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RESEARCH PAPER

Bone Morphogenetic Protein-2 Release from Composite Hydrogels of Oligo(poly(ethylene glycol) fumarate) and Gelatin

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ABSTRACT

Purpose Hydrogel composites of oligo(poly(ethylene glycol) fumarate) (OPF) and gelatin microparticles (GMs) were investigated as carriers of bone morphogenetic protein-2 (BMP-2) for bone tissue engineering applications.

Methods Hydrogel composites with different physical characteristics were prepared by changing the amount and type (acidic *vs.* basic) of gelatin incorporated in the OPF bulk phase. Composites with differing physical properties (degradation, swelling, and mechanical properties) and differing BMP-2 loading phase were investigated to determine the effect of these factors on BMP-2 release profiles over 28 days.

Results Overall, higher gelatin amount increased the degradation and swelling of composites, and acidic GMs further increased the degradation and swelling and reduced the compressive modulus of the composites. The most significant factor affecting the release of BMP-2 from composites was the loading phase of the growth factor: GM loading reduced the burst release, increased BMP-2 release during the later phases of the experiment, and increased the cumulative release in faster degrading samples.

Conclusions The results indicate that the physical properties and the BMP-2 release kinetics of hydrogel composites can be controlled by adjusting multiple parameters at the time of the hydrogel composite fabrication.

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KEY WORDS bone morphogenetic protein-2 · controlled release · gelatin microparticles · hydrogel composites · oligo(poly(ethylene glycol) fumarate)

ABBREVIATIONS

BMP-2	Bone morphogenetic protein-2
col-PBS	PBS containing 400 ng/ml collagenase type 1A
GM	Gelatin microparticle
HA	High acidic group
HB	High basic group
LA	Low acidic group
LB	Low basic group
OPF	Oligo(poly(ethylene glycol) fumarate)
pI	Isoelectric point
xxG	Gelatin loaded BMP-2
xxO	OPF loaded BMP-2

INTRODUCTION

Craniofacial bone injury or loss is a common result of trauma or tumor resection (1). However, current treatments are not ideal, as bone autografts result in significant morbidity to the patient and allografts risk disease transmission and have limited availability. Thus, biomaterials to regenerate bone are in high demand (2). Injectable biomaterials are favorable due to their noninvasive application and propensity for contouring to the existing bone surface, which is of unique interest to the clinical treatment of facial bone deformities (3).

Injectable biomaterials have been developed that meet the most basic design needs for craniofacial bone regeneration, i.e., *in situ* formed and biocompatible (3). However, the underlying mechanisms for augmenting craniofacial bone and maintaining the height of this bone long-term are not adequately established for tissue engineers to design and manufacture biomaterials that consistently meet the

requirements inherent to this specific application (4). Towards addressing this need, development is ongoing of an injectable, osteoinductive, composite hydrogel scaffold for bone augmentation.

Oligo(poly(ethylene glycol) fumarate) (OPF) is an *in situ* cross-linkable, cytocompatible, and minimally inflammatory injectable hydrogel that is appropriate for tissue engineering applications of many types (5). The incorporation of gelatin to form an OPF composite construct provides the hydrogel scaffolds with macroporosity and cell binding domains, which are essential to tissue formation. Composites of OPF and GMs offer advantages of both synthetic and naturally derived polymers including the reproducible manufacture and tunable properties of OPF and the biocompatibility and natural protein affinity of gelatin (5).

Based on the scope of the present work, bone morphogenetic protein-2 (BMP-2) was identified as the leading candidate to elicit an osteoinductive tissue response. BMP-2, a potent osteoinductive factor, promotes ectopic ossification as well as osteogenic differentiation of mesenchymal stem cells (6,7). In order to maximize the osteogenic effect of BMP-2 for tissue engineering, drug carriers have been designed to control and localize its release while also maintaining bioactivity of the protein *in vivo*. BMP-2 delivery has been characterized from various biomaterials including inorganic materials, synthetic and naturally derived polymers, and composites (8), and sustained BMP-2 release has been achieved (9–12).

This work is the first to investigate the release of BMP-2 from OPF hydrogels and the first to study the effect of increased gelatin loading and change in gelatin type on the physical properties of OPF-GM composites. Therefore to enable the design of such a biomaterial, the specific hypotheses of this work were as follows: (1) Both the amount and type (acidic *vs.* basic) of gelatin incorporated will affect the composite physical properties (degradation, swelling, and mechanical properties), (2) Modifying the composite physical properties will control the kinetics of BMP-2 release from composites (burst release, average rate of release during the various phases, and final cumulative release), and (3) Changing the loading phase of BMP-2 will further control the kinetics of BMP-2 release.

MATERIALS AND METHODS

Experimental Design

Composite scaffolds consisting of OPF and GMs were evaluated for their physical properties and for controlled release of BMP-2. In order to generalize the results and provide a model for the behavior of similar systems, a full factorial design was developed in which acidic (pI = 5.0) and basic (pI = 9.0) GMs were incorporated into composite scaffolds at two concentrations: 0.22 g GMs/g OPF and 0.44 g GMs/g OPF on a dry basis. Additionally, BMP-2 was loaded into two separate phases of the composite scaffolds: bulk OPF phase and GM phase.

OPF Synthesis and Characterization

OPF was synthesized according to an established procedure (5,13). Dichloromethane (EMD, Billerica, MA) was dried by refluxing in the presence of calcium hydride (Sigma Aldrich, St. Louis, MO) and distilled to yield the anhydrous product. PEG with nominal number average molecular weight of 3,350 Da (Sigma Aldrich, St. Louis, MO) was dried by distillation in toluene (Fisher Scientific, Waltham, MA) and dissolved in anhydrous dichloromethane. Triethylamine (Sigma Aldrich, St. Louis, MO) and fumaryl chloride (Acros, Geel, Belgium) were added dropwise to the PEG solution, and the reaction was allowed to proceed for 2 days. The product was purified by removal of dichloromethane, precipitation of salt, and recrystallization, washing, and subsequent drying of the OPF. The product was characterized by gel permeation chromatography and ^{1}H NMR.

