

# **Sustained presentation of BMP-2 enhances osteogenic differentiation of human adipose-derived stem cells in gelatin hydrogels**

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## Abstract

Human adipose-derived stem cells (hASCs) show great potential for healing bone defects. Bone morphogenetic protein-2 (BMP-2) has been reported to stimulate their osteogenic differentiation both *in vitro* and *in vivo*. Here, methacrylated gelatin (GelMA) hydrogels were evaluated as a system to deliver BMP-2 to encapsulated hASCs from two different donors, and BMP-2 delivered from the hydrogels was compared to BMP-2 presented exogenously in culture media. GelMA hydrogels were shown to provide sustained, localized presentation of BMP-2 due to electrostatic interactions between the growth factor and biomaterial after an initial burst release. Both donors exhibited similar responses to the loaded and exogenous growth factor; BMP-2 from the hydrogels had a statistically significant effect on hASC osteogenic differentiation compared to exogenous BMP-2. Expression of alkaline phosphatase was accelerated, and cells in hydrogels with loaded BMP-2 deposited more calcium at one, two, and four weeks than cells without BMP-2 or with the growth factor presented in the media. There were no statistically significant differences in calcium content between groups with 25, 50 or 100  $\mu\text{g/mL}$  loaded BMP-2, suggesting that using a lower growth factor dose may be as effective as a higher loading amount in this system. Taken together, these findings suggest that controlled delivery of BMP-2 from the GelMA enhances its osteogenic bioactivity compared to free growth factor presented in the media. Thus, the GelMA system is a promising biomaterial for BMP-2-mediated hASC osteogenesis.

**Keywords:** bone tissue engineering, drug delivery, growth factor, gelatin methacrylate, biomaterials

## Introduction

Human adipose-derived stem cells (hASCs) are multipotent adult stem cells that are easily isolated from adipose tissue and can differentiate down the osteogenic lineage both *in vitro*<sup>1</sup> and *in vivo*.<sup>2</sup> These cells can be obtained in high number from both young and elderly patients,<sup>3</sup> possess immunomodulatory properties,<sup>4</sup> and are highly proliferative *in vitro* allowing for cell expansion<sup>5</sup>. Compared to human bone marrow-derived mesenchymal stem cells (hMSCs), hASCs can more easily be obtained in larger numbers<sup>6</sup> and are more proliferative in culture.<sup>7</sup> Combined with their strong osteogenic capacity, these factors make hASCs a promising cell source for bone tissue engineering strategies.

Bone morphogenetic protein-2 (BMP-2) is a growth factor known for its role in bone tissue formation. With respect to hASCs, exogenous presentation of this growth factor in culture media has also been shown to induce osteogenesis by hASCs both *in vitro*<sup>8-10</sup> and when injected periodically *in vivo* into defects filled with hASC-laden scaffolds.<sup>10</sup> However, delivery of free growth factors, either in cell culture media or to a bone defect site, is problematic as they are typically unstable in physiologic conditions with very short half-lives. When growth factors are injected intravenously their half-life is on the order of minutes,<sup>11,12</sup> and, for BMP-2 specifically, it is on the order of hours in serum-containing culture medium at 37°C *in vitro*.<sup>13</sup> As a result, regular replenishment is required to maintain their bioactivity. Such replenishment is not only inconvenient, but the total amount of protein used in all doses may be cost-prohibitive. While extracellular matrix (ECM) proteins can bind BMP-2,<sup>14</sup> protecting and stabilizing the growth factor, this slows its diffusion, possibly keeping it from cells in the center of a tissue engineered construct.<sup>15</sup> Sustained presentation of the growth factor from a biomaterial directly to encapsulated cells would alleviate these concerns, as the BMP-2 would be available to cells in

the interior of the construct as well as those near its edges, while the biomaterial can serve the role of protecting and stabilizing the growth factor, thus maintaining its bioactivity. Such a strategy may readily be translated to the clinic as a construct loaded with cells and growth factor could be injected or implanted into a patient, and sustained release of the bioactive factor could preclude the need for future growth factor injections.

