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Effect of different sintering methods on bioactivity and release of proteins from PLGA microspheres

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Abstract

Macromolecule release from poly($_{\text{D,L}}$-lactide-co-glycolide) (PLGA) microspheres has been well-characterized, and is a popular approach for delivering bioactive signals from tissue-engineered scaffolds. However, the effect of some processing solvents, sterilization, and mineral incorporation (when used in concert) on long-term release and bioactivity has seldom been addressed. Understanding these effects is of significant importance for microsphere-based scaffolds, given that these scaffolds are becoming increasingly more popular, yet growth factor activity following sintering and/or sterilization is heretofore unknown. The current study evaluated the 6-week release of transforming growth factor (TGF)-β_3 and bone morphogenetic protein (BMP)-2 from PLGA and PLGA/hydroxyapatite (HAp) microspheres following exposure to ethanol (EtOH), dense phase carbon dioxide (CO_2), or ethylene oxide (EtO). EtO was chosen based on its common use in scaffold sterilization, whereas EtOH and CO_2 were chosen given their importance in sintering microspheres together to create scaffolds. Release supernatants were then used in an accelerated cell stimulation study with human bone marrow stromal cells (hBMSCs) with monitoring of gene expression for major chondrogenic and osteogenic markers. Results indicated that in microspheres without HAp, EtOH exposure led to the greatest amount of delivery, whilst those treated with CO_2 delivered the least growth factor. In contrast, formulations with HAp released almost half as much protein, regardless of EtOH or CO_2 exposure. Notably, EtO exposure was not found to significantly affect the amount of protein released. Cell stimulation studies demonstrated that eluted protein samples performed similarly to positive controls in PLGA-only formulations, and ambiguously in PLGA/HAp composites. In conclusion, the use of EtOH, subcritical CO_2, and EtO in microsphere-based scaffolds may have only slight adverse effects, and possibly even desirable effects in some cases, on protein availability and bioactivity.

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Keywords

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1. INTRODUCTION

In many tissue engineering applications, a considerable fraction of regenerative efficacy originates from physicochemical signals embedded within a scaffold. These signals can be natural or material-based such as collagens, minerals, synthetic substrates, or small molecules.[1, 2] Some applications, however, utilize the release of bioactive factors, such as macromolecules. Vascular endothelial growth factors (VEGFs),[3, 4] glial-derived neurotrophic factors (GDNFs),[5-7] insulin-like growth factors (IGFs),[8-10] BMPs,[11-15] and TGFs[8, 9, 16-18] have become increasingly popular in tissue engineering, as these stimulate angiogenesis, promote neural growth, increase biochemical production, and initiate stem cell differentiation into bone and cartilage, respectively. Thus, ample effort is placed on the determination of techniques to load and deliver said molecules the site of action.

Popular delivery vehicles for macromolecules are PLGA nano- or microparticles, which can be created by a number of methods.[19] These particles can be embedded within fibrous matrices, hydrogels, or collagen networks.[20, 21] Some applications, however, use sphere-shaped microparticles to create scaffolds constructed solely of the microspheres alone. Microsphere-based scaffolds have been the focus of our technology that sinters microspheres together using EtOH[22-25] or subcritical CO_2.[26] The scaffolds can be made into custom sizes and shapes[26] or incorporate nanoparticles to create nanocomposite materials.[24] An advanced variation of this microsphere-based scaffold design utilized two types of microspheres (loaded with either TGF-β or BMP-2) oriented in an opposing continuous-gradient fashion.[22, 23] The regions provided regenerative signals for cartilage and bone growth in a single seamless 3-D design.

Biochemical and histological results from i*n vitro*[23] and *in vivo*[22, 27] studies utilizing the dual-gradient design have provided supportive evidence of growth factor bioactivity, but there remain many questions about protein delivery and bioactivity after being subjected to processing conditions such as EtOH, CO_2, or EtO. These treatments are of particular importance because both EtOH and CO_2 are used to sinter microspheres together into a shape-specific scaffold (although others have used hydrogels to effectively encapsulate the microspheres in lieu of creating a macroporous scaffold[28]), and EtO is used to sterilize the scaffolds. In addition, the effect of mineral nanoparticle inclusion, which is intended to facilitate osteo- induction/conduction[29-35] and provide "raw materials" for the regenerating tissue, on protein delivery characteristics is unknown. Thus, an investigation on the overall effect of scaffold fabrication procedures on cartilage- and bone-promoting growth factor delivery and bioactivity is a critical step in refining the microsphere-based scaffold design. With such knowledge, future iterations could potentially utilize mineral incorporation in conjunction with bioactive factors, creating both material and signal gradients.

The current study evaluated the general release characteristics of BMP-2 and TGF-β_3 from PLGA microspheres subjected either to EtO alone, EtO with EtOH, EtO with CO_2, or EtOH alone. In addition, BMP-2 was also loaded in PLGA/HAp composite microspheres and exposed to the aforementioned conditions to elucidate the effects of minerals on protein availability. Release supernatants from experimental groups were utilized in an accelerated cell stimulation study with hBMSCs, after which gene expression of major chondrogenic

and osteogenic markers was measured and compared to positive and negative control treatments.

2. MATERIALS AND METHODS

2.1 Materials

PLGA copolymer (50:50 lactic acid : glycolic acid, acid end group, M_W ~38,000 Da) of intrinsic viscosity (i.v.) 0.34 dL/g was purchased from Lakeshore Biomaterials (Birmingham, AL). Poly(vinyl alcohol) (PVA; 88% hydrolyzed, 25,000 Da) was obtained from Polysciences, Inc. (Warrington, PA). Nanophase HAp (< 200 nm) and Pluronic F-127 (F-127) were purchased from Sigma Aldrich (St. Louis, MO). Recombinant human TGF-β_3 and BMP-2 (*E. coli* derived) were purchased from Peprotech, Inc. (Rocky Hill, NJ). hBMSCs from a single donor (29 year old, African American male) at P1, (i.e., plated once) were purchased from StemCell Technologies (Vancouver, Canada). HAp was purchased from Sigma Aldrich (St. Louis, MO).

2.2 Preparation of Microspheres

Three types of microsphere formulations were prepared: TGF-β_3-loaded, BMP-2-loaded, and BMP-2-loaded with 5% w/w HAp. To fabricate the microspheres, either TGF-β_3 or BMP-2 was reconstituted in 0.1% w/v bovine serum albumin (BSA) in phosphate buffered saline (PBS) (both from Sigma Aldrich). The reconstituted protein solutions were individually mixed with PLGA that was dissolved in dichloromethane (DCM) at 20% w/v, with an oil:water ratio of 10:1. For the BMP-2 formulation with 5% w/w HAp, nanophase HAp was added to the PLGA-protein-DCM solution such that the total solids (HAp + PLGA) content was 20% w/v. A loading mass of 30 ng TGF-β_3 or 60 ng BMP-2 per 1.0 mg of solids (PLGA or PLGA-HAp) was used, which represented concentrations employed in previous *in vivo* studies.[22] The final mixture was then sonicated over ice at 30% amplitude for 20 seconds. Using PLGA-protein (or PLGA-protein-HAp) emulsions, uniform protein-loaded PLGA microspheres were prepared using technology described in our previous reports.[22, 24-26, 36] Briefly, using acoustic excitation produced by an ultrasonic transducer, regular jet instabilities were created in the polymer stream that produced uniform polymer droplets of ~250 μm. An annular carrier non-solvent stream (0.5% w/v PVA in DI H_2O) surrounding the droplets was produced using a nozzle coaxial to the needle. The emanated polymer/carrier streams flowed into a beaker containing the non-solvent at 0.5% w/v with an additional 1.25% w/v F-127 in DI H_2O to prevent aggregation of the particles. Incipient polymer droplets were stirred for 3-4 hours to allow solvent evaporation, which were then filtered and rinsed with DI H_2O to remove residual PVA, and stored at −20 °C (Fig. 1A). Following 48 hours of lyophilization, the microspheres were subjected to individual treatments.

