

# Apatite-Polymer Composite Particles for Controlled Delivery of BMP-2: *In Vitro* Release and Cellular Response

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**Abstract** — Bone morphogenetic protein-2 (BMP-2) has the ability to induce osteoblast differentiation of undifferentiated cells, resulting in the healing of skeletal defects when delivered with a suitable carrier. We have applied a versatile delivery platform comprising a novel composite of two biomaterials with proven track records – apatite and poly(lactic-co-glycolic acid) (PLGA) – to the delivery of BMP-2. Sustained release of this growth factor was tuned with variables that affect polymer degradation and/or apatite dissolution, such as polymer molecular weight, polymer composition, apatite loading, and apatite particle size. The effect of released BMP-2 on C3H10T1/2 murine pluripotent mesenchymal cells was assessed by tracking the expression of osteoblastic makers, alkaline phosphatase (ALP) and osteocalcin. Release media collected over 100 days induced elevated ALP activity in C3H10T1/2 cells. The expression of osteocalcin was also upregulated significantly. These results demonstrated the potential of apatite-PLGA composite particles for releasing protein in bioactive form over extended periods of time.

**Index Terms** — Composite, apatite, PLGA, bone morphogenetic protein

## I. INTRODUCTION

Current treatment of bone defects due to trauma, cancer, or degenerative spine diseases involves the implantation of a bone graft. Autografts, which are

harvested from the patient's own body, are associated with problems of limited availability and pain at the harvest site. The use of allografts obtained from donors is also not desirable due to the risks of disease transmission and the costs of maintaining bone banks [1]. The ideal solution would be to regenerate bone to fill the defects. A group of potent growth factors known as bone morphogenetic proteins (BMPs) have been hailed as alternatives to bone grafts due to their ability to elicit new bone formation [2-8]. Clinical use of BMPs involves loading the protein solution onto collagen sponges and subsequent implantation [9, 10]. However, these conventional collagen carriers show rapid clearance of BMPs within ~ 2 weeks, whereas bone healing is a longer process, especially in higher mammals. The poor retention of BMPs in collagen sponges may explain the greater variability in higher mammals' response to BMP, ranging from full bone bridging within weeks to no bone union [11]. Hence, the motivation for our research is to develop new carriers to more efficaciously deliver these expensive therapeutic proteins to achieve bone healing.

To this end, we have devised a novel composite of two biomaterials with proven track records: poly(lactic-co-glycolic acid) and apatite [12]. The controlled release strategy is based on the use of a biodegradable polymer with acidic degradation products to control the dissolution of the basic apatitic component. Proteins are pre-adsorbed onto the apatitic component such that as the apatite dissolves, proteins are released. The release profile can be modified systematically by changing variables that affect polymer degradation or apatite dissolution, such as polymer molecular weight, polymer hydrophobicity, apatite loading, and apatite particle size.

In this paper, we describe the application of apatite-polymer composite particles to the delivery of BMP-2. The bioactivity of the released BMP-2 was assessed using a pluripotent murine embryonic fibroblast cell line, C3H10T1/2, which can be induced to differentiate along osteoblast, chondrocyte, adipocyte or myoblast lineages, depending on the signals provided. BMP-2 promotes the development of the osteoblast phenotype in these cells, as measured by increased expression of alkaline phosphatase (ALP), osteocalcin, and other osteoblastic markers [13-17].

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## II. EXPERIMENTAL

### A. Aseptic Preparation of RhBMP-2-Loaded Composite Microparticles

Recombinant human BMP-2 (rhBMP-2) was obtained from R&D Systems. Composite microparticles encapsulating rhBMP-2 were synthesized under aseptic conditions by a solid-in-oil-in-water (S/O/W) emulsion process [12]. A typical synthesis involved dissolving 250 mg of PLGA (Alkermes) in 2 ml of dichloromethane. The polymer solution was sterile filtered through a 0.45- $\mu\text{m}$  Teflon membrane into a vial containing carbonated apatite pre-adsorbed with rhBMP-2. The mixture was vortexed to create a solid-in-oil suspension, which was then transferred to 50 ml of 0.1 w/v% methyl cellulose solution that had been autoclaved. An S/O/W suspension was formed by homogenizing at 8000 rpm for 2 min at room temperature. To solidify the composite particles, dichloromethane was evaporated by heating the suspension at 30°C for 3 h. The particles were collected by centrifugation, washed with sterile water, and freeze-dried.