Gelatin Microparticle Preparation

GMs were synthesized according to an established method (14). GMs were fabricated from acidic and basic gelatin (Nitta Gelatin Co., Osaka, Japan) and cross-linked in 10 mM glutaraldehyde (Sigma Aldrich, St. Louis, MO) overnight. After drying, the GMs were sieved to obtain particles 50–100 μm in size.

Composite Scaffold Fabrication

Composite scaffolds were fabricated for degradation, swelling, and mechanical testing experiments according to the specifications in Table I. Fabrication techniques adhered to established protocols (15). OPF was mixed with PEG-DA cross-linker (Glycosan BioSystems, Inc., Alameda, CA) at a 2:1 weight ratio, which corresponded to a double bond ratio of 1.62 (double bond ratio is the ratio of the number of cross-linkable double bonds in OPF to those in PEG-DA). The polymers were dissolved in PBS to yield a final polymer concentration of 0.2 g/ml. Pre-swollen GMs were added to the polymer solution and vortexed. For cross-linking, equal parts of initiator solutions, 0.3 M ammonium persulfate (Sigma Aldrich, St. Louis, MO) and 0.3 M *N,N,N',N'*-tetramethylethylenediamine (Sigma Aldrich, St. Louis, MO) were added to yield a final concentration of 25 mM. The solution was injected into Teflon molds and incubated at 37°C for 8 min. After cross-linking, composite scaffolds were transferred to plastic cassettes in the shape of plate

wells. The composite scaffold geometry used in this work was specific to the study of bone augmentation in rats. Therefore, the cassettes were designed to fit an equilibrium swollen, disk shaped, composite scaffold (d: 6 mm, h: 2 mm) to match the shape of an implant that would be used for the study of bone augmentation *in vivo* (16), and the size of the implant was adjusted to be suitable for implantation in rats (17). Each scaffold along with its cassette was then transferred to the experimental media.

Degradation and Swelling

The physical characterization of composite scaffolds was performed using established methods (18). Composite scaffolds were fabricated as described above and transferred to PBS containing 400 ng/ml Collagenase 1A (Sigma Aldrich, St. Louis, MO) (col-PBS). Scaffolds were maintained at 37°C on a shaker table at 70 rpm with media changes after 1 day and continuing twice weekly. Scaffolds were evaluated immediately after fabrication and at 1 day, 1 week, and 6 weeks ($n=4$). A number of scaffolds were dried immediately after fabrication and weighed (W_i). At each time point the scaffolds were blotted to remove excess PBS on the surface, weighed (W_s), dried overnight, and reweighed (W_d). Mass loss was determined by comparing the dry mass of the scaffold at day 0 to the dry mass of the scaffold at each time point according to the following equation, mass loss $=(W_i-W_d)/W_i$. Sol fraction was measured as the percent mass loss from the samples after 24 h incubation (19). Mass swelling ratio for each time point was determined by the ratio of the mass of PBS absorbed in the scaffold to the dry mass of the scaffold according to the following equation, mass swelling ratio $=(W_s-W_d)/W_d$.

Mechanical Testing

The compressive modulus of cross-linked composite scaffolds was determined by established methods (20). After cross-linking, scaffolds were swollen in PBS at 37°C for 24 h to reach equilibrium swelling. The compressive modulus was determined using a Thermomechanical Analyzer (TMA 2940, TA Instruments, New Castle, DE). Mechanical testing was performed with load applied at 0.1 N/min ($n=3$). Stress and strain data were plotted, and the compressive modulus was determined from the slope of the linear region of the stress–strain curve.

Table I Specifications for Composite Scaffolds for the Degradation, Swelling, and Mechanical Testing Experiments

	Gelatin amount	Gelatin type
LA	Low (0.22 g/g OPF)	Acidic (pI = 5.0)
LB	Low	Basic (pI = 9.0)
HA	High (0.44 g/g OPF)	Acidic
HB	High	Basic
BLANK	N/A	N/A

Low corresponds to 0.22 g GM/g OPF, and high corresponds to 0.44 g GM/g OPF. Acidic and basic refer to the type of gelatin that results from two different processing techniques that result in gelatin with isoelectric point of 5.0 or 9.0, respectively. Blank hydrogels were fabricated without GMs

BMP-2 Release

Composite scaffolds were fabricated according to the method described for the physical characterization, and BMP-2 (PeproTech, Rocky Hill, NJ) was loaded at 40 ng/ml based on cross-linked scaffold volume in all groups. BMP-2 loaded per GM was equivalent in groups with both GM amounts. Therefore, in groups with high loading only half of the GMs were loaded with BMP-2 while the remaining fraction was pre-swollen in PBS without BMP-2. Release was quantified according to established methods (21). A small fraction of incorporated BMP-2 was ^{125}I-radiolabeled (Perkin Elmer, Waltham, MA), and the quantified release of this portion was scaled to determine the total released amount. BMP-2 was loaded into the GM phase by dripping a solution of BMP-2 in PBS (5 µl PBS/mg GM) and storing the GMs at 4°C overnight. BMP-2 was loaded into the OPF phase by adding a solution of BMP-2 directly to the mixture of pre-swollen blank GMs and OPF precursor at the time of fabrication immediately prior to adding initiators. Composite scaffolds were placed 1 per well in 24 well plates with 2 ml of PBS containing 400 ng/ml Collagenase 1A to mimic the degree of enzymatic degradation of GMs that would occur *in vivo* (21). Release was quantified at time points of 1, 2, 3, 6, 10, 13, 17, 20, 24, and 28 days by collecting the supernatant and analyzing the level of radioactivity compared to a standard curve made from ^{125}I-radiolabeled BMP-2 stock solution using a gamma counter (Cobra II Autogamma, Packard, Meridian, CT) ($n=4$–6; some samples were lost during removal of buffer). Percent cumulative release was determined by normalizing the cumulative amount of growth factor released at each time point to the total amount loaded in each sample (determined by taking the sum of release at each time point and the amount of BMP-2 remaining in the composite scaffold after 28 days).