2.3 Description of Experimental Groups

Microsphere formulations (TGF-β_3-loaded, BMP-2-loaded, and BMP-2-loaded with 5% w/w HAp) were each subjected to three different experimental conditions plus one control (Table 1). The experimental conditions represented those imposed on the microspheres during scaffold fabrication and sterilization: EtOH exposure alone, subcritical CO_2 exposure followed by EtO sterilization, and EtOH exposure followed by EtO sterilization. The exposure time of EtOH, and pressure for CO_2, was adjusted accordingly for each type of microsphere (described below and in Table 1), based on what was required for proper microsphere sintering.[23-26] A negative control group for each type of microsphere formulation was also included, where only EtO exposure was performed.

2.4 Ethanol Treatment

Approximately 20 mg of microspheres were loaded into 1.5 mL microcentrifuge tubes (Fisher Scientific, Pittsburgh, PA). Then, 1.0 mL of EtOH (200 proof, obtained in-house) was added to the tube. The samples were allowed to sit for 60 minutes, after which the ethanol was decanted and the microspheres were lyophilized for 48 hours. For PLGA microspheres containing 5% w/w HAp, EtOH exposure was performed for 90 minutes, as previous investigations showed that sintering microspheres that contain nanoparticles required longer durations than observed with pure PLGA.[24, 27]

2.5 Subcritical CO_2 Exposure

Approximately 20 mg of microspheres were loaded into 1.5 mL microcentrifuge tubes (Fisher Scientific). The tubes were placed in a custom pressure vessel with a maximum pressure rating of 400 bar, as we described in a previous report.[26] Vessels were purged of air and filled with CO_2 at a rate of ~1 psi/sec until reaching 218 psi (~15 bar). The samples were maintained at this pressure for 60 minutes, at which point the chamber was evacuated at a rate of ~1 psi/sec using an automated back pressure regulator (Waters Technologies Corporation; Palatine, IL). For PLGA microspheres containing 5% w/w HAp, the samples were subjected to a pressure of 363 psi (~25 bar) for 60 minutes, and the chamber was evacuated at a rate of ~1 psi/sec.

2.6 Ethylene Oxide Sterilization

After EtOH or subcritical CO_2 exposure, microsphere tubes were loaded into a sterilization chamber (Anproline AN74i, Anderson Sterilizers, Haw River, NC) and exposed to EtO for 12 hours, followed by a 2-hour ventilation stage. For one EtOH-treated group, EtO exposure was omitted as noted above. This group was included to elucidate the effects of EtOH alone on release characteristics, in the event that EtOH is considered in the future as both a sintering and sterilizing agent. Another group was treated with just EtO, to evaluate the effects of EtO sterilization alone, without sintering (control).

2.7 Measurement of Glass Transition Temperature

Glass transition temperatures (T_g) for PLGA and PLGA/Hap composite microspheres subjected to various treatments were determined by differential scanning calorimetry analysis (DSC, Q200, TA Instruments, USA). **Samples (1-5 mg)** were equilibrated at 0 °C and purged with pure dry nitrogen at a flow rate of 40 ml/min. The samples were then heated to 70 °C at 5 °C/min, after which they were cooled back to 0 °C at the same rate. Afterward, the second heating cycle followed at a 5 °C/min temperature ramp speed to 70 °C. The T_g was obtained by calculating the onset, end, and inflection of the glass transition. For the analysis, samples were crimped in standard aluminum pans. An empty pan, sealed in the same way as the samples, was used as a reference.

2.8 Protein Release

Protein release was conducted as previously-described, with the exception of placing samples in small tubes, as opposed to well plates.[22] Following exposure to EtO/EtOH/ CO_2 (Table 1), **approximately 20 mg of microspheres** (n = 3) were loaded into 1.5 mL microcentrifuge tubes (Fisher Scientific). The total mass of TGF-β_3 theoretically loaded in each sample was 600 ng, whereas the total mass of BMP-2 theoretically loaded in each sample was 1200 ng. The tubes were filled with 1.1 mL PBS and placed in an incubator at 37 °C with 5% CO_2. The tubes were not agitated continuously, as this was most representative of the microsphere environment in a scaffold formulation: static and in close proximity to one another. At each sampling time point, the tubes were lightly inverted, then centrifuged at 5,000 rpm for 30 seconds. A 1.0 mL sample of PBS surrounding the

microspheres was removed from each tube and gently pipetted into a concentrated BSA-in-PBS solution such that the final BSA concentration was 0.1% w/v. BSA was included as a carrier protein to aid in the prevention of TGF-β_3 and BMP-2 aggregation and to bind to container surfaces during sampling and storage. The aliquots were split into two parts (one for ELISA, one for cell culture) and frozen at −80 °C until study completion (Fig. 1B). Sampling was done at 12 hours, then at 1, 2, 3, 5, 7, 10, 14, 18, 22, 26, 30, 34, 38, and 42 days. Release profiles were determined via sandwich ELISAs. Enzyme-linked immunosorbent assay (ELISA) development kits for TGF-β_3 and BMP-2 were purchased from R&D Systems (Minneapolis, MN) and Peprotech, Inc., respectively. External standardization was performed with each ELISA kit to account for discrepancies in sensitivity to the *E. coli* derived proteins. Separate ELISAs confirmed that BSA use in diluents did not inhibit protein detection. Protein release was represented as total mass and percent (normalized to total protein released at 42 days).

2.9 Cell Seeding and Culture

Frozen hBMSCs at P1 (i.e., plated once) were thawed and plated at a density of 10,000 cells/cm^2 on tissue culture-treated well plates. The culture medium for hBMSCs consisted of Dulbecco's Modified Eagle medium (DMEM; low glucose), 1% penicillin–streptomycin (P/S), and 10% fetal bovine serum (FBS-MSC certified) (all from Invitrogen Life Technologies, Carlsbad, CA). Cells were allowed to attach to the plates for 24 hours. At this time (denoted as "Day 0"), the medium was refreshed, along with an additional growth factor treatment. The growth factor treatments were the protein-containing supernatants from the completed release study, administered daily for 12 days, corresponding to the first 12 release sampling intervals (30 days, 90% total protein released). During daily medium refreshing and growth factor treatments, the volume ratio of medium to growth factor aliquot was 1:1 to ensure cell viability (Fig. 1B). Negative and positive control groups were also included. The negative control consisted of 0.1% BSA in PBS without protein.