### B. Evaluation of In Vitro Release

Release studies were conducted in Eagle's basal medium (BME; Sigma) supplemented with 10 v/v% heat-inactivated fetal bovine serum (Invitrogen) and 1 v/v% antibiotics (100 IU/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin; ATCC). This medium will be referred to as 'complete BME'. RhBMP-2-loaded composite particles were suspended at a concentration of 20 mg/ml in complete BME and incubated at 37°C. At pre-determined time intervals (1, 4, 7, 10, 14, 18, 22, 26, 30 days, up to 100 days), the samples were centrifuged, and the supernatant was withdrawn completely and replaced with an appropriate volume of fresh medium. The collected supernatant was filtered and stored at -20°C until evaluation by a BMP-2 sandwich enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems). The assay consisted of adding 50  $\mu\text{l}$  of standards and test samples to a 96-well plate coated with anti-BMP-2 monoclonal antibody. The plate was incubated at room temperature for 2 h and washed. Biotinylated anti-BMP-2 antibody was then added, and the plate was further incubated for 2 h. Unbound antibody was removed by washing, following which streptavidin-horse radish peroxidase was introduced. After 30 min, color was developed by the addition of hydrogen peroxide and tetramethylbenzidine. Absorbance was read at 450 nm using a UV-Vis microplate reader (VersaMax, Molecular Devices) with wavelength correction at 570 nm. The concentration of the protein released at each time point was used to construct cumulative release profiles.

### C. Cellular Response

A murine pluripotent embryonic fibroblastic cell line, C3H10T1/2, was obtained from ATCC. The cell line was maintained in complete BME. Cells were expanded in T-75 flasks at a density of 2000 cells/cm<sup>2</sup> with two medium

renewals per week. Subculturing was performed before cells reached confluence due to the sensitivity of these cells to contact and the post-confluence inhibition of cell division. 0.25% trypsin/0.53 mM EDTA (ATCC) was used to dissociate cells, which were then plated onto 24-well plates for *in vitro* experiments. Cells from the 11th to 15th passages were used.

#### 1) Effect of Release Medium Collected at Each Time Point

Different sets of rhBMP-2-loaded composite microparticles were incubated at 37°C in complete BME at a concentration of 20 mg/ml. Blank composite particles prepared under the same processing and material conditions but without rhBMP-2 served as controls. The medium was renewed every 3–4 days as described in Section II-B. The release medium collected at each time point was stored at -20°C until incubation with cells. As a standard for comparison, Helistat<sup>®</sup> collagen sponges (Integra Life Sciences) were soaked with rhBMP-2 solution for 10–15 min based on protocols in clinical use [9, 18]. Loaded and blank (control) sponges were then transferred into complete BME for release studies.

Alkaline phosphatase (ALP) activity was determined by a modification of the protocol of Lowry *et al.* [19]. C3H10T1/2 cells were seeded on 24-well plates at a density of 6000 cells/cm<sup>2</sup> (~ 12,000 cells/well). The cells were allowed to adhere overnight for 16 h. The medium was aspirated and replaced with 0.5 ml per well of release medium from a specific time point. For each time point, 5 wells were prepared. In addition, complete BME enriched with 0–2  $\mu\text{g}/\text{ml}$  of rhBMP-2 was incubated with cells. For each rhBMP-2 concentration, 5 wells of cells were tested, each containing 0.5 ml of enriched BME. This experiment was intended to establish the bioactivity of rhBMP-2 as well as to evaluate dose-dependent cellular response.