Methacrylated gelatin (GelMA) is a good candidate material for both cell encapsulation and sustained, localized BMP-2 presentation. A prepolymer solution can be crosslinked into a hydrogel in the presence of UV light under conditions that support viability of encapsulated cells,<sup>16</sup> and the hydrogel can take the shape and size of a mold *in vitro* or defect *in vivo*. Further, since the gelatin is derived from collagen, it contains adhesive peptide sequences that cells can bind to via integrins,<sup>17</sup> meaning that the material does not have to be modified for cell adhesion, and matrix metalloproteinase (MMP)-sensitive degradation sites, so that the material can be degraded by cell-secreted enzymes.<sup>18</sup> An additional important characteristic of GelMA is its ability to form polyionic complexes with growth factors including BMP-2.<sup>19</sup> The isoelectric point of BMP-2 is 8.5, meaning it is positively charged at physiological pH. Gelatin type B, obtained by processing collagen-containing tissues in an alkaline solution, typically has an isoelectric point near 5.0, meaning it is negatively charged at physiological pH.<sup>19</sup> Thus, the BMP-2 can ionically interact with the GelMA, which has been shown to delay its release from gelatin matrices both *in vitro* and *in vivo*.<sup>20</sup> While delivery of growth factors, including BMP-2, from gelatin matrices has been studied extensively, glutaraldehyde<sup>19,20</sup> or genipin<sup>21,22</sup> were often used as crosslinking agents, neither of which enables encapsulation of viable cells. Utilizing the capacity to encapsulate growth factor and cells within photopolymerizing GelMA while maintaining high cell viability, transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) has been incorporated

into these hydrogels with chondrocytes and shown to enhance their proliferation.<sup>23</sup> Recently, BMP-4 release from GelMA microparticles was characterized,<sup>24</sup> but its osteogenic bioactivity was not evaluated, and release profiles of proteins delivered from microparticles are different from those delivered from a bulk hydrogel.<sup>25</sup> BMP-2 release from GelMA/alginate coacervate hydrogels has also been characterized, and the growth factor was shown to be osteogenic to encapsulated human bone marrow-derived mesenchymal stem cells (hMSCs).<sup>26</sup> However, BMP-2 release profiles from bulk GelMA hydrogels have not yet been reported.

Here, BMP-2 presented in a sustained, localized fashion from GelMA hydrogels was compared with exogenously delivered BMP-2 in media for its ability to drive *in vitro* hASC osteogenic differentiation. The exogenously supplied BMP-2 concentrations used, 100 ng/mL and 300 ng/mL, are in the range of what is used in the literature to drive hASC osteogenic differentiation.<sup>5,9,10</sup> BMP-2 amounts loaded directly into the hydrogels were similar to the total amounts of exogenous growth factor delivered over four weeks. Delivery of BMP-2 from the GelMA was shown to support hASC proliferation and differentiation and enhance the osteogenic bioactivity of the growth factor. To our knowledge, this is the first report comparing the osteogenic response of hASCs encapsulated in gelatin-based hydrogels to BMP-2 exogenously presented and to BMP-2 presented in a sustained manner from the hydrogels. This bioactive factor delivery system with encapsulation of a highly clinically relevant stem cell population may have great utility in treating bone defects.