2.10 Positive Control Selection for Cell Culture

The positive control concentration was derived from the average burst concentration between microsphere groups for each protein formulation (Fig. 1C). Specifically, the average burst concentration was calculated for each formulation (either TGF-β_3, BMP-2, or BMP-2 with HAp), rounded up to the nearest 10 ng/mL (Table 2), and then divided by 2 (to allow for a 1:1 medium dilution with eluted protein samples). Positive control concentrations were delivered to cells on a daily basis. Positive control samples were also reconstituted in 0.1% BSA in PBS and subjected to one freeze-thaw cycle, just as release supernatants.

2.11 Quantitative Polymerase Chain Reaction

In preparation for RT-PCR, samples (n = 3) at 0 and 12 days were first homogenized in 1 mL Trizol reagent (Invitrogen) and the RNA was isolated according to the manufacturer's guidelines. Isolated RNA was cleaned with an RNeasy spin column method (Qiagen, Valencia, CA) and converted to cDNA using a TaqMan High Capacity kit (Applied Biosystems, Foster City, CA) in a BioRad ThermoCycler. TaqMan Gene expression assays from Applied Biosystems for glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Hs99999905_m1), bone gamma-carboxyglutamate protein (BGLAP, Osteocalcin, Hs01587813_g1), collagen type I (COL1A1, Hs00164004_m1), runt-related transcription factor 2 (RUNX2, Hs00231692_m1), aggrecan (ACAN, Hs00202971_m1), collagen type II, (COL2A1, Hs00156568_m1), and sex determining region Y-box 9 (SOX9, Hs00165814_m1) were used in conjunction with an Applied Biosystems 7500 Fast Real-time PCR System. A $2^{-\Delta\Delta Ct}$ method was used to evaluate the relative level of expression for

each target gene.[37] For quantification, the negative control (at day 0) constructs were designated as a calibrator group, and GAPDH expression as an endogenous control.

2.12 Statistical Analyses

Statistical analyses were performed using a single factor analysis of variance (ANOVA) followed by a Tukey's Honestly Significant Difference *post hoc* test in PASW 18.0 software (SPSS Incorporated, Chicago, IL), when significance was detected below the $p = 0.05$ value. All quantitative results (numerical values and representative diagrams) are expressed as the average ± standard deviation.

3. RESULTS

3.1 Glass Transition Temperatures

Glass transition temperatures for PLGA/HAp composite microspheres were about 10 °C lower than pure PLGA microspheres following EtO + CO_2 ($p < 0.05$), EtO + EtOH ($p < 0.05$), and EtOH only ($p < 0.005$) treatments, whereas there was no statistically significant difference in T_g between PLGA and PLGA/HAp composite microspheres that received only EtO exposure. Similarly, there were no statistically significant differences among T_g in PLGA only microspheres, or among T_g PLGA/HAp composite microspheres (Fig. 2).

3.2 TGF-β3 Release from PLGA Microspheres

Total protein release from microspheres loaded with TGF-β_3 was markedly different among experimental groups (**data not shown**). Microspheres with only EtO exposure demonstrated a release of ~175 ng of TGF-β_3 by 7 days, with another small burst at 14 days. Net release leveled off afterward, with a small burst at 42 days. The release behavior for microspheres treated with EtO/CO_2 was similar, with a maximum of ~150 ng of TGF-β_3 by 42 days. For microsphere groups treated with EtOH, however, the total amount of TGF-β_3 liberated was almost 3 times as much by 42 days. The release behavior for EtO/EtOH and EtOH only-treated groups exhibited more distinct third release phases at approximately 21 days, with ~400 and ~350 ng delivered by 42 days. The standard deviations related to groups treated with EtOH were considerably larger than the EtO and EtO/CO_2 groups. In all of the groups, 40% of TGF-β_3 was released by 24 hours, and at least 60% by 14 days (Fig. 3).

3.3 BMP-2 Release from PLGA Microspheres

Net protein delivery from microsphere groups loaded with BMP-2 followed similar patterns to those loaded with TGF-β_3 (**data not shown**). The total mass of BMP-2 liberated in the EtO-treated group was greater than the EtO/CO_2 group (~700 ng versus ~400 ng by 42 days), with the EtO-treated group exhibiting a third phase of release at 21 days. For microsphere groups treated with EtO/EtOH and EtOH only, the total amounts of BMP-2 delivered were ~950 and ~800 ng, respectively. The initial burst behavior for EtOH-treated groups was dissimilar to EtO and EtO/CO_2 groups. Specifically, for EtO and EtO/CO_2 microspheres, ~80% of BMP-2 had been released within 7 days, whereas only ~50% of the protein was delivered by the same time in EtOH-treated groups (Fig. 3). As with TGF-β_3, the standard deviations in EtOH-treated groups were relatively large compared to the other groups.

3.4 BMP-2 Release from PLGA/HAp Microspheres

Total BMP-2 release from composite PLGA/HAp microspheres was far less than that of BMP-2 from non-composite microspheres, with as little as ~150 ng from EtO-treated groups, and as much as ~300 ng from EtO/CO_2-treated groups (**data not shown**). A third

phase of release was not observed for any of the composite PLGA/HAp groups. The net mass and percent of protein liberated was essentially unchanged after 21 days (Fig. 3).

3.5 Bioactivity of TGF-β3 from PLGA Microspheres

For TGF-β_3-stimulated hBMSCs, expression of aggrecan, collagen II, and Sox9 was monitored at 0 and 12 days. For aggrecan (Fig. 4A), only the EtOH-treated group demonstrated a statistically significant increase in gene expression relative to the day 0 value ($p < 0.05$). For collagen II expression (Fig. 4B), only the negative control was shown to have a significantly increased expression at 12 days compared to the day 0 value ($p < 0.05$). There were no statistically significant differences among treatment groups at 12 days for Aggrecan or Collagen II. With regard to Sox9 expression (Fig. 4C), only the positive control was statistically different from the negative control (lower) at 12 days ($p < 0.05$), but positive control expression was not significantly different from the experimental groups.

3.6 Bioactivity of BMP-2 from PLGA Particles

For BMP-2-stimulated hBMSCs, expression of osteocalcin, collagen I, and Runx2 was monitored at 0 and 12 days. Expression of osteocalcin (Fig. 5A) was decreased for all samples at 12 days relative to the day 0 value ($p < 0.0005$). Only the EtO-treated group was found to be significantly greater than the positive control ($p < 0.05$). For collagen I expression (Fig. 5B), only the EtO and EtO/CO_2 groups were found to have increased expression relative to the day 0 value ($p < 0.05$). All of the groups except for the positive control were found to have increased Runx2 expression (Fig. 5C) relative to the day 0 value ($p < 0.01$). The negative control and experimental groups had a significantly increased expression of Runx2 compared to the positive control at day 12 ($p < 0.005$).

3.7 Bioactivity of BMP-2 from PLGA/HAp Particles

For BMP-2-stimulated hBMSCs (from HAp microspheres), expression of osteocalcin, collagen I, and Runx2 was monitored at 0 and 12 days. Osteocalcin expression (Fig. 6A) was decreased at 12 days, compared to the day 0 value, in groups treated with BMP-2 released from composite microspheres ($p < 0.0005$), but expression was not significantly different among experimental and control groups at 12 days. Collagen I expression (Fig. 6B) in the negative control and experimental groups was less than the positive control at 12 days ($p < 0.001$). Notably, expression of BMP-2 in the positive control was also greater than day 0 ($p < 0.0005$). Runx2 expression (Fig. 6C) was similar between the positive and negative control groups at 12 days, and expression in these groups was also greater than the day 0 value ($p < 0.0005$). All four experimental groups had decreased Runx2 expression at day 12 compared to the positive and negative controls ($p < 0.0005$).