After 4 days of culture, the cells were rinsed twice with phosphate-buffered saline (PBS; Gibco) and the plates were blotted dry. The cells were lysed according to the protocol provided by R&D Systems. 100  $\mu\text{l}$  of 0.1% Triton X-100 solution (Sigma) containing 150 mM of NaCl and 3 mM of NaHCO<sub>3</sub> (pH 9.3) were added to each well. The plates were incubated at 37°C for 30 min. 25  $\mu\text{l}$  of the cell lysate were assayed in duplicate for ALP activity using 100  $\mu\text{l}$  of *p*-nitrophenyl phosphate substrate solution (pNPP; Sigma) in 96-well plates. The absorbance at 405 nm was read every 5 min for 30 min at 37°C. ALP cleaves the phosphate off pNPP to form *p*-nitrophenol. Using Beer's Law and an extinction coefficient of 18.45 cm<sup>2</sup>/L/mmol for *p*-nitrophenol, the amount (in nmol) of *p*-nitrophenol formed per minute was determined. ALP activity was normalized by the protein concentration of the lysate as determined by total protein assay (Pierce) and expressed as nmol/min-mg protein. ALP activity induced by the medium collected at each time point was plotted as a function of time. Data were plotted as mean  $\pm$  standard deviation. Statistical analysis was performed using Student's t-test. A significance level of  $p < 0.05$  was used.

## 2) Effect of Prolonged Exposure to Release Medium

Based on results from *in vitro* release and induced ALP activity at each time point, a set of composite microparticles with a sustained release profile of bioactive rhBMP-2 was chosen. This set of composite particles was constructed of 59 kD PLGA and 0.08 mg of carbonated apatite per mg of PLGA. RhBMP-2 loading in the particles was 145 ng per mg carrier. The effect of prolonged exposure of C3H10T1/2 cells to release medium from this set of particles was studied. As before, the composite microparticles were incubated at 37°C in complete BME at a concentration of 20 mg/ml. C3H10T1/2 cells were seeded in 24-well plates at a density of 6000 cells/cm<sup>2</sup>. Twice per week, the medium in the wells was aspirated and replaced with 0.5 ml of filtered release medium collected from the particles. Release medium from blank composite particles served as controls. For comparison, complete BME systems enriched with fixed concentrations of rhBMP-2 (10 and 100 ng/ml; 0.5 ml per well) were used. Cell culture was conducted for 8 weeks in all groups.

At 1, 2, 3, 4, 6 and 8 weeks, cells in 4 wells for each group were lysed, and the ALP activity of the cell lysate was measured. At each week, the osteocalcin level of the conditioned medium of 4 wells in each group was assayed by sandwich ELISA (Biomedical Technologies, Inc.). The procedure for this ELISA was similar to that of the BMP-2 assay. Statistical analyses of ALP and osteocalcin levels were performed using Student's t-test. A significance level of  $p < 0.05$  was used.

## III. RESULTS AND DISCUSSION

### A. Effect of Material and Processing Parameters on the *In Vitro* Release of RhBMP-2 from Composite Microparticles

#### 1) Polymer Molecular Weight

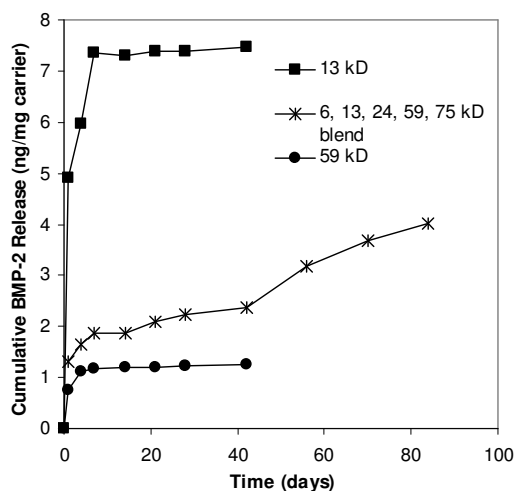


Fig. 1. Effect of PLGA molecular weight on rhBMP-2 release. Composite particles were loaded with 65 ng of rhBMP-2 per mg of carrier.

PLGA of lower molecular weight degraded more

quickly, and hence, enhanced apatite dissolution and rhBMP-2 release from composite microparticles (Fig. 1). By using an equi-portion blend of PLGA of different molecular weights, the production of acidic degradation products was equalized over time, leading to a more gradual and sustained release of rhBMP-2.

#### 2) Apatite Particle Size

RhBMP-2 was adsorbed onto carbonated apatite of two particle sizes: 1  $\mu\text{m}$  and 7  $\mu\text{m}$ . The apatite-rhBMP-2 complexes formed were subsequently incorporated into separate sets of composite microparticles. Apatite particle size was found to have a pronounced effect on rhBMP-2 release (Fig. 2). The smaller apatite particles presented a larger proportion of rhBMP-2 on their surface than within their pores due to their higher surface/volume ratio. As the apatitic surface was eroded by acidic degradation products, 1  $\mu\text{m}$ -sized particles released larger amounts of rhBMP-2 than 7  $\mu\text{m}$ -sized particles (Fig. 2).