## Methods

### *Gelatin modification*

GelMA was synthesized as previously described.<sup>27</sup> Briefly, gelatin type B (Sigma Aldrich, St. Louis, MO) dissolved in phosphate buffered saline (PBS, Hyclone, Logan, UT) at

10% w/v was heated to 50°C. Methacrylic anhydride (Sigma Aldrich) was added dropwise to the stirring solution at 0.8 mL per gram of gelatin. The reaction continued at 50°C with stirring for one hour. Then, an equal volume of warmed PBS was added to the reaction mixture, and the solution was dialyzed using a membrane with MWCO 12000-14000 Da (Spectrum Laboratories, Rancho Dominguez, CA) against ultrapure deionized water (diH<sub>2</sub>O) at 37°C for seven days, filtered through a 0.22µm membrane, frozen and lyophilized until dry. Modification of lysine residues of the gelatin backbone with methacrylate groups was characterized by <sup>1</sup>H NMR (Varian Inc., Palo Alto, CA) using the quantification method described in Ovsianikov et al.<sup>28</sup> Percent modification was shown to range from 81.0% to 93.6% depending on the batch.

#### *Growth factor release*

Radiolabeled recombinant human BMP-2 (<sup>125</sup>I labeled BMP-2, Perkin Elmer, Waltham, MA) was used to determine BMP-2 release from the GelMA hydrogels. Lyophilized GelMA was dissolved at 10% w/v in DMEM-LG containing 0.25% Irgacure 2959 photoinitiator (Sigma Aldrich). Recombinant human BMP-2 (Department of Developmental Biochemistry, University of Würzburg, Germany) was dissolved at 1µg/mL in a reconstitution buffer of diH<sub>2</sub>O containing 4mM HCl and 0.01% w/v bovine serum albumin (BSA, Fisher Scientific). For groups to be loaded with growth factor, BMP-2 in buffer solution was added to achieve final concentrations of 25 µg/mL, 50 µg/mL or 100 µg/mL. Reconstitution buffer was added to the GelMA solution for groups that received less or no BMP-2 to ensure all groups had the same ratio of GelMA to BMP-2 reconstitution buffer. Each solution also received 20 µL/mL <sup>125</sup>I BMP-2 in PBS, and 20 µL droplets of GelMA/BMP-2/<sup>125</sup>I BMP-2 solution were dispensed onto a glass plate and covered with a quartz plate on 0.75 mm spacers. Samples were irradiated with a UV lamp (Lumen Dynamics, Mississauga, Ontario, Canada) with a 320-500 nm filter at 3.0 mW/cm<sup>2</sup> for 45

seconds to crosslink. Each hydrogel was then placed in a low retention microcentrifuge tube containing 1 mL PBS and kept at 37°C. After two hours, and then again on days 1, 3, 5, 8, 11, 18, 25, 33 and 51, the PBS was removed and replaced with fresh solution. To quantify the amount of BMP-2 in the release samples, 50  $\mu$ L of PBS releasate was mixed with 150  $\mu$ L of Optiphase Hi Safe II liquid scintillation cocktail (Perkin Elmer) and the resulting luminescence was measured on a 1450 MicroBeta Trilux Scintillation Counter (Perkin Elmer). Radiolabeled  $^{125}$ I BMP-2 from the same batch was diluted in PBS and also kept at 37°C and used to create standard curves. Hydrogels were broken down in 1% collagenase type IA (Sigma Aldrich) to confirm the amount of BMP-2 remaining at the end of the release experiment. Release experiments were conducted with N=6 replicates per condition.

#### *Stem cell isolation and expansion*

hASCs were generously provided by the Stem Cell Biology Laboratory at the Pennington Biomedical Research Center (Baton Rouge, LA). Cells were isolated following an Institutional Review Board-approved protocol as previously described.<sup>29</sup> Fresh human subcutaneous lipoaspirate from patients undergoing elective liposuction surgery was processed by digestion in collagenase type IA (Sigma Aldrich), and then density centrifugation was used to isolate the stromal vascular fraction. These cells were plated in tissue culture flasks at 3500 cells/cm<sup>2</sup> in Dulbecco's Modified Eagle's Medium (DMEM)-F12 with 10% fetal bovine serum (FBS; HyClone), 100 U/mL penicillin and 100  $\mu$ g/ml streptomycin (BioWhittaker, Suwanee, GA) and cultured in a humidified 37°C, 5% CO<sub>2</sub> incubator. The adherent cell population was cryopreserved in medium containing 80% FBS, 10% DMEM, and 10% dimethylsulfoxide (DMSO; Sigma-Aldrich).