4. DISCUSSION

This was the first study to investigate the release and relative bioactivity of TGF-β_3 and BMP-2 following various processing and sterilization factors used in microsphere-based scaffold technology.[23, 25, 26] The results have implications on the efficacy of growth factor incorporation for stem cell differentiation in a 3-D scaffolding environment.

Glass transition temperature data provided a clear distinction between microspheres containing HAp and those without. A reason for all groups with HAp having a decrease in T_g is not readily discernable, but may be due to HAp disrupting the polymer network within the microsphere, thus enabling more efficient penetration of CO_2 and EtOH during the solvent treatments. Even though a previous investigation found that a 50 minute ethanol exposure lowered the glass transition temperature (T_g) of a ~50 kDa 50:50 PLGA, [25] the current study demonstrated no statistically significant differences in T_g between EtOH-

treated groups (both PLGA and PLGA/HAp composite) and their controls. The discrepancy may have been due to differing molecular weights.

During protein release, a noticeable difference occurred with regard to whether microspheres were treated with EtOH. The fact that EtOH-treated groups released more TGF-β_3 and BMP-2 from PLGA microspheres suggested that perhaps a pre-solubilization of the polymer allowed for diffusion of protein from the innermost layers of the microspheres toward the perimeter. When placed in release medium, the outermost layer of the microspheres may have been "primed" with protein, hence the large amount dumped in the first week of release. Such a phenomenon came with higher variability. EtOH exposure as a co-solvent has been shown to adversely affect drug loading and simultaneously increase the "burst" release from PLGA microparticles by concentrating drug at the surface of the vehicle.[38] Such an increased mass released with EtOH-exposure, or any treatment, did not occur with composite PLGA/HAp microspheres. With T_g data supporting a disruption in the polymer network from mineral incorporation, a low protein release was more likely a consequence of either a poor entrapped mass of protein during fabrication. Alternatively, the physicochemical properties of HAp might have created an environment that hindered protein liberation from the bulk of the microsphere, physically or chemically,[39-42] which has been seen before with BMP-2.[43]

Similarly to HAp formulations, CO_2 treatments also leveled off in net mass released by 21 days, and did not exhibit a third phase of release. The absence of a third phase is contradictory to theoretical macromolecule release profiles from microspheres,[44] which was most aptly demonstrated by the profile for TGF-β_3 EtO/EtOH group. An absence of this phase may have been indicative of protein retention inside the vehicle.[39-42] The mass of released BMP-2 relative to TGF-β_3 was in approximate correspondence with the loading ratio of 2:1 (60 ng BMP-2/mg or and 30 ng TGF-β_3/mg) or the microsphere formulations, even though the shape of the release for each of these proteins was somewhat different. Different release profile shapes may indicate that although the proteins are approximately the same size, their diffusion from the microsphere core was altered due to the environmental factors. Overall, however, using the simple estimate that equilibrium is reached when the Fourier number for diffusion is approximately equal to unity, $D*t/L^2 \approx 1$, the magnitude of the diffusion coefficients for TGF-β_3 and BMP-2 can be estimated.[45] Here, L represents the characteristic length of the microspheres (the radius, ~125 μm), D is the diffusion coefficient (cm^2/sec), and t is the characteristic timescale (about 7 days to reach the lag phase in release). This yields a diffusion coefficient of ~3 x 10^{-10} cm^2/sec, which is on the order of typical diffusion coefficients of proteins in polymers.[46-49] It should be noted that some of the solvent treatments utilized in this study were polar molecules (EtO and EtOH), which might have disrupted protein conformation or unfolding, leading to aggregation, which would also affect diffusivity of the macromolecules from the vehicles. Future studies should consider the extent of protein aggregation due to EtO and EtOH exposure.

In addition to atypical behavior in the release profiles, cell differentiation studies indicated that the released proteins might have exhibited a diminished bioactivity. To put bioactivity into context, both positive and negative controls were included for each type of formulation. Aggrecan expression was similar between virtually all of the groups at 12 days, which only demonstrated that perhaps TGF-β_3 did not stimulate aggrecan expression in the prescribed experimental conditions. Collagen II expression, however, was dramatically increased at 12 days in the negative control compared to the day 0 value, lending to the notion that TGF-β_3 in the treatment groups had a similar effect as TGF-β_3 in the positive control. The ability of the released proteins to affect Sox9 expression was ambiguous. BMP-2 from non-composite microsphere formulations showed a similar performance to the positive control with regard

to osteocalcin and collagen I expressions, but showed a distinctly different performance from the positive control with respect to Runx2 expression. A similar confounding result was seen with regard to Runx2 expression from composite-HAp microspheres, where experimental expression was different from both positive and negative controls. It is difficult to identify circumstances, however, when the experimental treatment groups would alter protein structure or function in such a way that selectively affects the expression of certain genes. Likewise, it is important to identify that gene expression not readily affected by the treatment groups does not necessarily mean inactive proteins, but potentially protein concentrations that were not ideal. Coupled with T_g data, some experimental groups with HAp may have had lower entrapped protein concentrations after microsphere drying. Additionally, positive control concentrations were representative of the average experimental concentrations, and were provided continually throughout culture. The experimental design attempted to mitigate this problem by using a minimal medium:release aliquot dilution of 1:1. Thus, while a perfectly ideal positive control is not realistic, an effort was made to provide a reasonable value to serve the purpose of placing experimental group results in perspective.

Other factors that could have affected gene expression relate to the components of the release supernatants during cell feeding. Specifically, release samples contained the acidic breakdown products of PLGA, lactic acid and glycolic acid, which was evidenced by medium color change during feedings, and would certainly differ from *in vivo* conditions, where buffering and removal would in part contribute to differences. During breakdown, the pH within each microsphere can become as low as 1.5.[50] This acidic microenvironment might have been an influence on selective gene expression or overall bioactivity of TGF-β_3 or BMP-2. Similarly, in groups with PLGA/HAp microspheres, a small amount of HAp was certainly present in the release medium as well, which also might have affected gene expression. Since PLGA degradation is highly acidic,[50, 51] using other buffers such as calcium carbonate or sodium bicarbonate may serve to mitigate acid-related adverse effects. Lastly, although ELISA cannot be taken as an absolute indication of bioactivity, it is an indication that much of the structural integrity of the proteins was retained for at least non-composite microspheres.