Diffusion Analysis

The release kinetics were analyzed according to the Ritger-Peppas equation, $M_t/M_\infty = kt^n$, where M_t/M_∞ is the fraction of BMP-2 released, k is a constant for the system, t is release time, and n is an exponent that describes the mechanism of diffusional release. The parameters k and n were determined from the initial portion of log-log plots of the fraction of BMP-2 released *versus* time ($M_t/M_\infty \leq 0.6$). For a

disk-shaped scaffold with aspect ratio 3, a value for n of approximately 0.44 is characteristic of Fickian diffusion. Values either above or below 0.44 are indicative of anomalous diffusion (22).

Statistics

Values of percent mass remaining, mass swelling ratio, compressive modulus, release rates, and final cumulative release were analyzed by ANOVA followed by Tukey's post-hoc test ($p<0.05$) for statistical significance.

RESULTS

OPF Characterization

OPF was synthesized with a number average molecular weight of 7,500±200 Da and a weight average molecular weight of 36,300±600 Da. Incorporation of fumarate monomers into the OPF oligomer was confirmed using ^{1}H NMR as demonstrated previously (13).

Degradation

The degradation profiles of the composite scaffolds are compared in Fig. 1 at time points of 1 day, 1 week, and 6 weeks where degradation was measured as the dry mass remaining in the scaffold at each time point compared to the dry mass of the scaffold at the time of fabrication. All the groups tested including the blank control experienced 33–44% mass loss after 1 day (sol fraction). Based on these data there was no significant difference in sol fraction between groups.

By 1 week of culture, HA samples experienced more significant degradation than all other groups (76±6%). LB and HB samples experienced significant degradation at 1 and 6 weeks compared to the previous time point (LB: 1d 36±4%, 1w 53±2%, 6w 61±3%; HB: 1d 37±6%, 1w 58±6%, 6w 70±5%) while LA and HA samples underwent degradation from 1 day to 1 week but not between 1 week and 6 weeks (LA: 1d 41±4%, 1w 57±8%, 6w 62±4%; HA: 1d 44±9%, 1w 76±6%, 6w 75±6%). Based on gelatin type alone, there was no difference in degradation at 6 weeks (LA: 62±4%, LB: 61±3%; HA: 75±6%, HB: 70±5%). Considering the effect of GM amount, HA and HB degraded more than LA and LB at 6 weeks. Considering the control samples without GMs, after 1 week of culture blank scaffolds degraded less (44±5%) than all other groups except LB, and by 6 weeks of culture, the blank group had degraded less than all other groups (50±4%).

Finally, all groups including the blank control experienced some level of degradation between 1 day and 6 weeks indicating that the OPF hydrogels were indeed degradable with or without the inclusion of GMs.

Swelling

The mass swelling ratios of the composite scaffolds are compared in Fig. 2. For equilibrium swelling, which is reached after 1 day of culture, the blank group exceeded all others in mass swelling ratio (13.6±0.6). Dose dependence on GM amount was not observed. By 1 week of culture the HA group had significantly higher swelling than all other groups (26.2±4.3). Swelling of LB, HB, and blank samples increased at 1 and 6 weeks compared to the previous time point (LB: 1d 11.2±0.7, 1w 15.6±0.6, 6w 18.0±1.0; HB: 1d 11.2±0.8, 1w 15.9±1.4, 6w 21.8±0.9; Blank: 1d 13.6±0.6, 1w 15.3±0.9, 6w 18.3±0.5) while swelling of LA and HA samples increased from 1 day to 1 week but not between 1 week and 6 weeks (LA: 1d 11.5±1.1, 1w 15.4±2.1, 6w 17.6±1.4; HA: 1d 11.4±1.3, 1w 26.2±4.3, 6w 22.1±2.5). By 6 weeks of culture, the HA and HB groups swelled more than all others demonstrating a GM dose dependence for mass swelling. All groups demonstrated some increase in swelling between 1 day and 6 weeks.

Mechanical Testing

The compressive moduli of the composite scaffolds are compared in Fig. 3. The compressive moduli ranged from 13.6 to 18.1 kPa. At equilibrium, the compressive modulus of the blank group exceeded that of the LA and HA groups. However, none of the composites with GMs demonstrated a significant difference in the values of their compressive moduli.

BMP-2 Release

The release profiles of BMP-2 from the various groups are displayed in Fig. 4. Percent cumulative release of BMP-2 is shown over 4 weeks where GM loading and OPF loading are compared in each panel for a specific GM amount and gelatin type. Clearly a distinct release profile results from the two phases of loading, and this effect is confirmed statistically by dividing the release into various phases as shown in Table II. In each panel of Fig. 4, OPF loading results in higher day 1 burst release (phase 1). Burst release ranged from 38.0% to 39.4% for OPF loading (LAO: 39.4±1.8%, LBO: 39.1±0.8% HAO: 39.1±7.3%, HBO: 38.0±0.7%) and from 24.8% to 27.5% for GM loading (LAG: 24.8±3.8%, LBG: 27.5±3.2%, HAG: 24.9±2.0%, HBG: 26.2±1.5%). BMP-2 release for OPF loading was higher than GM loading only in high GM groups from day 1 to 3 (phase 2). Another obvious difference in each panel of Fig. 4, GM loading results in higher release from day 3 to 17 (phase 3).