On thawing, cells were expanded by plating at 3500 cells/cm<sup>2</sup> in DMEM-F12 (HyClone) medium containing 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin (MP Biomedicals, Solon, OH), and 1 ng/mL fibroblast growth factor-2 (FGF-2; generously provided by the NCI BRB Preclinical Repository). After 80–90% confluency was reached, cells were trypsinized and frozen in liquid nitrogen in cryopreservation medium containing 90% FBS and 10% DMSO. Cells were expanded a second time following the procedure described above and used at passage 3 for all experiments. Cells were used from two donors, both females, ages 36 and 44, with body mass indices of 25 and 27.

#### *Hydrogel formation*

10% GelMA solutions were prepared as described earlier, and hASCs were uniformly suspended in this solution at 10 million cells/mL. For sample groups with loaded growth factor, BMP-2 was mixed into the prepolymer solution at the concentrations indicated in Table 1. BMP-2 reconstitution buffer was added to groups that received less or no BMP-2 solution to ensure all groups had the same ratio of GelMA to BMP-2 buffer. Hydrogels were formed by crosslinking the cell- and BMP-2-laden GelMA solutions as described above and then placed in 24-well culture plates and cultured in DMEM-low glucose (Sigma Aldrich) containing 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin, 10 mM β-glycerophosphate disodium salt (EMD Millipore, Billerica, MA) and 120 nM L-ascorbic acid phosphate magnesium salt (Wako Chemicals, Osaka, Japan) in a humidified 37°C, 5% CO<sub>2</sub> incubator. In the conditions receiving exogenous growth factor supplementation, BMP-2 was added to the media at the concentrations presented in Table 1. Control hydrogels were made without cells and cultured under the same conditions to measure any calcification that was not cell-mediated. For all groups, culture medium was replaced every 3 days.



### *Three-dimensional osteogenesis assays*

After one, two or four weeks of culture, hydrogels were placed in 1 mL of Cellytic-M cell lysis buffer (Sigma Aldrich) and homogenized for 60 seconds using an OMNI-TH handheld homogenizer (Omni International, Kennesaw, GA). DNA was quantified with a Picogreen kit (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions using a Synergy H1 Multi-Mode plate reader (BioTek, Winooski, VT) to measure fluorescence. ALP activity was quantified by mixing equal volumes of buffer containing homogenized hydrogels with a p-nitrophenyl phosphate substrate solution (pNPP, Sigma Aldrich), and after 30 minutes at 37° C, measuring conversion of pNPP to 4-nitrophenol by reading absorbance at 405 nm on a VersaMax plate reader (Molecular Devices, Sunnyvale, CA). One unit of ALP is defined as the amount that hydrolyzes 1  $\mu$ mole of pNPP per minute at 37 °C.<sup>30</sup> Calcium content was measured using a cetylpyridinium chloride assay kit (Pointe Scientific, Inc., Canton, MI) according to the manufacturer's instructions, reading absorbance at 575 nm on a VersaMax plate reader. For all assays, there were N=4 samples per condition at each time point.

### *Histology*

After four weeks of culture, samples were embedded in OCT compound (Fisher Scientific) and frozen in liquid nitrogen. Ten  $\mu$ m thick sections were cut using a Microm HM 505E cryostat (Thermo Scientific, Walham, MA), placed on Superfrost Plus microscope slides (Fisher Scientific) and kept frozen until staining. Before Alizarin Red S staining, slides were equilibrated to room temperature, fixed in acetone for 20 minutes and then stained with 2% Alizarin Red S (pH = 4.15, Sigma Aldrich) for 5 minutes. Slides were rinsed with acetone and mounted with Permount mounting medium (Fisher Scientific). Photomicrographs were acquired with an Olympus BX61VS microscope (Olympus, Center Valley, PA) with a Pike F-505 camera

(Allied Vision Technologies, Stadtroda, Germany). N=4 samples from each condition were sectioned and stained, with representative images shown.