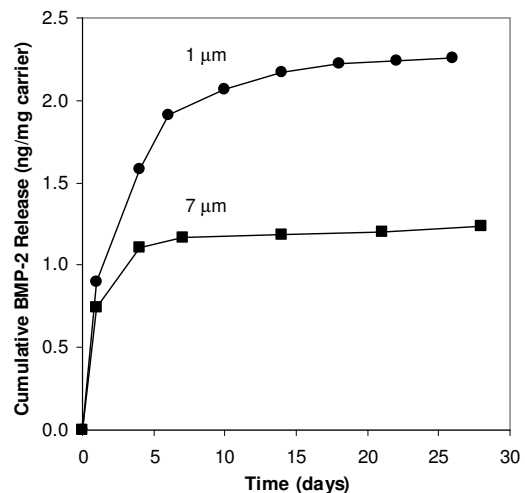


Fig. 2. Effect of apatite particle size on rhBMP-2 release. Composite particles were fabricated from 59 kD PLGA and contained 65 ng of rhBMP-2 per mg of carrier.

#### 3) Apatite Loading

When the amount of rhBMP-2 was held constant while the loading of apatite in composite microparticles was varied from 18 to 45 mg per 250 mg of polymer, rhBMP-2 release was found to decrease with increasing apatite loading (Fig. 3). For the release of a fixed amount of protein, a higher apatite loading requires a larger amount of apatite to be dissolved, corresponding to a greater extent of polymer degradation. In addition, apatite served as a buffer, mitigating the acidity within the composite particles and diminishing the autocatalytic effect of pH on polymer hydrolysis. Hence, the apatite served towards dampening the protein release.

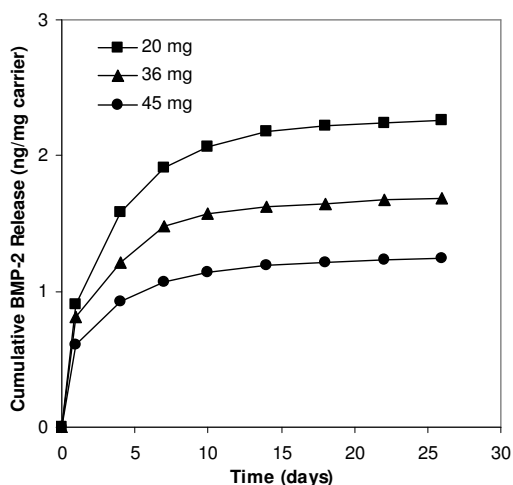


Fig 3. Effect of apatite loading (per 250 mg of polymer) on rhBMP-2 release. Composite particles were fabricated from 59 kD PLGA and contained 65 ng of rhBMP-2 per mg of carrier.

#### 4) Polymer Hydrophobicity

Increasing polymer hydrophobicity by the addition of PLA led to a surprising observation: rhBMP-2 release was enhanced with increasing polymer hydrophobicity (Fig. 4).

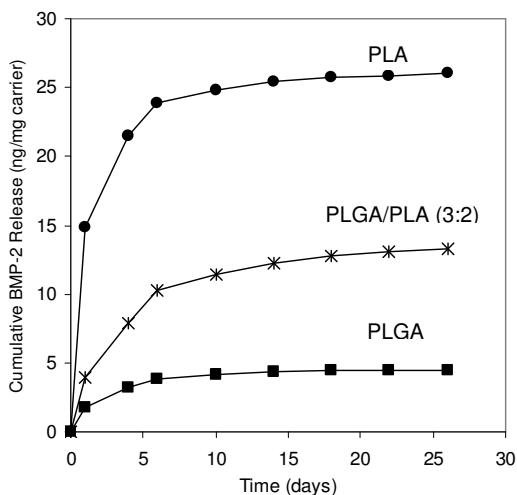


Fig. 4. Effect of polymer hydrophobicity on rhBMP-2 release. Composite particles contained 145 ng of rhBMP-2 per mg of carrier. Molecular weights of PLGA and PLA were 59 kD and 25 kD, respectively.

