{et al.} have successfully used a co-precipitation 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.\textsuperscript{13,33-35} The incorporation of the proteins significantly decreased the passive release of BSA as compared to a superficial deposition of the protein. BMP-2 co-precipitated onto the titanium implants in the presence of calcium phosphate retained its biological activity \textit{in vitro} and \textit{in vivo} after being released from its carrier.\textsuperscript{34,35} So far, the co-precipitation 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, non-cell-mediated mechanisms of growth factor delivery. 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 (i) that the burst release of proteins from ceramic biomaterials found with conventional protein adsorption can be minimized by applying the co-precipitation of proteins to calcium phosphate ceramics and (ii) 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. [3H]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, USA). Bovine serum albumin (BSA) was from Sigma Aldrich (Basle, Switzerland). The XTT cell proliferation kit and 1,25(OH)₂D₃ 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 ceramics (pore size 150-200\(\mu\)m, porosity 75\% \pm 5\%) composed of 80\% hydroxyapatite (HA) and 20\% \(\beta\)-tricalcium phosphate (\(\beta\)-TCP) were purchased from Progentix BV (MB Bilthoven, The Netherlands). Ceramic cylinders with a diameter of 14mm were prepared and discs with a thickness of 0.5mm were cut with a diamond saw. The BCP discs were cleaned with ethanol and UV sterilized (4 hours) directly before use.

Preparation of \[^3\text{H}]\text{BSA}\)

Fifty milligram bovine serum albumin (BSA) was labeled with tritium ([\(^3\text{H}\)]) by the reductive methylation procedure of Tack et al.\(^{42}\). The reaction involves a brief exposure of the protein to formaldehyde and sodium boro-[\(^3\text{H}\)]-hydride (Cat. No. NET023H100MC, Perkin Elmer, Switzerland) which specifically labels the \(\alpha\)-amino groups of NH\(_2\)-terminal residues and \(\varepsilon\)-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 non-incorporated free label. The protein concentration within the purified preparation was determined using a total protein quantification assay.

Immobilization of \[^3\text{H}]\text{BSA} \text{onto BCP Ceramics}\)

BCP ceramics were coated with tritium labeled bovine serum albumin ([\(^3\text{H}\)]BSA) either by adsorption or by protein co-precipitation. [\(^3\text{H}\)]BSA was superficially adsorbed to the surface of the materials by immersion of BCP discs in an aqueous protein solution (total volume of 2.5ml) for 48 hours at 37\(^\circ\)C in 24-well plates using a custom made holder (Coating 1: Adsorption). To co-precipitate proteins and calcium phosphate onto BCP ceramics, the materials were first immersed for 24 hours at 37\(^\circ\)C in 5-times concentrated simulated body fluid (Na\(^+\) 733.5mM, Cl\(^-\) 720.0mM, HPO\(_4^{2-}\) 5.0mM, SO\(_4^{2-}\) 2.5mM) under high-nucleation conditions in the presence of Mg\(^{2+}\) (7.5mM) 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\(^+\) 140.0mM, Ca\(^{2+}\) 4.0mM, Cl\(^-\) 144.0mM, HPO\(_4^{2-}\) 2.0mM) for 48 hours at 37\(^\circ\)C in the presence of [\(^3\text{H}\)]BSA. [\(^3\text{H}\)]BSA and calcium phosphate were thereby co-
precipitated onto the BCP ceramics (Coating 2: Co-precipitation). The co-precipitation of \[^3\text{H}\]BSA was performed in volumes of 2.5ml and 12ml supersaturated calcium phosphate solution, respectively. In a third coating procedure the co-precipitation technique was at first performed without addition of \[^3\text{H}\]BSA to create a protein free layer of crystalline calcium phosphate. Subsequently, \[^3\text{H}\]BSA was adsorbed to the ceramics which had been precipitated with calcium phosphate beforehand (Coating 3: CaP precipitation/adsorption). Volumes of 2.5ml and 12ml of supersaturated calcium phosphate solution and aqueous protein solution were used. With all coating techniques, \[^3\text{H}\]BSA was added to the coating solutions to a final concentration of 0.1, 1, and 10µg/ml coating solution. For the first 60 minutes 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 \textit{ddy} mice. After equilibration of the BCP discs in \(\alpha\)-MEM supplemented with 10% FBS (not heat inactivated) and 1% Pen/Strep (100 U/ml and 100µg/ml, respectively), \(1\times10^6\) cells were seeded on each BCP disc. The cells were cultured in \(\alpha\)-MEM containing 10% FBS and 1% Pen/Strep with CSF-1 (30ng/ml) for differentiation towards the monocyte/macrophage lineage and with CSF-1 (30ng/ml) and RANKL (50ng/ml) for differentiation towards the osteoclast lineage. The media was changed in 3 day intervals. From day six of culture, the media were acidified by adding 15meq/l H\(^+\) according to Arnett \textit{et al.}\textsuperscript{38-40} in order 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 \(1\times10^{-10}\) 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 10min. Afterwards, the samples were washed with demineralized water and incubated with 0.5ml TRAP staining solution for
5min. The samples were rinsed again with demineralized water and incubated with Hoechst 33342 nuclear stain (concentration 1mg/ml, Fluka Switzerland) for 10 minutes. Finally, the ceramics were washed with PBS and t 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 biphasic calcium phosphate 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 10nm gold was deposited using a Baltec SCD 004 unit (Baltec, Baltzers, Liechtenstein). For imaging, the following microscope parameters were used: voltage: 10kV, working distance: ~10mm, electron source: secondary electrons.