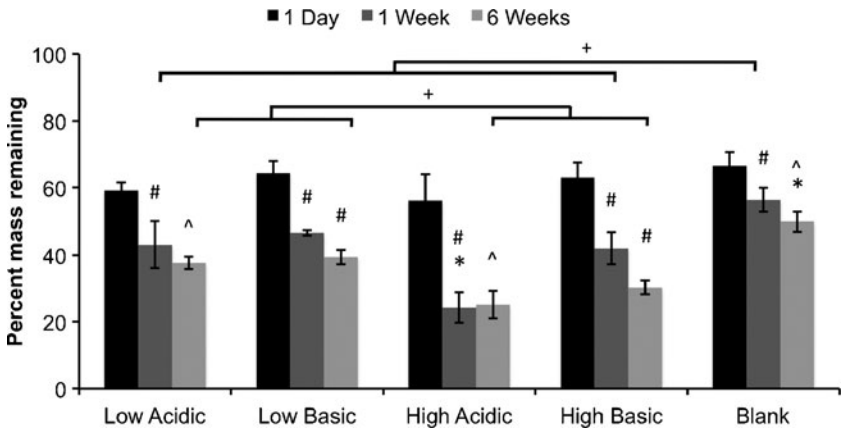

Fig. 1 Degradation of composite hydrogel scaffolds over a period of 6 weeks displayed as percent of the original dry mass of the hydrogel remaining. Low corresponds to 0.22 g GMs/g OPF, and high corresponds to 0.44 g GMs/g OPF. Acidic and basic refer to the type of gelatin that results from two different processing techniques that result in gelatin with isoelectric point of 5.0 or 9.0, respectively. Blank hydrogels were fabricated without GMs. Significant differences ($p < 0.05$) are designated by (+) for a difference between groups at the same time point, (*) for a difference from all other groups at the same time point, (#) for a difference in the same group from the previous time point, and (^) for a difference in the same group from day 1. Error bars represent ± 1 standard deviation. Sol fraction is represented as the percent of mass lost at day 1, but there was no difference between groups for this measure.

A relatively linear region of the plot during this time period demonstrates this effect graphically. BMP-2 release for GM loading was higher than OPF loading in all groups except HBG from day 17 to 28 (phase 4). This continues the trend of release being delayed by GM loading. For both loading phases, release continues for at least 28 days, and the release rate at the final time point is less than 1% per day in all groups.

In terms of GM amount, only one difference was observed, and that was in phase 4 where percent BMP-2 released per day from LBG exceeded that HBG. The most significant effect of gelatin type was for phase 3 of release for GM loading; LAG and HAG both had higher BMP-2 release in phase 3 compared to their basic GM counterparts, LBG and HBG, respectively. Finally, in phase 4, release from LBG exceeded that of LAG.

The final percent cumulative release of BMP-2 from each group is displayed in Table III. Differences based on gelatin type include higher final release of BMP-2 from LAG compared to LBG and higher final release from HAG compared to HBG demonstrating a trend of higher release from acidic GMs for GM loading. Additionally, final percent cumulative release was higher from GM loading compared to OPF loading for LAG and HAG samples.

Diffusion Analysis

A summary of the diffusion analysis is shown in Table IV. The parameter n identifies the transport mechanism that dominates the release of BMP-2 for each group. N values for all groups are below 0.44, which is the value of n that

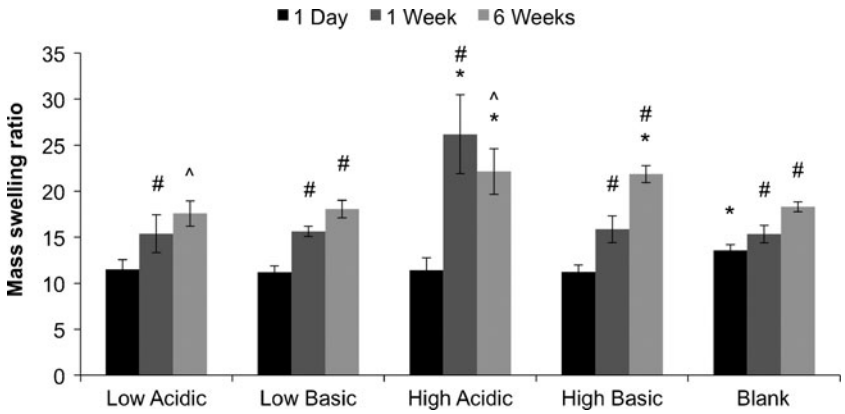

Fig. 2 Mass swelling ratio of composite hydrogel scaffolds over a period of 6 weeks. Low corresponds to 0.22 g GM/g OPF, and high corresponds to 0.44 g GM/g OPF. Acidic and basic refer to the type of gelatin that results from two different processing techniques that result in gelatin with isoelectric point of 5.0 or 9.0, respectively. Blank hydrogels were fabricated without GMs. Significant differences ($p < 0.05$) are designated by (*) for a difference from all other groups at the same time point, (#) for a difference in the same group from the previous time point, and (^) for a difference in the same group from day 1. Error bars represent ± 1 standard deviation. Equilibrium swelling is reached after 1 day.

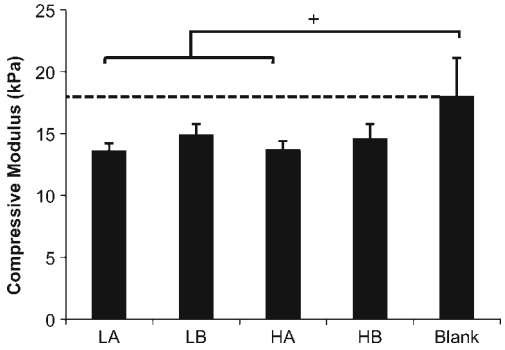

Fig. 3 Compressive moduli of composite hydrogel scaffolds at equilibrium swelling. Significant differences ($p < 0.05$) between groups are designated by (+). Error bars represent ± 1 standard deviation. The *dashed line* represents the compressive modulus of blank hydrogels for comparison.

corresponds to Fickian diffusion for a disk of aspect ratio 3 (22). Therefore, anomalous diffusion is the dominant mechanism of diffusion in all the groups investigated in the present study. For GM loading, n values range from 0.34 to 0.41, and for OPF loading, n values range from 0.21 to 0.29. K values range from 0.24 to 0.27 for GM loading and 0.39 to 0.40 for OPF loading. R^2 values show the suitability by which the data fit the diffusion model.

DISCUSSION

In this study, the release of BMP-2 from OPF-GM composite constructs was evaluated in order to determine the effect of the type and amount of gelatin and the growth factor loading phase on the construct physical properties and the BMP-2 release profile. The goal of this work was to investigate such factors in order to further the development of injectable scaffolds for bone tissue engineering. A full factorial study was designed to investigate GM amount, gelatin type, and BMP-2 loading phase. These factors are easily varied during the material design and fabrication process, and evidence was available to suggest that each factor would significantly affect BMP-2 release profiles thereby providing precise control over the performance of the constructs (23,24).