Polymer hydrophobicity was expected to reduce water penetration and polymer degradation, and hence, decrease protein release. The unexpected result could be explained by considering the release of structurally intact, biologically active protein versus the total release of protein. The determination of rhBMP-2 concentration by ELISA is highly specific; rhBMP-2 molecules have to be of the appropriate conformation for binding to antibodies. Denatured rhBMP-2 molecules are not detected, as confirmed by our previous experience with the evaluation

of rhBMP-2 concentration in citrate buffers of low pH – the low pH led to rhBMP-2 denaturation and poor detection by ELISA. Therefore, higher polymer hydrophobicity could have led to a less aggressive pH environment within the composite microparticles, contributing to less protein denaturation and greater release of bioactive rhBMP-2.

#### B. Effect of Conditioned Medium Collected at Each Time Point

The induction of ALP activity in C3H10T1/2 cells by different concentrations of rhBMP-2 over 4 days was examined. Increasing the concentration of rhBMP-2 from 0 ng/well to 200 ng/well (400 ng/ml) was found to elevate ALP activity (Fig. 5). At rhBMP-2 concentrations beyond 200 ng/well, the effect reached saturation. Thus, there appeared to be an optimum dosage of rhBMP-2 for evoking ALP expression, which was related to the commitment of undifferentiated cells to the osteoblast lineage.

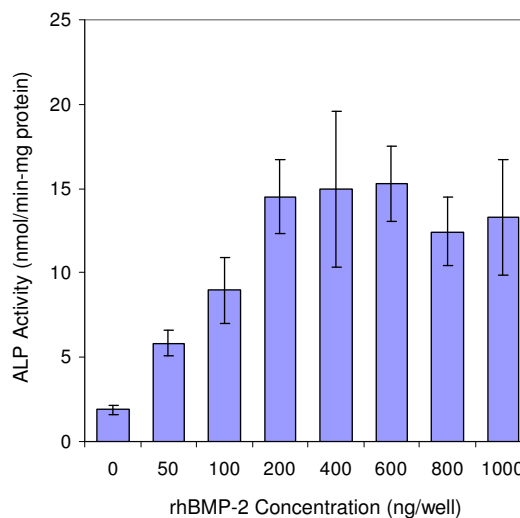


Fig. 5. Effect of rhBMP-2 concentration on induced ALP activity in C3H10T1/2 cells. Each well contained 0.5 ml of medium; 50 ng/well is equivalent to 100 ng/ml of rhBMP-2.

Levels of ALP activity induced by release media collected from rhBMP-2-loaded composite microparticles are plotted in Figs. 6–8. An asterisk denotes statistical significance of  $p < 0.05$  by Student's t-test for  $n = 5$ . Cumulative release is given in units of 'ng/well' to reflect the actual amounts to which the cells were exposed, and to facilitate comparison with Fig. 5.

Fig. 6 shows that the release media collected over a period of 100 days was able to induce elevated ALP expression in C3H10T1/2 cells. These encouraging results suggested that the released rhBMP-2 retained at least part of its biological activity and that the release of bioactive rhBMP-2 over extended periods of time was possible. The experiment was halted at 100 days, but we speculate that this set of composite microparticles could release bioactive rhBMP-2 for even longer periods of time since the

cumulative release and bioactivity of rhBMP-2 appeared to be experiencing an upturn at 100 days (Fig. 6).

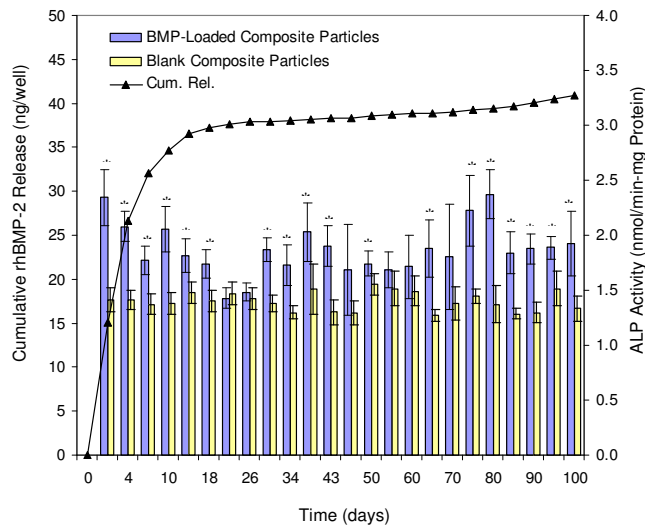


Fig. 6. ALP activity induced by release medium collected at each time point from rhBMP-2-loaded composite microparticles. Particles contained 145 ng of rhBMP-2 per mg of carrier, and were fabricated from 59 kD PLGA and 0.08 mg of carbonated apatite per mg of PLGA.