**Cell Fixation and Critical Point Drying (CPD)**

To analyze the behavior of the cells on the ceramics by SEM, cells were fixed as described by Baxter *et al.* In brief, the ceramics with the cells were washed with 0.1M 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 \[^3\text{H}\]BSA Uptake**

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

**Analysis of the Release of \[^3\text{H}\]BSA from BCP Ceramics**

The passive and the cell-mediated release of \[^3\text{H}\]BSA from the BCP discs was monitored over a period of 19 days. BCP discs coated with \[^3\text{H}\]BSA were equilibrated in α-MEM culture media for 24 hours. Afterwards, the discs were
incubated in α-MEM culture media for another 18 days to investigate the spontaneous release of [³H]BSA. To induce the cell-mediated release of [³H]BSA 1x10⁶ 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 [³H]BSA, 500µl of the collected culture supernatants were added to 3 ml Ultima Gold™ scintillation fluid and [³H] 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 [³H]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) x 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, USA). 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 [³H]BSA by BCP Ceramics
The uptake of [³H]BSA by BCP ceramics was quantified after dissolution of the ceramics in 2ml 1M 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 0.1µg/ml [³H]BSA the coating efficacy was similar irrespective of whether [³H]BSA was adsorbed or co-precipitated onto BCP ceramics [Adsorption (volume 2.5ml): 14.1 ± 5.2%, Co-precipitation (volume 2.5ml): 14.5 ± 2.7%, Co-precipitation (volume 12ml): 17.0 ± 3.4%]. At a concentration of 1µg/ml and 10µg [³H]BSA per ml coating solution there was a statistically significant difference found in coating efficacy between adsorption and co-precipitation.
with 2.5ml and 12ml coating volume [Adsorption (volume 2.5ml, 1µg \[^{3}\text{H}]\text{BSA/ml}}): 26.3 \pm 1.5\%, Co-precipitation (volume 2.5ml, 1µg \[^{3}\text{H}]\text{BSA/ml}}): 21.4 \pm 4.6\%, Co-precipitation (volume 12ml, 1µg \[^{3}\text{H}]\text{BSA/ml}}): 20.7 \pm 3.2\%, Adsorption (volume 2.5ml, 10µg \[^{3}\text{H}]\text{BSA/ml}}): 31.8 \pm 3.1\%, Co-precipitation (volume 2.5ml, 10µg \[^{3}\text{H}]\text{BSA/ml}}): 23.2 \pm 4.0\%, Co-precipitation (volume 12ml, 10µg \[^{3}\text{H}]\text{BSA/ml}}): 24.4 \pm 5.8\%; p<0.05 Adsorption (volume 2.5ml) vs. Co-precipitation (volume 2.5ml and 12ml)]. As shown in table 1, adsorption of \[^{3}\text{H}]\text{BSA} subsequent to CaP precipitation of the ceramics (CaP precipitation/adsorption) at a concentration of 1µg \[^{3}\text{H}]\text{BSA} per ml coating showed similar coating efficacy as the co-precipitation of \[^{3}\text{H}]\text{BSA}. The coating efficacy of CaP precipitation/adsorption of \[^{3}\text{H}]\text{BSA} was significantly lower as compared to direct adsorption of \[^{3}\text{H}]\text{BSA} onto BCP ceramics.