### *Statistical analysis*

All quantitative data is expressed as mean  $\pm$  standard deviation. Statistical analysis was performed with one-way analysis of variance (ANOVA) with Tukey honestly significant difference post hoc tests using Minitab software (Minitab Inc., State College, PA). A value of  $p < 0.05$  was considered statistically significant.

## **Results**

### *BMP-2 Release*

All BMP-2 loading concentrations in the GelMA hydrogels examined exhibited similar cumulative release profiles (Figure 1a). An initial burst of growth factor diffused out within the first two hours: 44.2% in the group loaded with 25  $\mu\text{g/mL}$  (load25), 45.7% in the group loaded with 50  $\mu\text{g/mL}$  (load50) and 43.3% of the loaded growth factor in the group loaded with 100  $\mu\text{g/mL}$  (load100). Within the first 24 hours, another 15.1% (load25), 13.0% (load50), and 9.4% (load100) was released (Figure 1c). The release rate then slowed, with between 0.5% and 3.0% of the loaded growth factor released at each time point after 24 hours (Figure 1e) as the release profiles approached plateaus of 64.1-73.0% of total BMP-2 released out to seven weeks (Figure 1a). Hydrogel degradation with collagenase confirmed that 35.9%, 27.0% and 29.4% of the BMP-2 remained in the groups loaded with 100  $\mu\text{g/mL}$ , 50  $\mu\text{g/mL}$  and 25  $\mu\text{g/mL}$ , respectively. While the percentages released were similar across loading groups, the total amount of growth factor released at each time point varied with the amount loaded (Figure 1 b,d,f) as expected.

### *Cell viability and proliferation*

Cells in hydrogels from all groups showed clear proliferation over time, but there were no statistically significant differences in DNA content between groups at any time point for both donors (Figure 2 a-b). Similarly, when the data from the two donors was pooled, there remained no significant differences in average DNA content between groups at any time point, further supporting the trends seen (Figure S1a). H&E staining supports the DNA data, also suggesting similar levels of cellularity of the constructs in all groups after 4 weeks (Figure S2).

### *Osteogenic Differentiation*

There were statistically significant differences in ALP activity (Figure 2 c-d), as well as ALP activity normalized to DNA content (Figure 2 e-f), between hydrogels cultured with exogenous or loaded BMP-2, for both donors. Since there were no statistically significant differences in DNA content, relative differences between groups were similar for both ALP and ALP/DNA. For conditions with no BMP-2 (control) or exogenous BMP-2 (exo100 and exo300), ALP activity increased from one to two weeks, and then decreased from two to four weeks. For all conditions with loaded BMP-2 (load25, load50 and load100), except for donor A load25, ALP activity decreased steadily from one to two to four weeks. After one week in culture, cells from donor B cultured with 300 ng/mL exogenous BMP-2 (exo300) showed statistically significant increases in ALP and ALP/DNA compared to cells cultured without BMP-2 (control). Donor A showed the same statistically significant increases in ALP/DNA after two and four weeks in culture, and increases in ALP activity for both 100 ng/mL (exo100) and 300 ng/mL (exo300) exogenous BMP-2 compared to the control group at these time points. However, at the early one week time point the magnitude of increase in ALP activity compared to control was greater for cells in hydrogels with loaded BMP-2. For donor A, conditions with 50 and 100 µg/mL loaded BMP-2 (load50 and load100) showed significantly higher ALP activity and