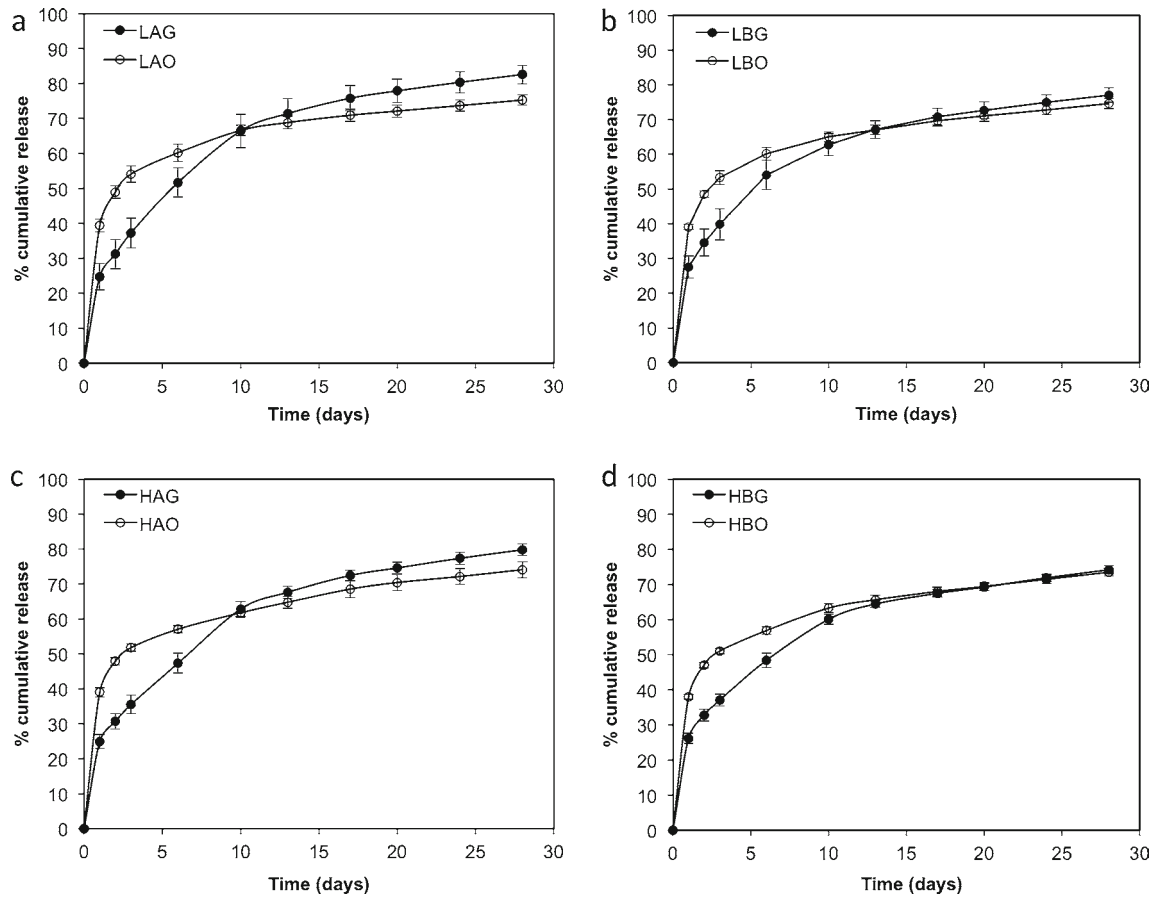

Fig. 4 Percent cumulative release of BMP-2 over 4 weeks measured at 1, 2, 3, 6, 10, 13, 17, 20, 24, and 28 days. Percent cumulative release was determined by normalizing the cumulative amount of BMP-2 released at each time point to the total amount loaded in each sample (determined by taking the sum of release at each time point and the amount of BMP-2 remaining in the composite scaffold after 28 days). To be consistent between panels, filled circles represent GM loading, and open circles represent OPF loading. Error bars represent ± 1 standard deviation.

Table II BMP-2 Release Divided Into 4 Phases, Phase 1 (0–1 Day), Phase 2 (1–3 Days), Phase 3 (3–17 Days), and Phase 4 (17–28 Days)

The table shows the percent release/day for each group in each of the 4 phases as a percentage of the total amount of BMP-2 loaded in each scaffold. Values are shown with ± 1 standard deviation. Significant differences ($p < 0.05$) are designated for gelatin amount (#), gelatin type (^), and loading phase (*)

	Phase 1 (%/day)	Phase 2 (%/day)	Phase 3 (%/day)	Phase 4 (%/day)
GM loading				
LA	24.8 ± 3.8	6.3 ± 0.6	2.8 ± 0.8 ^ *	0.6 ± 0.1 *
LB	27.5 ± 3.2	6.1 ± 0.7	2.2 ± 0.2 *	0.8 ± 0.1 # ^ *
HA	24.9 ± 2.0	5.3 ± 0.5	2.6 ± 0.2 ^ *	0.7 ± 0.1 *
HB	26.2 ± 1.5	5.5 ± 0.4	2.2 ± 0.1 *	0.6 ± 0.0
OPF loading				
LA	39.4 ± 1.8 *	7.4 ± 0.8	1.2 ± 0.3	0.4 ± 0.0
LB	39.1 ± 0.8 *	7.1 ± 0.9	1.2 ± 0.1	0.4 ± 0.0
HA	39.1 ± 7.3 *	6.4 ± 0.3 *	1.2 ± 0.1	0.5 ± 0.1
HB	38.0 ± 0.7 *	6.5 ± 0.1 *	1.2 ± 0.1	0.5 ± 0.0

Previous studies have evaluated the effect of PEG chain length, OPF cross-linking density, and OPF cross-linking time on hydrogel physical properties (15,18,23,25). The inflammatory response and degradation of OPF hydrogels was evaluated when implanted subcutaneously and within cranial defects in the rabbit (25). Furthermore, the effect of PEG chain length on osteogenic differentiation of encapsulated mesenchymal stem cells was measured (26). However, studies have not investigated the release of BMP-2 from OPF hydrogels or the effect of increased gelatin loading or change in gelatin type on the physical properties of OPF-GM composites.