From the perspective of current techniques for making microsphere-based scaffolds, it seems as though the approach of EtOH treatment[22-25] for microsphere sintering delivered the most protein over the course of 6 weeks, as opposed to CO_2 exposure.[26] While the variability of BMP-2 and TGF-β_3 delivery in EtOH formulations was larger, this might perhaps be a small sacrifice compared to losing a considerable fraction of protein to encapsulation or long-term retention and aggregation inside the vehicle. In addition, there is currently no definitive indication that EtOH sintering, or any treatment, is detrimental to cellular stimulation, as gene expression is only a "snapshot" of fluctuating transcription and translation of proteins relevant to differentiation. In addition, changing gene expression may also be due to other factors not explicitly targeted within the scope of this manuscript, such as response to PLGA attachment, HAp presence, or cell feeding conditions. For the purposes of sterilization, there was not enough evidence to conclude that EtO drastically affected protein liberation or bioactivity under the experimental conditions, which was supported by T_g and RT-PCR data. For all formulations, however, the timeline of growth factor release may not necessarily correspond to cellular differentiation requirements *in vivo*. The release pattern may also not be commensurate with local and systemic immune responses during the earliest stages of wound healing, as this might adversely affect the overall efficacy of protein delivery in several ways.[27] The duration of release, however, was favorable in the context of osteochondral wound healing, as this process requires great lengths of time to complete *in vivo*.

Future work should focus on developing methods to properly quantify the actual amount of entrapped protein, the physical protein distribution within the microsphere formulations, whether solvent treatments lend to protein aggregation, and physical protein-HAp interactions. The aforementioned studies should provide insight into questions whether HAp inhibits BMP-2 loading during fabrication, as supported by T_g data, and whether including HAp in formulations provides a regenerative benefit over only BMP-2 incorporation. Cell differentiation studies with more frequent time points might also further contextualize the bioactivity of the proteins. In addition, methods to remove polymer byproducts or HAp particles from the release supernatant, or add these factors to positive control groups, will also give a more accurate reproduction of the release medium and protein microenvironment during breakdown of the particles *in vitro*.

5. CONCLUSIONS

This was the first study to investigate several high-interest processing conditions on the release and relative bioactivity of growth factors that are used in a continuously-graded scaffold technology.[22-25] Release profiles indicated that microspheres released protein with a diffusional profile during the first week, and in some instances, provided a third phase of release near 3 weeks. Formulations that were exposed to EtOH generally released more protein, and had larger variations in release profiles. Formulations with HAp released less protein, which may have been related to a poor initial protein entrapment, as supported by T_g data. Cell bioactivity assays indicated that in some instances, the experimental groups had an effect on hBMSC gene expression, but optimal concentrations may not have been employed with the current experimental design. In addition, the breakdown products of microspheres may have affected cell stimulation under the prescribed experimental conditions. The overall results indicated, however, that protein release from microspheres exposed to key processing and sterilization methods required to formulate microsphere-based scaffolds, was entirely feasible with PLGA or PLGA/HAp formulations, and especially those treated with EtOH during the sintering step, which makes this technology a candidate for scaffolds with continuous growth factor[23, 25] and material composition and stiffness[24] gradients.

Acknowledgments

Biography

Nathan Dormer, Ph.D. Nathan Dormer is currently a Senior Scientist for Orbis Biosciences in Kansas City, KS. Orbis uses a proprietary technology called Precision Particle Fabrication (PPF) to produce microspheres and microcapsules with a high monodispersity, which are used in the formulation and controlled release of peptides, proteins, small molecule drugs, and nutrient and flavor oils. Dr. Dormer received his B.S. in Chemical Engineering and his Ph.D. in Bioengineering, both from the University of Kansas. Upon completion of his Ph.D.,

he was awarded the William and Marnie Argersinger Prize for the University's most outstanding dissertation, which focused on osteochondral tissue engineering.


Vineet Gupta, B.S. Vineet Gupta is a currently a graduate student in The University of Kansas' Bioengineering program specializing in Biomaterials & Tissue Engineering. His doctoral work focuses on monodisperse microsphere fabrication with high hyaluronic acid content for healing in osteochondral defects. Previously, Mr. Gupta received his B.S. in Biophysics from Panjab University in Chandigarh, India.

Aaron Scurto, Ph.D. Aaron Scurto is presently an associate professor in Chemical & Petroleum Engineering at The University of Kansas. Dr. Scurto's research focuses on the relationship between solvents and catalysts or metal complexes, with an emphasis on homogeneous catalysts in primarily enantio-selective (chiral) reactions. Among the various solvents of interest are supercritical fluids such as CO_2, and a new class of solvents called ionic liquids. Dr. Scurto received his Ph.D. from Notre Dame before completing an NSF postdoctoral fellowship at RWTH-Aachen University, and becoming a postdoctoral fellow at MIT.

Cory Berkland, Ph.D. Cory Berkland currently holds joint professorships in Chemical & Petroleum Engineering and Pharmaceutical Chemistry at The University of Kansas. He earned his B.S. in Chemical Engineering from Iowa State, and his Ph.D. from The University of Illinois. Dr. Berkland's work merges engineering and biological sciences, outlining methods for regenerating tissues, developing biosensors, and effectively delivering therapeutic agents. Accomplishing such tasks requires specifically designing biomedical or drug delivery devices that possess the required physicochemical properties. His lab focuses on developing precision engineering technologies at the macro- to molecular scale for novel or improved biomedical and drug delivery devices.
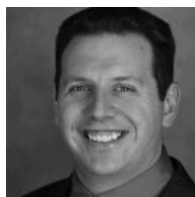
Michael Detamore, Ph.D. Michael Detamore is a Professor of Chemical & Petroleum Engineering and Director of the Biomaterials and Tissue Engineering Laboratory at the University of Kansas. He earned his B.S. in chemical engineering from the University of Colorado and his Ph.D. in bioengineering from Rice University. He is the recipient of the NSF CAREER Award and the Coulter Foundation Translational Research Award, and was a Fulbright Scholar and Visiting Professor at NUI Galway in Ireland in 2011. His research interests are biomaterials, biomechanics, stem cells and tissue engineering. Central research themes include umbilical cord stem cells and gradients in tissue engineering.

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STUDY HIGHLIGHTS

- Protein-loaded PLGA microspheres were subjected to post processing conditions.

- Released protein was used in cell stimulation studies.

- Ethanol exposure was found to increase amount of released protein.

- Hydroxyapatite and carbon dioxide decreased amount of released protein.