When a higher apatite loading was used in the composite microparticles, rhBMP-2 release was dampened, and the capacity for ALP induction declined within the first week (Fig. 7). In contrast, when rhBMP-2 release was augmented and prolonged by using a 3:2 blend of PLGA and PLA in the composite microparticles, ALP expression was significantly elevated throughout the course of the study (Fig. 8).

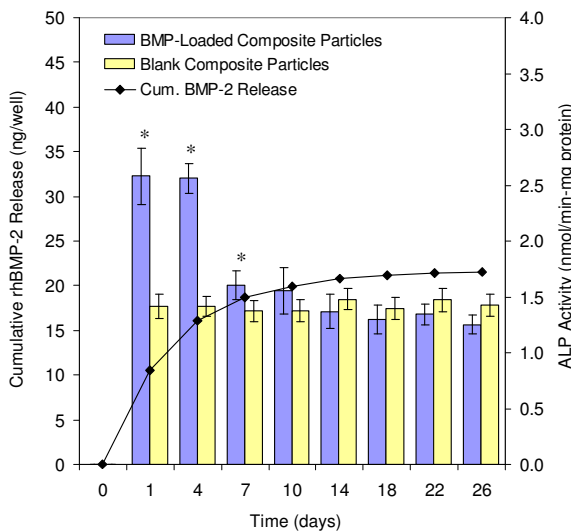


Fig. 7. ALP activity induced by release medium collected at each time point from rhBMP-2-loaded composite microparticles. Particles contained 145 ng of rhBMP-2 per mg carrier, and were fabricated from 59 kD PLGA and 0.18 mg of carbonated apatite per mg of PLGA.

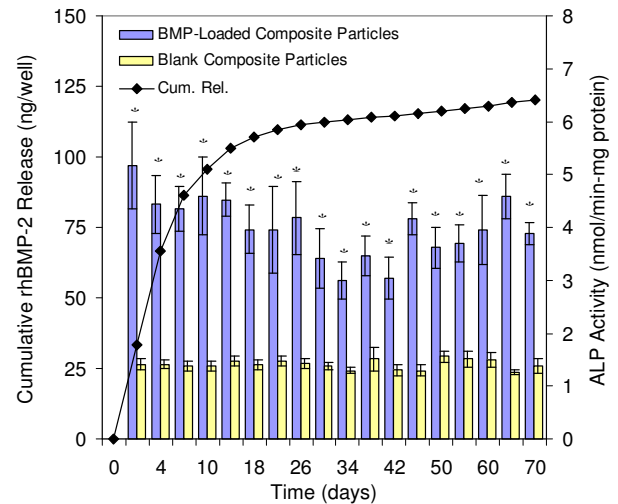


Fig. 8. ALP activity induced by release medium collected at each time point from rhBMP-2-loaded composite microparticles. Particles contained 145 ng of rhBMP-2 per mg carrier, and were fabricated from a 3:2 blend of 59 kD PLGA and 25 kD PLA. Apatite loading was 0.08 mg of carbonated apatite per mg of polymer.

However, the induction of ALP activity was not a simple function of the amount of rhBMP-2 released. This was made apparent by our experiments with Helistat<sup>®</sup> collagen sponges, which were the conventional BMP carriers used in spinal fusions. As depicted in Fig. 9, rhBMP-2 release from Helistat<sup>®</sup> sponges was approximately twice the magnitude of that from composite microparticles shown in Fig. 8.