ALP/DNA after one week in culture than any of the control or exogenous BMP-2 groups (control, exo100 and exo300). For donor B, at the same one week time point all of the conditions with loaded BMP-2 (load25, load50, load100) showed significantly higher ALP and ALP/DNA than the control group or the cells in hydrogels cultured with 100 ng/mL exogenous BMP-2 (exo100). For both donors, these differences were much less dramatic after two and four weeks, with donor B showing no significant differences in ALP or ALP/DNA between any groups at four weeks. Pooling the donor data showed similar trends. Groups load50 and load100 exhibited significantly higher ALP and ALP/DNA activity after one week compared to control, and then by two and four weeks, lower ALP and ALP/DNA activity compared to hydrogels with 300 ng/mL of exogenous BMP-2 (exo300), but this latter trend was not statistically significant (Figure S1b and c).

Hydrogels in all groups from both donors showed increasing mineralization over time (Figure 3). In contrast, hydrogels without cells incubated in DMEM-LG for the same amount of time achieved an average of only  $3.8 \pm 0.8$   $\mu\text{g}$  calcium per sample at one week,  $4.6 \pm 0.6$   $\mu\text{g}$  at two weeks, and  $5.9 \pm 1.1$   $\mu\text{g}$  at four weeks, one to two orders of magnitude lower than the cell-laden hydrogels. At the early one week time point, for both donors the conditions with 50 and 100  $\mu\text{g/mL}$  loaded BMP-2 (load50 and load100) had significantly higher calcium content than the control group and the cells in hydrogels cultured with 100 ng/mL exogenous BMP-2 (exo100). This significant difference held at two and four weeks for donor B, while for donor A only the 100  $\mu\text{g/mL}$  loaded BMP-2 condition (load100) was significantly higher than control and exogenous BMP-2 (control, exo100, and exo300) after four weeks. After four weeks the differences between the control group and the 100  $\mu\text{g/mL}$  loaded BMP-2 group (load100) were large, with 61.3  $\mu\text{g}$  compared to 100.0  $\mu\text{g}$  calcium for donor A and 61.8  $\mu\text{g}$  compared to 124.3  $\mu\text{g}$

calcium for donor B. These trends were again consistent when the donor data was pooled. After one, two or four weeks, the largest amount of calcium was detected in the load100 group, which was significantly higher than the control, exo100 and exo300 (Figure S1d).

When normalized to DNA, hydrogels with loaded BMP-2 continued to show the highest calcium content (Figure 3 c,d). After two and four weeks, the normalized calcium content of hydrogels with cells from donor B and loaded with 50 $\mu$ g/mL or 100 $\mu$ g/mL was significantly higher than the normalized calcium content of the control hydrogels or those with exogenously presented BMP-2 (control, exo100, exo300). For donor A, the trends were similar, with significantly higher normalized calcium content of in groups load50 and load100 compared to control and exo100 after four weeks. Analogous results were seen when the normalized calcium data was pooled across both donors: at all time points hydrogels with loaded BMP-2 had higher average normalized calcium content than those with no or exogenously presented BMP-2 (Figure S1e). These differences were statistically significant after four weeks for hydrogels with the highest amount of loaded BMP-2 (load100), compared to control hydrogels or those with exogenously presented BMP-2. For hydrogels loaded with 50  $\mu$ g/mL BMP-2 (load50), normalized calcium content was higher than either control hydrogels or those cultured with 100 ng/mL exogenous BMP-2 (exo100) at four weeks.

For both donors at all time points, there were no statistically significant differences in calcium content between the groups with BMP-2 loading (load25, load50 and load100), although in all cases load100, which contained the highest concentration of loaded BMP-2, had the highest calcium content. Further, when the donor data were pooled, there was no significant difference between load25, load50 and load100 within each time point, except for load25 being lower than load100 after four weeks (Figure S1d). Alizarin Red S staining for calcium corroborated these

findings (Figure 4). After four weeks, all groups showed visible calcification nodules, indicating bone-like mineralization. Load100, which had significantly more quantified calcium content than the control and exogenous BMP-2 groups at four weeks, also showed the strongest Alizarin Red S staining for both donors.