\textit{Table 1: Uptake of \[^{3}\text{H}]\text{BSA} onto biphasic calcium phosphate ceramics}

<table>
<thead>
<tr>
<th>Coating technique</th>
<th>V (ml)</th>
<th>Uptake [^{3}\text{H}]-\text{BSA} (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adsorption</td>
<td>2.5</td>
<td>26.3 \pm 1.5**</td>
</tr>
<tr>
<td>Adsorption</td>
<td>12.0</td>
<td>28.0 \pm 2.4**</td>
</tr>
<tr>
<td>Co-precipitation</td>
<td>2.5</td>
<td>21.4 \pm 4.6</td>
</tr>
<tr>
<td>Co-precipitation</td>
<td>12.0</td>
<td>20.7 \pm 3.2</td>
</tr>
<tr>
<td>CaP precipitation/adsorption</td>
<td>2.5</td>
<td>20.9 \pm 1.8</td>
</tr>
<tr>
<td>CaP precipitation/adsorption</td>
<td>12.0</td>
<td>22.5 \pm 1.4</td>
</tr>
</tbody>
</table>

The table depicts the value of protein uptake using \[^{3}\text{H}]\text{BSA} at a concentration of 1µg per ml coating solution. Mean values \pm standard deviation (n = 6 for each group), *p<0.05 vs. Co-precipitation and same volume of coating solution, **p<0.05 vs. CaP precipitation/adsorption and same volume of coating solution.

\textit{Passive and Cell-mediated Release of \[^{3}\text{H}]\text{BSA from BCP Ceramics}}

The passive and the cell-mediated release of \[^{3}\text{H}]\text{BSA} from BCP ceramics was monitored over 19 days. Within 19 days, 65% to 73% of \[^{3}\text{H}]\text{BSA} adsorbed to the ceramics was passively released into the medium. Co-precipitation of \[^{3}\text{H}]\text{BSA} onto the material significantly decreased the passive release to 13% to 21% (p<0.001 vs. Adsorption) of the total protein bound to the ceramics. Co-precipitation improved the retention of \[^{3}\text{H}]\text{BSA} from passive release independent of whether the protein was co-precipitated to BCP ceramics at
concentrations of 0.1µg, 1µg, or 10µg per ml coating solution (Table II). With adsorption and co-precipitation of [³H]BSA, the passive release proceeded almost completely within the first 4 days of incubation (Fig. 1). Adsorption of BCP ceramics with [³H]BSA at a concentration of 1µg/ml (volume 2.5ml) resulted in a release of 63.0% ± 6.0% of the protein within 4 days as compared to 71.7 ± 5.3% within 19 days of incubation. The analysis of the release kinetics after co-precipitation of [³H]BSA at a concentration of 1µg/ml (volume 2.5ml) 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 [³H]BSA (CaP prec./Ads.) did not modify the release kinetics of the protein as compared to adsorption of [³H]BSA directly onto the ceramics. With the CaP precipitation/adsorption of [³H]BSA at a concentration of 1µg/ml (volume 2.5ml) 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, co-precipitation, CaP precipitation/adsorption showed similar release kinetics independent of whether the respective technique was used with a coating volume of 2.5ml or 12ml (Fig. 1; Table 2).