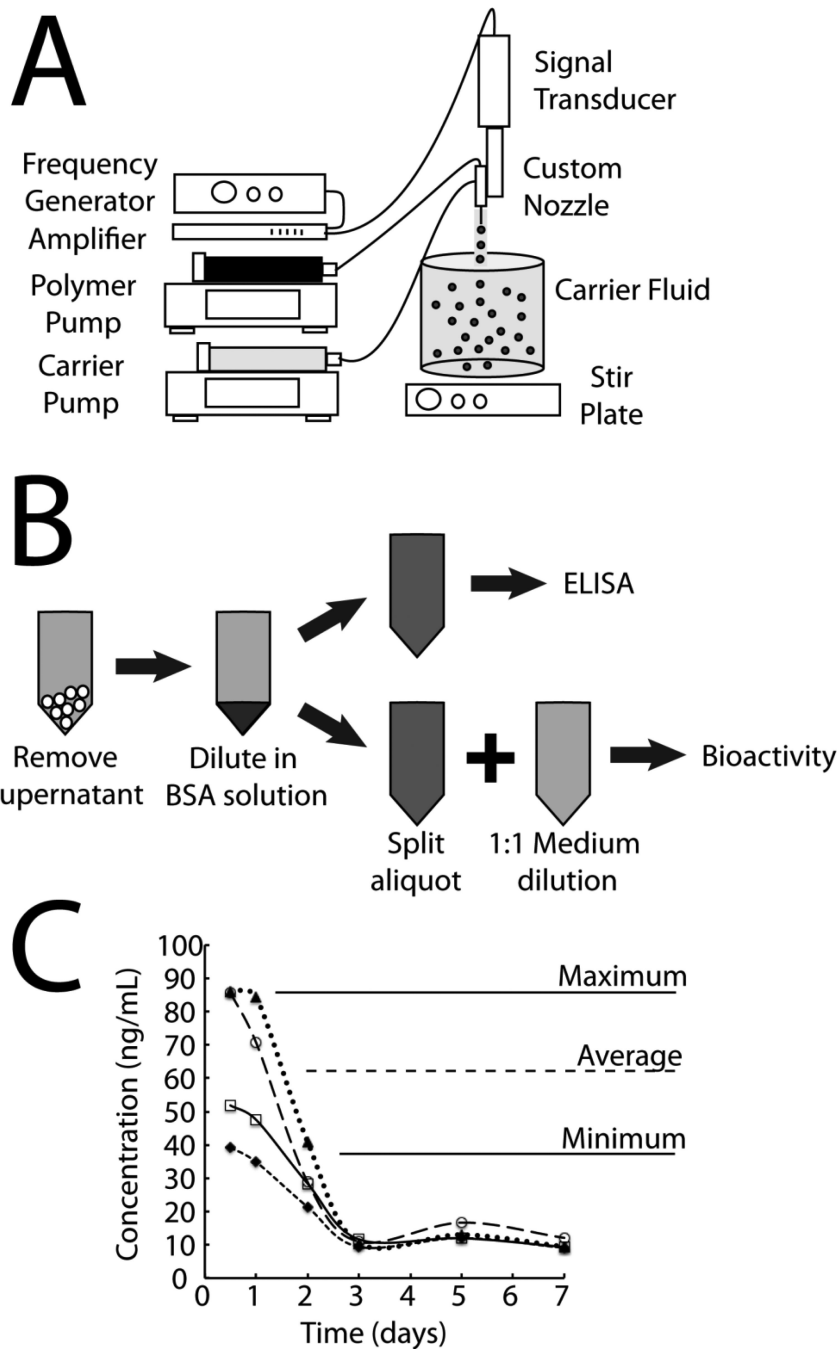- Hydroxyapatite presence decreased the glass transition temperature of microspheres.