The degradation and swelling behavior of composite hydrogels have previously been shown to directly correlate to TGF-β1 release from the constructs (27). Here, the effect of gelatin type on the degradation and swelling of the composite hydrogels was evaluated. Clearly, acidic GMs accelerated the degradation of composite scaffolds compared to basic GMs, but the effect of gelatin type on degradation was equalized by 6 weeks. After 6 weeks, the only factor affecting composite degradation was the amount of GMs incorporated in the construct, which demonstrated a dose dependent effect (HA, HB > LA, LB > Blank) and would be expected if the cause of accelerated degradation scales with the amount of gelatin incorporated.

Composite scaffolds loaded with acidic GMs initially degraded more quickly due to several potential mechanisms. From previous studies it is known that acidic GMs undergo complete degradation in 9 days when cultured in col-PBS, while basic GMs require 20 days to completely degrade (21). Therefore, substantial degradation of acidic GMs would have occurred within the first week of culture and subsequent diffusion of gelatin fragments from the composite scaffolds into the surrounding media would have contributed to the reduced dry weight of the scaffolds. Furthermore, the enhanced degradation of the acidic *vs.* basic GMs could have led to additional effects. GM degradation and the resulting reduced cross-linking density would have resulted in increased swelling of the GMs, which is known to apply disrupting mechanical forces on the OPF matrix leading to enhanced degradation of the OPF network itself (27). Finally, the introduction of pores into the OPF hydrogel from GM degradation and dissolution would have promoted hydrolysis of the polymer (27). It should be noted that there was no difference in sol fraction between groups, and thus it can be concluded that none of the factors investigated in this experiment affected polymer incorporation into the network since all properties of the polymers were controlled including double bond ratio.

Future studies could evaluate the response of hydrogels to either differences in porogen swelling alone or changes in porosity alone to further elucidate the mechanisms controlling degradation. However these results demonstrate that the degradation is clearly caused by both enzymatic digestion of gelatin and hydrolysis of OPF, and composite degradation can be precisely tuned by varying either GM amount or gelatin type.

Previously, burst release of TGF-β1 was shown to coincide with composite swelling over the first 24 h indicating diffusional control of growth factor release by the bulk OPF

Table III Final Percent Cumulative Release of BMP-2

Values are shown with ± 1 standard deviation. Significant differences ($p < 0.05$) are designated for gelatin type (^) and loading phase (*)

	LA	LB	HA	HB
GM loading				
Final percent cumulative release	82.6 ± 2.7 ^ *	77.0 ± 2.0	79.8 ± 1.7 ^ *	74.2 ± 1.1
OPF loading				
Final percent cumulative release	75.3 ± 1.5	74.6 ± 1.4	74.1 ± 2.3	73.5 ± 1.1

Table IV Analysis of the Type of Transport Mechanism Governing BMP-2 Release from Composite Scaffolds Assuming a Cylindrical Geometry

	n	k	r^2	Transport mechanism
GM loading				
LA	0.41	0.24	0.992	Anomalous
LB	0.37	0.27	0.993	Anomalous
HA	0.36	0.24	0.993	Anomalous
HB	0.34	0.26	0.997	Anomalous
OPF loading				
LA	0.29	0.40	0.996	Anomalous
LB	0.29	0.39	0.995	Anomalous
HA	0.21	0.40	0.964	Anomalous
HB	0.22	0.39	0.968	Anomalous

phase surrounding the dispersed GMs (27). Therefore, equilibrium swelling was expected to be an important parameter affecting growth factor release in this study. Blank samples experienced higher equilibrium swelling compared to all other groups, which was expected as cross-linked OPF has a higher mass swelling ratio than 10 mM cross-linked GMs. Thus, a composite with a higher proportion of OPF would be expected to uptake more water (23).

Acidic GMs accelerated the mass swelling of composite scaffolds compared to basic GMs, and this is in agreement with the degradation profiles for these groups. This provides more evidence for the conclusion that enzymatic degradation of acidic GMs occurs at a faster rate, leading to increased GM swelling and disruption of the OPF network, thus increasing the swelling of the OPF bulk phase. Electrostatic repulsion within hydrogel composites is another likely contributor to increased swelling in the acidic groups. OPF hydrogels undergo ester hydrolysis resulting in the formation of acidic carboxylic acid groups (5). With acidic GMs dispersed throughout the matrix a repulsive force is likely to result between negative charges in the OPF chains and those present on the GM surface leading to increased swelling. The same repulsive forces would not be present in OPF hydrogels loaded with positively charged GMs, and therefore, the gels would swell to a lesser extent.

Similar to the degradation behavior, there was a GM dose dependence on swelling at 6 weeks. Whether the two parameters have a causal relationship is unknown although several possibilities for their interaction have been presented. Nonetheless, both parameters are meaningful to characterize the composites, and in this case the results reinforce one another in their description of the physical behavior of the system.

This study demonstrates an advantage of OPF-GM composite scaffolds in that they are fabricated by combining two materials that have unique degradation and swelling behavior in aqueous solution depending on numerous factors including enzymatic activity, temperature, and mixing. Therefore, degradation and swelling of OPF-GM composite scaffolds can be tuned by varying the mass ratio of the two materials in the composite as well as factors that affect each material separately, e.g., gelatin type.