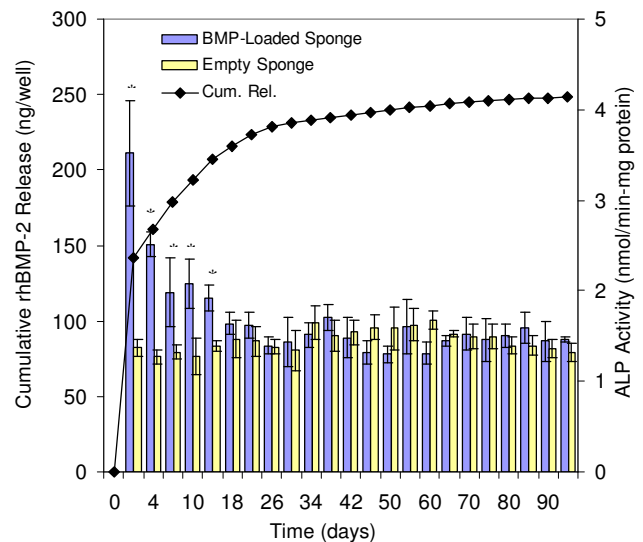


Fig. 9. ALP activity induced by release medium collected at each time point from rhBMP-2-loaded Helistat sponges. Sponges contained 200 ng of rhBMP-2 per mg carrier.

Nevertheless, the level of ALP expression was lower, and tapered off after 2 weeks, despite the continued release of rhBMP-2. It is unclear what caused the lowered

bioactivity of rhBMP-2 released from these sponges. The experiment was repeated to ascertain the validity of these results, and the same drop-off in ALP activity was observed for both trials.

### C. Effect of Prolonged Exposure to Conditioned Medium

#### 1) Measurement of Induced Alkaline Phosphatase Activity

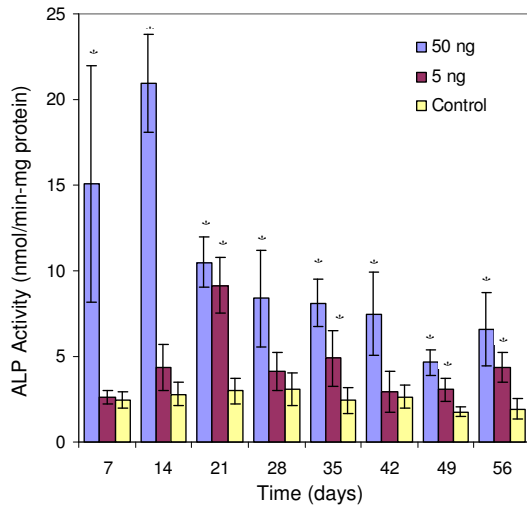


Fig. 10. Effect of length of exposure to rhBMP-2 on ALP activity. rhBMP-2 concentrations of 0 (control), 5 and 50 ng/well were used.

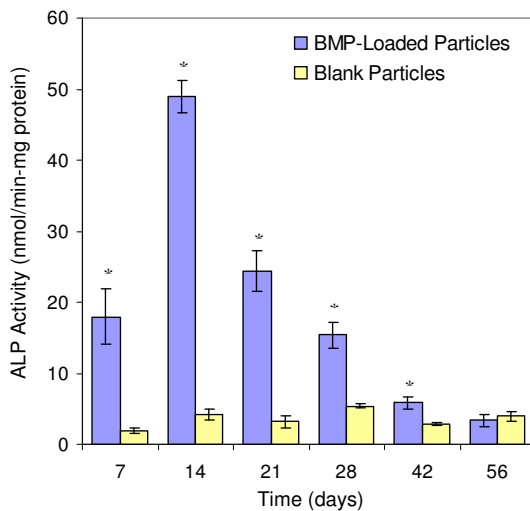


Fig. 11. Effect of prolonged exposure to release medium collected from rhBMP-2-loaded composite microparticles on ALP activity. Particles contained 145 ng of rhBMP-2 per mg of carrier, and were fabricated from 59 kD PLGA and 0.08 mg of carbonated apatite per mg of PLGA.

Compared against controls with no exposure to rhBMP-2, prolonged exposure of C3H10T1/2 cells to the growth factor resulted in elevated levels of ALP expression (Fig. 10). However, the increase was not monotonic; ALP activity reached a maximum at ~ 2 weeks and then dropped to levels that were maintained over the remainder of the

experiment. Similar profiles were reported by Shea *et al.*, who studied the effect of BMP-7 on C3H10T1/2 cells and observed a peak in ALP activity at 8 days [20]. Fig. 10 also shows a dose-dependent response to rhBMP-2; 50 ng of rhBMP-2 per well induced higher levels of ALP activity than 5 ng/well.