Figure 1.
Particle fabrication and release experiment. (A) Precision particle fabrication apparatus made microparticles with high monodispersity. (B) At each sampling time point, supernatant was mixed with a concentrated BSA solution, split into two aliquots, and used in ELISA and cell stimulation. (C) The positive control protein concentration was defined as the average concentration during the burst release for TGF-β_3, BMP-2, or BMP-2 from PLGA/HAp composite microspheres. The positive control concentration was provided to hBMSCs daily for 12 days, whereas treatment aliquots contained decreasing concentrations with time.
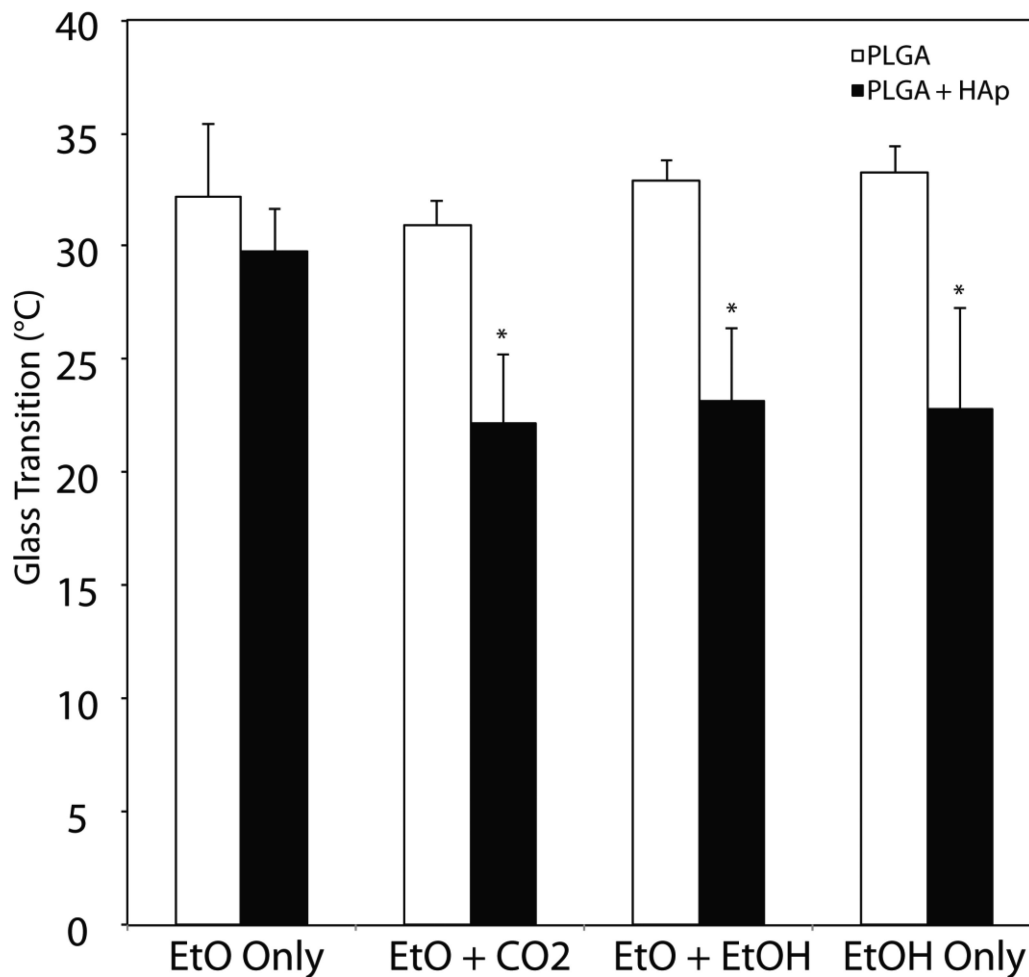
Figure 2.
Glass Transition Temperature (T_g). Glass transition temperatures for PLGA/HAp composite microspheres were lower than PLGA only microspheres following EtO + CO_2, EtO + EtOH, and EtOH only treatments, whereas there was no statistically significant difference in T_g between PLGA and PLGA/HAp composite microspheres that received only EtO exposure. Similarly, there were no statistically significant differences among T_g in PLGA only microspheres, or among T_g PLGA/HAp composite microspheres. All values are expressed as the average ± standard deviation (n = 3), $p < 0.05$, * = statistically significant difference from the control at that time point.
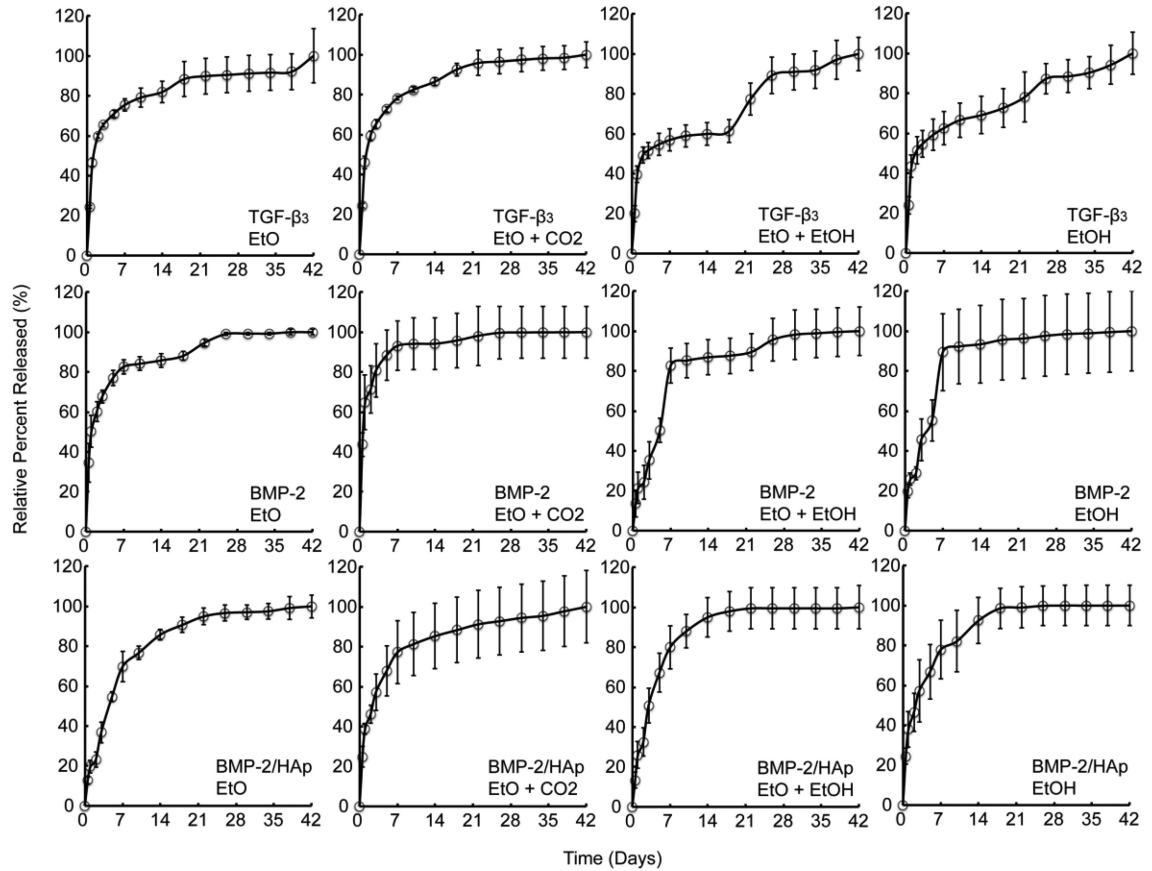
Figure 3.
Percent protein released. Results demonstrated that EtOH-treated microspheres without HAp released a lower percentage of protein than control or CO_2-treated groups before 7 days, but ultimately delivered a larger percentage in the remaining 35 days. The shape of the release profiles was similar between all formulations, with ~90% of the protein being released by 30 days. Data is represented as percentage mass released ± standard deviation. The percent released was calculated relative to the total mass liberated at 42 days. All values are expressed as the average ± standard deviation (n = 3), $p < 0.05$, @ = statistically significant difference from week 0 value, # = statistically significant difference from the negative control at that time point, and * = statistically significant difference from the positive control at that time point.
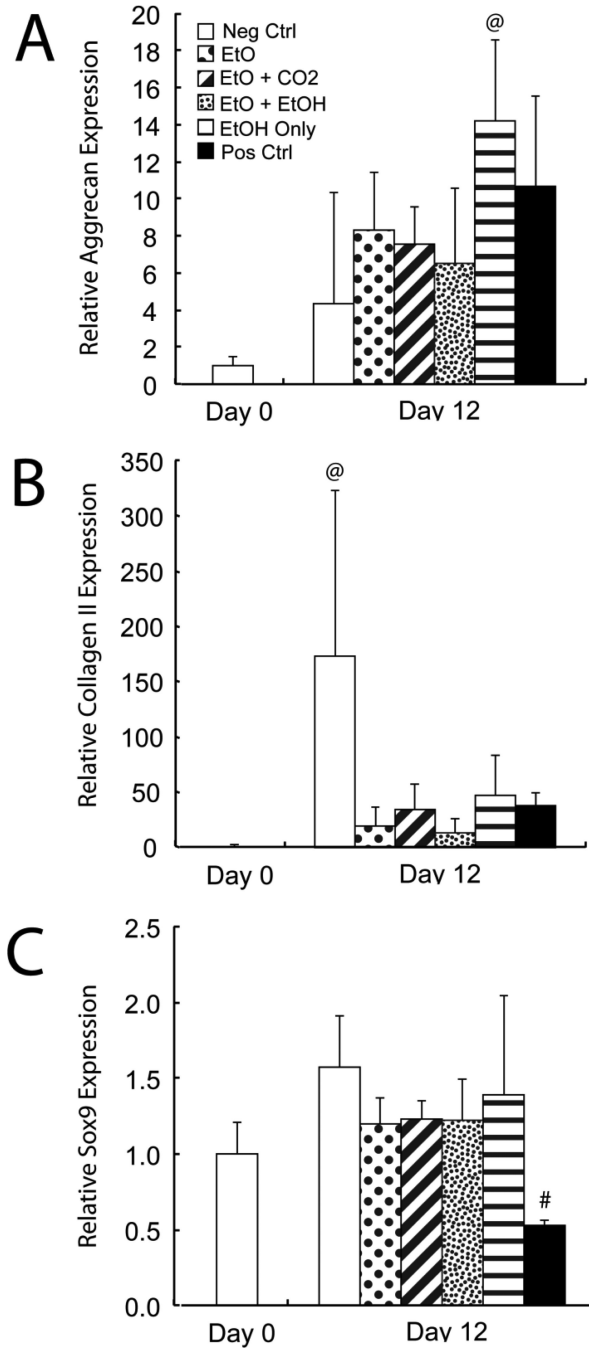
Figure 4.