As expected, the results of mechanical testing were consistent with the degradation and swelling behavior, as the type of gelatin incorporated had a significant impact on the compressive moduli of composite scaffolds compared to the blank samples at the two levels of GM amount that were tested. Previously, effects of microsphere incorporation on mechanical properties of hydrogels have been illustrated where incorporation of rigid, hydrophobic microspheres increased compressive moduli of various hydrogels by acting as reinforcing nodes within the matrix owing to the better mechanical properties of the microspheres compared to the bulk material. This reinforcing effect only held at relatively low microsphere concentrations, as increasing the concentration eventually led to weakening of the mechanical properties, showing the presence of competing effects (28,29). Furthermore, GM incorporation into calcium phosphate cement constructs resulted in a significant weakening of the compressive mechanical properties due to the inferior mechanical properties of the GMs (30). In the present study, altering the mass of GMs incorporated into OPF composites had no effect on the compressive moduli of the constructs likely because the materials have similar mechanical properties. However incorporating acidic GMs weakened the constructs compared to blank OPF hydrogels, and the reason for this effect is based on electrostatic interaction between the GMs and OPF. At physiologic pH, acidic gelatin has a net negative charge, as does OPF due to the formation of carboxylic acid groups during degradation. The result of this like-charge interaction is a destabilization of the interface between the GM surface and OPF that limits the transfer of mechanical load across the construct. On the other hand, basic gelatin has a positive charge at physiologic pH, which acts to stabilize the interface between the two materials and strengthens the composites. The values for the compressive moduli of the OPF hydrogels in Fig. 3 are of the same order of magnitude of OPF hydrogels tested in other studies (31).

In previous work, OPF hydrogels were evaluated for mesenchymal stem cell encapsulation and immobilization of cell binding peptides such as RGD and osteopontin-derived peptide, and these scaffolds were effective for modulating cell binding, proliferation, and differentiation (32,33). Building on this work, it was postulated that incorporation of soluble bioactive agents such as growth factors into the scaffolds would induce a more pronounced tissue response, and in order to control the release of growth

factors, a GM drug delivery vehicle was incorporated. Subsequent studies explored and utilized the phenomenon of electrostatic binding of charged proteins to gelatin for controlled release of TGF-β1 from acidic GMs (23,34). Not only did this composite system enable precise control of local growth factor administration it also enabled the fabrication of scaffolds that become porous in a time-dependent manner through GM degradation. For example, OPF-GM composite scaffolds delivering TGF-β1 supported osteochondral tissue formation in several studies (35,36). Furthermore, studies have investigated the effects of parameters such as gelatin amount and loading phase on TGF-β1 release from OPF-GM composites and GM type on IGF-1 release from GMs alone (23,24). Although some of the same parameters from the present work were investigated in the studies mentioned, these results may not be applicable when a different growth factor with different amino acid sequence, zeta potential, size, and structure is used.

Here, it was observed that the burst release of BMP-2 from the OPF loading phase was significantly higher than burst release from the GM loaded phase. The difference observed between the two loading phases could be due to a higher barrier to release present in the case of GM loading, as the protein must diffuse through the gelatin and undergo desorption from the GM surface, thereby increasing the effective diffusion coefficient of BMP-2 in the construct. BMP-2 release for OPF loading was higher than GM loading only in high GM groups in phase 2 (days 1–3), which could be explained by the higher swelling of HA compared to LA at 1 week, supporting the theory that the effective diffusion coefficient is lower in the case of OPF loading. Although swelling of HB does not differ significantly from that of LB, the same mechanism is expected to apply, and the difference in BMP-2 release between the two types of loading is less for basic groups than for acidic. The effect of changing the loading phase was observed previously; changing TGF-β1 from OPF phase to GM phase reduced the burst release and increased the rate of release during phase 3 in col-PBS (24).

Higher release due to GM loading during phase 3 in the present study was likely caused by complexation of BMP-2 to gelatin of both types. Noncovalent binding of various proteins to GMs is an established phenomenon affected by specific protein, gelatin type, GM cross-linking, and protein dose (21). Growth factor adsorption to cross-linked gelatin is governed by multiple mechanisms including electrostatic, hydrophobic, and hydrogen-bonding interactions. While bFGF and TGF-β1 exhibit pronounced electrostatic bonding to acidic gelatin (34), VEGF and BMP-2 exhibit mainly hydrophobic and/or hydrogen-bonding interactions (34), which are weaker, even though VEGF and BMP-2 have basic isoelectric points. Therefore, in the case of BMP-2, the adsorption mechanism is somewhat nonspecific compared to other proteins such as bFGF, TGF-β1, and IGF-1(37,38). BMP-2 is coated with oligomannose groups that reduce its zeta-potential for electrostatic attraction to negatively charged materials (38). Even so, release of bFGF, TGF-β1, VEGF, and BMP-2 was strongly governed by gelatin hydrogel degradation when implanted into the back subcutis of mice demonstrating diffusional control of release (38). When BMP-2 was loaded into GMs cross-linked using the same chemistry, a much stronger interaction was observed demonstrated by delayed release compared to gelatin hydrogel disks under the same culture conditions (21,39). Gelatin type was established as a factor that significantly affected BMP-2 release kinetics leading to the hypothesis in this work that varying the type of gelatin between acidic and basic would contribute to varying release kinetics from OPF-GM composites (21). During phase 3 of release for GM loading, acidic GMs resulted in higher BMP-2 release compared to basic. This result is likely affected by the binding of BMP-2 to acidic GMs as well as increased swelling and faster degradation of composite scaffolds with acidic GMs since diffusion would be expected to increase with these changes in the scaffold architecture. In phase 4, release from LBG exceeded that of LAG providing further evidence to the effect of delayed swelling and degradation of LBG composites. Differences in final percent cumulative release likely result from accelerated degradation and swelling of samples with acidic GMs as described earlier followed by the concomitant increase in the diffusion of BMP-2 resulting from the changes in scaffold mesh size.

In terms of GM amount, it was expected that higher loading of GMs would result in increased release during at least one phase of the study since this phenomenon was observed for the release of TGF-β1, although the GM amounts tested were lower than in the present study (23), but this effect was not observed even though higher loading enhanced the rate of composite degradation. In fact, the opposite occurred as LBG exceeded HBG in phase 4. The HBG group may exhibit a lower BMP-2 release rate because it contains a higher concentration of GMs, which increases the effective diffusion coefficient of the construct.