### Table 2: Cumulative passive and cell-mediated release of [³H]-BSA from BCP ceramics

<table>
<thead>
<tr>
<th>Coating technique</th>
<th>V (ml)</th>
<th>Incubation</th>
<th>BMC</th>
<th>BMC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>α-MEM</td>
<td>+CSF-1, -RANKL</td>
<td>+CSF-1, +RANKL</td>
</tr>
<tr>
<td>Adsorption</td>
<td>2.5</td>
<td>71.1 ± 5.3</td>
<td>80.5 ± 8.2</td>
<td>87.7 ± 6.9*</td>
</tr>
<tr>
<td>Adsorption</td>
<td>12.0</td>
<td>84.4 ± 2.6</td>
<td>93.0 ± 3.2*</td>
<td>91.6 ± 3.2*</td>
</tr>
<tr>
<td>Co-precipitation</td>
<td>2.5</td>
<td>15.5 ± 2.5</td>
<td>17.4 ± 2.8</td>
<td>29.3 ± 5.3*</td>
</tr>
<tr>
<td>Co-precipitation</td>
<td>12.0</td>
<td>12.5 ± 2.0</td>
<td>14.7 ± 3.0</td>
<td>37.8 ± 6.1*</td>
</tr>
<tr>
<td>CaP precipitation/adsorption</td>
<td>2.5</td>
<td>82.1 ± 9.3</td>
<td>87.4 ± 3.2</td>
<td>91.9 ± 3.1*</td>
</tr>
<tr>
<td>CaP precipitation/adsorption</td>
<td>12.0</td>
<td>81.8 ± 0.9</td>
<td>82.0 ± 2.0</td>
<td>92.3 ± 4.2*</td>
</tr>
</tbody>
</table>

The cumulative release of [³H]-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 towards monocytes/macrophages (BMC +CSF-1, -RANKL) and osteoclasts (BMC +CSF-1, +RANKL). Mean values ± standard deviation (n = 6 for each group), *p<0.05 vs. α-MEM of the same group, †p<0.05 vs. BMC, +CSF-1, -RANKL of the same group, ‡p<0.001 vs. α-MEM of the group, §p<0.001 vs. BMC, +CSF-1, -RANKL of the same group.
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 towards monocytes/macrophages or osteoclasts [Fig. 2(C, D); Table 2].

In BCP ceramics co-precipitated with $[^3]$H-BSA, BMCs were able to induce a cell-mediated release of $[^3]$H-BSA if differentiated towards osteoclasts. [Fig. 2(E, F); Table II]. From day 7 the cell-mediated release of co-precipitated $[^3]$H-BSA by osteoclasts gave rise to a sustained liberation of $2.6 \pm 0.5\%$ to $7.3 \pm 2.0\%$ of the protein bound to the materials per 3 days period. The addition of calcitonin to BMCs differentiated towards 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 co-precipitated $[^3]$H-BSA. There was no modification of the passive release kinetics by monocytes/macrophages found.

**Figure 1:** The graphs show the passive release kinetics of $[^3]$H-BSA coated onto BCP discs at a concentration of 1µg/ml using coating volumes of 2.5ml (A) and 12ml (B) during the course of 19 days of incubation. Mean values are represented ± standard deviation (n = 6 for each group), $^*p<0.001$ vs. adsorption and vs. 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 $[^3]$HBSA loaded to the ceramics at a concentration of 1µg/ml. Adsorption (A, B): Release of $[^3]$HBSA adsorbed to the surface of the ceramics with a volume of 2.5ml (A) and 12ml (B). CaP precipitation/adsorption (C, D): Release of $[^3]$HBSA from ceramics that first underwent precipitation of calcium phosphate and were subsequently adsorbed with $[^3]$HBSA at a volume of 2.5ml (C) and 12ml (D). Co-precipitation (E, F): Release of $[^3]$HBSA co-precipitated to the ceramics with 2.5ml (E) and 12ml (F) calcium phosphate solution. Mean values are represented ± SD (n = 6 for each group), $^*$p≤0.001 vs. 1µg/ml $[^3]$HBSA (α-MEM) and vs. 1µg/ml $[^3]$HBSA (BMC, +CSF-1, -RANKL); $^#p≤0.001$ vs. 1µg/ml $[^3]$HBSA (α-MEM), vs. 1µg/ml $[^3]$HBSA (BMC, +CSF-1, -RANKL), and vs. 1µg/ml $[^3]$HBSA (BMC, +CSF-1, +RANKL, +CT)
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 Fig. 3(A) and 3(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 co-precipitated with [3H]BSA (not shown).