Chondrogenic gene expression of hBMSCs. For cells treated with TGF-β_3 groups. (A) Aggrecan expression demonstrated that treatment groups performed similar to both the positive and negative controls. (B) Collagen II expression showed treatment groups performed similar to the positive control. (C) Sox9 expression demonstrated that experimental groups performed similar to negative control, but were not different from the positive control. All values are expressed as the average $\pm$ standard deviation (n = 3), $p <$ 0.05, @ = statistically significant difference from week 0 value, # = statistically significant difference from the negative control at that time point, and * = statistically significant difference from the positive control at that time point.
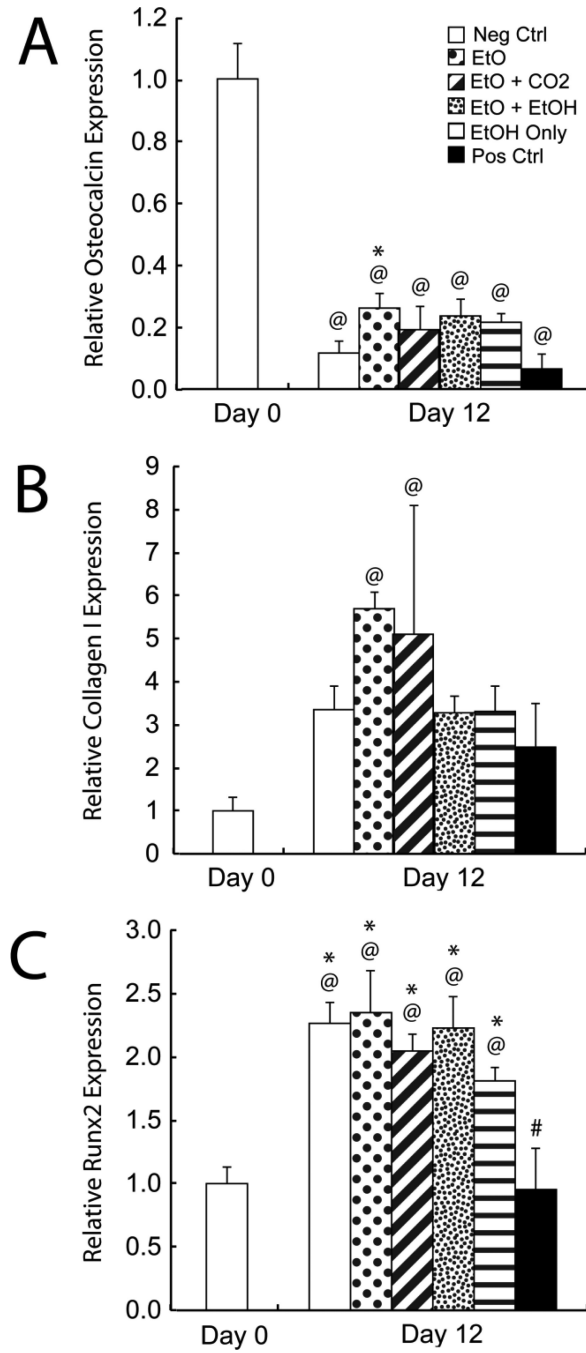
Figure 5.
Osteogenic gene expression of hBMSCs for BMP-2. For cells treated with BMP-2 groups. (A) Osteocalcin and (B) Collagen I expression demonstrated that treatment groups behaved similar to both the positive and negative controls. (C) Runx2 expression demonstrated that experimental groups performed similar to negative control. All values are expressed as the average $\pm$ standard deviation (n = 3), $p < 0.05$, @ = statistically significant difference from week 0 value, # = statistically significant difference from the negative control at that time point, and * = statistically significant difference from the positive control at that time point.
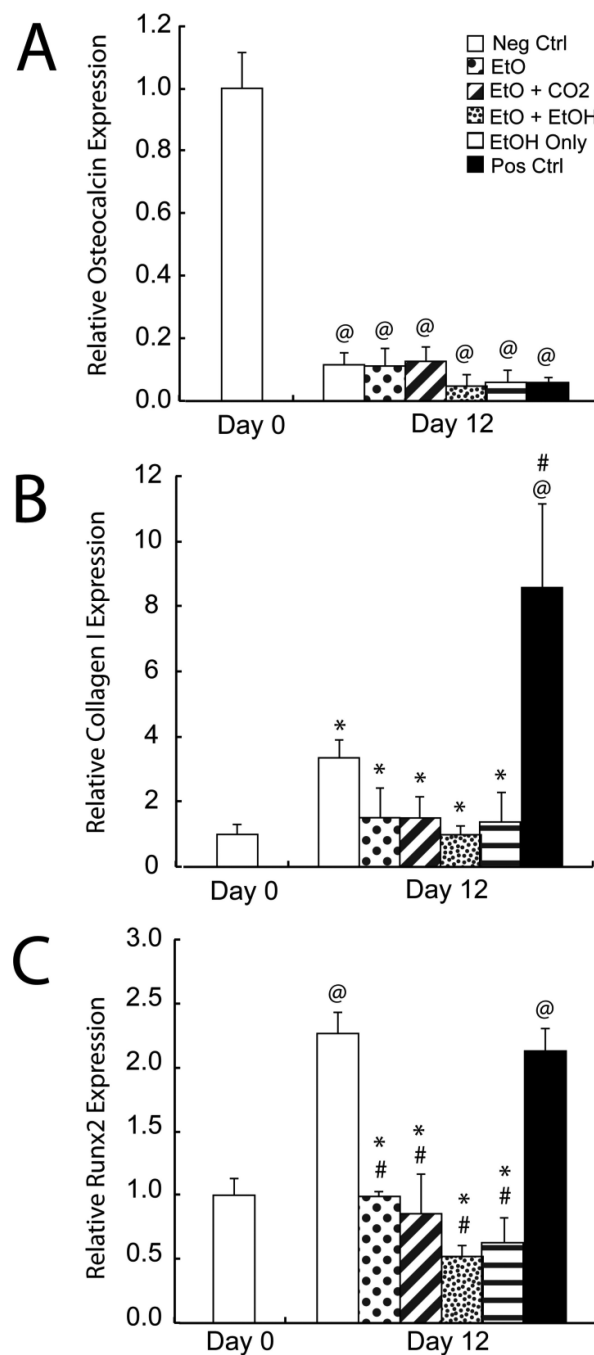
Figure 6.
Osteogenic gene expression of hBMSCs for BMP-2/HAp. For cells treated with BMP-2 from HAp composite scaffolds. (A) Osteocalcin expression demonstrated that treatment groups performed similar to both the positive and negative controls. (B) Collagen I expression shows treatment groups behaved similarly to the negative control. (C) Runx2 expression demonstrated that experimental groups had expression that was unlike either positive or negative controls. All values are expressed as the average ± standard deviation (n = 3), $p < 0.05$, @ = statistically significant difference from week 0 value, # = statistically significant difference from the negative control at that time point, and * = statistically significant difference from the positive control at that time point.

Table 1

Experimental reatment conditions for protein-loaded microspheres.

Formulation	Treatment	EtOH or CO_2 Conditions
	EtO	-
TGF-β_3	EtO + CO_2	15 Bar, 60 minutes
	EtO + EtOH	60 minutes
	EtOH	60 minutes
	EtO	-
BMP-2	EtO + CO_2	15 Bar, 60 minutes
	EtO + EtOH	60 minutes
	EtOH	60 minutes
	EtO	-
BMP-2 + HAp	EtO + CO_2	25 bar, 60 minutes
	EtO + EtOH	90 minutes
	EtOH	90 minutes

Table 2

Selection of positive control growth factor concentrations for cell stimulation studies.

Formulation	Treatment	Burst (ng/mL)	Average (ng/mL)	Positive (ng/mL)
	EtO	52		
TGF-β_3	EtO + CO_2	39	66	40
	EtO + EtOH	86		
	EtOH	86		
	EtO	252		
BMP-2	EtO + CO_2	187	181	90
	EtO + EtOH	126		
	EtOH	159		
	EtO	23		
BMP-2 + HAp	EtO + CO_2	83	55	30
	EtO + EtOH	34		
	EtOH	80		