The culture of C3H10T1/2 cells with release medium collected from rhBMP-2-loaded composite microparticles produced a similar effect. ALP activity was considerably raised in the first 2 weeks, and then slowly declined to levels comparable to the controls at the end of 8 weeks (Fig. 11).

#### 2) Measurement of Osteocalcin Expression

Osteocalcin is a later stage marker of osteoblast differentiation associated with matrix maturation and mineralization. RhBMP-2 was found to upregulate osteocalcin expression in C3H10T1/2 cells in a time- and dose-dependent manner (Fig. 12). Control cells with no exposure to rhBMP-2 showed low osteocalcin levels, particularly at the start of the experiment. 5 ng of rhBMP-2 per well produced a weak response, which became statistically insignificant ( $p > 0.05$ ) at 7 weeks. A more robust response was obtained with 50 ng of rhBMP-2 per well. Osteocalcin levels experienced doubling in the first 3 weeks, held steady for the next 4 weeks, and then peaked again at week 8.

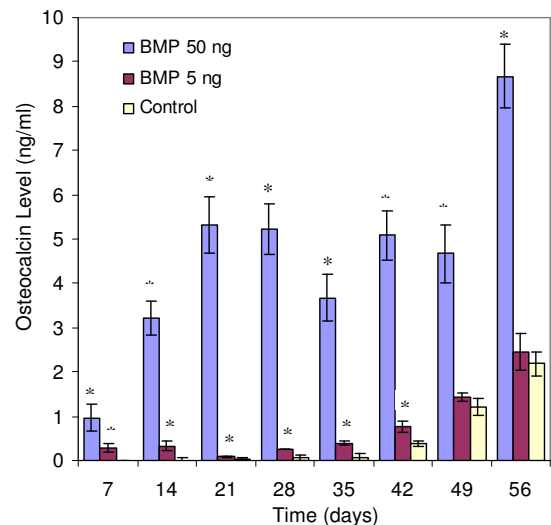


Fig. 12. Effect of length of exposure to rhBMP-2 on osteocalcin levels. Concentrations used were 0 (control), 5 and 50 ng of rhBMP-2 per well.

Release medium collected from rhBMP-2-loaded composite microparticles had a remarkable effect on osteocalcin upregulation. Osteocalcin levels increased steadily with time over 4 weeks, then showed an exponential rise from weeks 4 to 7 (Fig. 13). The level reached at week 7 was 10-fold that of the response to rhBMP-2-enriched BME (50 ng/well). These results suggested that the cells were undergoing a significant

amount of mineralization activity, indicative of an osteoblast phenotype.

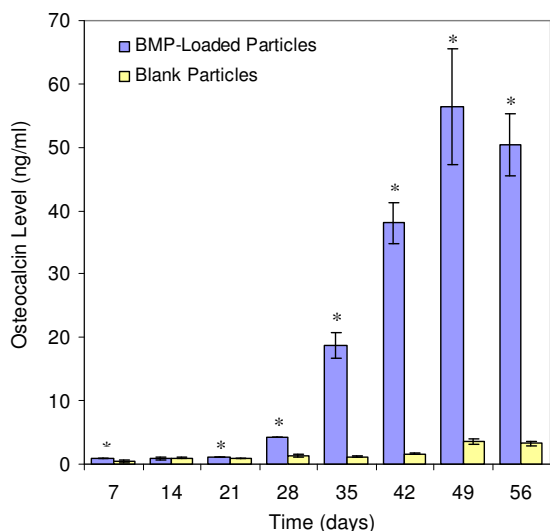


Fig. 13. Effect of prolonged exposure to release medium collected from rhBMP-2-loaded composite microparticles on osteocalcin levels. Particles contained 145 mg of rhBMP-2 per mg carrier, and were fabricated from 59 kD PLGA and 0.08 mg of carbonated apatite per mg of PLGA.

#### IV. CONCLUSIONS

We have prepared composite microparticles of a biodegradable polymer (PLGA) and an inorganic substrate (apatite) as delivery vehicles for rhBMP-2. These composite particles were found to be capable of releasing bioactive rhBMP-2 over 100 days. The released growth factor induced elevated levels of alkaline phosphatase as well as osteocalcin in C3H10T1/2 cells, indicating the differentiation of these cells into osteoblasts.

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