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 towards TRAP-positive (red) osteoclasts (D). Multi-channel 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 100µm.
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 5-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; co-precipitation 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 co-precipitation technique (I, J).
**Scanning Electron Microscopy Analysis**

Scanning electron microscopy demonstrated that the adsorption of $[^3]$HBSA to the materials did not change the surface morphology of the materials. By SEM, no differences in the structure of the original ceramics and ceramics adsorbed with $[^3]$HBSA were detectable [Fig. 4(A-D)]. The surface morphology of BCP ceramics subjected to the co-precipitation protocol, however, was markedly modified. The immersion of the material in 5-times concentrated simulated body fluid created an amorphous layer of calcium phosphate on the surface [Fig. 4(E, F)]. Subsequent co-precipitation of $[^3]$HBSA 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 $[^3]$HBSA first to create a protein free layer of crystalline calcium phosphate and were subsequently adsorbed with the protein showed similar surface morphologies as compared to ceramics co-precipitated with $[^3]$HBSA [Fig. 4(I, J)].

SEM analysis following the culture of murine BMCs on the ceramics showed that BMCs differentiated towards monocytes/macrophages were not able to resorb the crystalline or amorphous layer of calcium phosphate created by the co-precipitation 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. 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
associated with stem cell use and may be advantageous to MSCs in clinical settings.

Figure 5: Scanning electron microscopy was performed after 19 days of culture of murine bone marrow cells on BCP ceramics co-precipitated with [3H]BSA in the presence of calcium phosphate. (A-C) BMCs which grown on the ceramics and differentiated towards monocytes/macrophages were not able to resorb the amorphous phase of the crystalline layer of calcium phosphate created by the co-precipitation 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.
Efficient delivery of growth factors from carrier biomaterials depends critically on the release kinetics of the proteins that constitute the carrier. Woo et al. 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 to an immediate release model. In the present study, $[^{3}\text{H}]\text{BSA}$ was co-precipitated together with calcium phosphate onto biphasic calcium phosphate ceramics, in attempt to achieve a sustained delivery of the protein from BCP ceramics. The co-precipitation technique was compared to a conventional protein adsorption procedure. In accordance with previous findings, adsorption of $[^{3}\text{H}]\text{BSA}$ to biphasic calcium phosphate ceramics resulted in a rapid passive release of a large proportion of the protein bound to the material. On average 62% of $[^{3}\text{H}]\text{BSA}$ was released passively within the first 4 days. By applying the co-precipitation technique, approximately 80% of $[^{3}\text{H}]\text{BSA}$ 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 co-precipitation technique was in the range of previously described coating techniques exhibiting slow release kinetics. Kim and Valentini 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 the present study approximately 16% of immobilized rhBMP-2 was passively released within the first four 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. 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, non-cell-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.
suggestion is supported by a recent in vitro study by Lee et al.\textsuperscript{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 as compared to 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 co-precipitated 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 towards osteoclasts. BMC’s differentiated towards monocytes/macrophages did not modify the release kinetics of $[^{3}H]$BSA. Additionally, SEM analyses of BCP ceramics co-precipitated 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 micro-architecture was markedly modified by the co-precipitation technique as compared to the adsorption of $[^{3}H]$BSA. In order 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 co-precipitation 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 co-precipitation technique may be the diminished coating efficacy under certain coating conditions, which was found to be decreased by a factor of 1.2 to 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, co-precipitation of [$^3$H]BSA greatly enhanced protein retention by reducing the burst release of the protein by a factor of 4 to 4.9. Therefore, the decreased coating efficacy of protein co-precipitation is easily outweighed by the greatly ameliorated efficacy of protein delivery of this procedure.

In conclusion, co-precipitation 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. Due to the physiologic-like release mode, the co-precipitation 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.

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