The BMP-2 concentration in this work was determined from previous dosage studies. Porous PLGA scaffolds loaded with BMP-2 at a concentration as low as 30 ng/mm^3 resulted in a significant increase in new bone volume in rat calvarial defects (40). When BMP-2 concentrations of 10, 20, or 40 ng/mm^3 were loaded into composites of polypropylene fumarate (PPF) and GMs the critical concentration to produce significantly higher bone volume in rat cranial defects was 40 ng/mm^3 (39). Without prior evidence of the critical concentration of BMP-2 required to produce significant bone within OPF-GM composites, the PPF-GM system was identified as the most similar known system for determining the BMP-2 concentration that should be

applied. Therefore, for this work BMP-2 was loaded at 40 ng/mm^3 final scaffold volume determined at equilibrium swelling of the hydrogel. Since 40 ng/mm^3 was the specific concentration of interest, and previous studies demonstrated that the loading concentration of BMP-2 into 10 mM acidic GMs was not a factor affecting its release, only a single BMP-2 concentration was investigated (21). The OPF-GM system developed in this work achieves control over BMP-2 release to a level of precision that will allow appropriate release kinetics to induce bone formation *in vivo*.

One limitation of this study was that the change in porosity of the hydrogel composites was not directly measured since the task of determining porosity of hydrogels is not readily accomplished in a practical experiment. Much is known about the effect of porosity in tissue engineering scaffolds regarding suitable porosity for cell proliferation, migration, and differentiation, neovascularization, extracellular matrix deposition, and nutrient and oxygen diffusion (41). Parameters of scaffold pore size have been investigated to determine the optimum size for formation of different types of tissue including bone and osteoid but a consensus has not been reached. Even so, it is generally accepted that pore sizes on the order of hundreds of microns are best for bone osteoconduction although *de novo* bone formation readily occurs in pores of smaller size (42,43). In order to generate OPF scaffolds with suitable pore size and pore distribution, enzymatically digestible GMs were incorporated. Particles were sieved to a diameter of 50–100 μm and pre-swollen prior to composite fabrication giving a porogen size conforming to the general parameters established for bone tissue engineering as described above.

Another limitation of this study was not measuring the bioactivity of released BMP-2. It is accepted that organic solvents, high temperatures, and digestive enzymes denature proteins quite readily. A suitable drug delivery vehicle for tissue engineering strategies should ideally help to maintain growth factor bioactivity or at the very least act as an inert carrier. Of course the potential factors affecting loss of activity increase markedly when any scaffold is implanted, but the exact difference in activity is unknown. In this work, loss of BMP-2 activity was not evaluated because previous studies investigating release of BMP-2 from GMs *in vivo* did not exhibit any indication of the loading process inhibiting bioactivity (39). Furthermore, VEGF bioactivity released from GMs has been recorded (44), and several other growth factors have been released from OPF hydrogels without any evidence of the bioactivity being affected (35–37). Specifically, the bioactivity of TGF-β3 and IGF-1 released from OPF-GM composites was determined using *in vitro* cell assays, and the bioactivity of each growth factor was only minimally affected over 28 days of release (45).

Previous research demonstrated release of TGF-β1 from OPF-GM composites that depended on hydrogel mesh size (27). As the hydrogel composites investigated in the present work were degrading within the time span of BMP-2 release, dynamic changes would have been occurring within the hydrogel matrix that would have affected release characteristics. As the OPF matrix degraded, the mesh size would increase, which would lead to an increasing diffusion coefficient with time, and since Fickian diffusion is characterized by a constant diffusion coefficient (46), anomalous diffusion was observed. There was a difference in the value of n resulting from different BMP-2 loading. The values of n for GM loading were closer to 0.44, the value for Fickian diffusion, indicating a less significant variation in release from Fickian diffusion behavior (22). In order to explain this phenomenon, the relative rates of diffusion in OPF, diffusion in gelatin, and degradation of gelatin should be considered. First, diffusion through OPF would be affected to a similar extent by an increasing mesh size over time for either type of loading. For GM loading, diffusion through the gelatin matrix and desorption from the surface of GMs would delay release, and both of these factors would contribute to an increase in the overall effective diffusion coefficient of the construct. Finally, the effect of GM degradation would only be significant nearing the end of the time period over which the Ritger-Peppas model was applied; therefore, enhanced release of BMP-2 from GM degradation would be limited. Based on these observations, additional factors contribute to the overall apparent diffusion coefficient for GM loading compared to OPF loading. Therefore, the weighted impact of the observed increase in hydrogel mesh size would be reduced for GM loading resulting in less variation in the effective diffusion coefficient of these constructs, which provides a system that more closely approaches Fickian diffusion behavior. The diffusional analysis should be considered when comparing OPF-GM composites to other drug delivery systems as it allows a simple comparison of the type of drug delivery that can be achieved.

CONCLUSION

The objective of this work was to investigate factors affecting the physical properties and drug delivery behavior of an injectable, composite hydrogel scaffold. In order to accomplish this goal, several hypotheses were formulated and addressed in detail by implementing a full-factorial experimental design. Of the factors investigated, GM amount and gelatin type had significant implications on the physical properties of the composite scaffolds as indicated by differences in the degradation and swelling behavior and the mechanical compressive properties of the scaffolds. The physical properties of the scaffolds could be fine-tuned in a predictable manner, and the resulting composites exhibited

unique properties that could be leveraged for tissue engineering applications. Furthermore, the effect of changing physical properties of the scaffolds on the release of BMP-2 was assessed. No difference in the burst release of BMP-2 was observed based on physical parameters alone, yet other differences in the overall release profiles were apparent for both factors investigated. Finally, it was assessed whether changing the loading phase of BMP-2 affected its release. The change in loading phase demonstrated a significant effect in every phase of release and enabled tuning of the system regardless of the physical composition. The composite scaffolds designed in this work exhibit numerous advantages for bone augmentation applications in tissue engineering. The ability to tune the physical properties of the scaffolds and the release kinetics of BMP-2 provides an opportunity to begin optimizing the bone augmentation capability of these scaffolds *in vivo*. Additionally, by placing the results in the context of drug delivery mechanisms in general, the results of this research will supplement the expanding knowledge base for drug release from swelling hydrogels and composite systems for bone tissue engineering.

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