## Discussion

In this work, GelMA hydrogels were used to control BMP-2 presentation to encapsulated hASCs, with the goal of enhancing hASC osteogenic differentiation compared to exogenous delivery of the growth factor in the media. *In vitro* release curves show that GelMA hydrogels loaded with 25-100  $\mu\text{g/mL}$  of BMP-2 retained growth factor for seven weeks or longer, providing sustained, localized presentation of the growth factor. The significant burst of growth factor released from the GelMA system after two hours shows that diffusion dominates the initial release kinetics. However, electrostatic interactions between the growth factor and locally charged regions of the GelMA were sufficient to delay release, retaining the BMP-2 within the hydrogel. While groups with different loading amounts released different absolute amounts of BMP-2, their release kinetics as a percentage of total loaded protein were similar, suggesting that for these concentrations of BMP-2, the number of charged sites on the GelMA far exceeds the number of BMP-2 molecules that can interact with them. The GelMA hydrogel system also offers several simple ways to tune BMP-2 release kinetics, such as changing the concentration or methacrylation level of GelMA to affect the crosslinking density and pore size of the hydrogel. Increasing GelMA concentration or methacrylation level may prolong hydrogel degradation and slow BMP-2 diffusion during the burst phase, while decreasing them may accelerate hydrogel degradation and lead to more BMP-2 released at earlier time points.<sup>31</sup> Alternatively,

incorporating methacrylated heparin into the hydrogels may endow them with stronger affinity interactions with the BMP-2 and further delay its release.<sup>32</sup>

The sustained, localized BMP-2 presentation from the GelMA hydrogels led to a statistically significant improvement in hASC osteogenesis in both donors studied. The improvement was seen compared to cells in hydrogels cultured both in the absence of BMP-2 and with exogenously supplemented BMP-2. This was clear from the one week ALP activity, which typically increases during the first 1-2 weeks of osteogenic differentiation before decreasing as later stage mineralization occurs.<sup>33</sup> Groups with loaded BMP-2 had the highest ALP activity at one week, and then exhibited a steady drop to two and then to four weeks, indicating that ALP activity reached its peak early. In contrast, groups with no BMP-2 or exogenous BMP-2 showed an increase in ALP activity from one to two weeks, before the protein expression decreased at four weeks. This suggests that the sustained, localized BMP-2 presentation from GelMA hydrogels may have accelerated osteogenic differentiation in this system. This is further supported by the calcium content data, as groups with loaded BMP-2 had higher calcium content even at early time points, suggesting that mineralization was also accelerated. Since this enhanced calcium content was maintained at later time points, BMP-2 loading was shown to lead to more overall mineralization.

It is surprising that exogenous BMP-2 had no effect on hASC mineralization in this work. The ability of loaded BMP-2 from the same batch to drive hASC osteogenic differentiation, and, in other work from our lab, to drive hMSC osteogenesis,<sup>26,34</sup> demonstrates the bioactivity of the growth factor used. Since exogenous BMP-2 presentation did lead to increases in hASC ALP activity at two or four week time points compared to hASCs cultured without BMP-2, it is possible that mineralization would have been increased compared to the control group after a

longer culture period. Additionally, while many published studies suggest that exogenously presented BMP-2 does drive hASC osteogenic differentiation,<sup>8-10,35,36</sup> there are other reports that contradict this finding.<sup>37-39</sup> The data presented here suggest that BMP-2 stability in the culture conditions used in each experiment may play a key role in moderating its effect. For example, more frequently changing culture medium to replace the degraded growth factor or culturing in a defined medium without the addition of serum to decrease the presence of enzymes that may break down BMP-2 could enhance the osteogenic bioactivity of this growth factor.

Notably, despite the four-fold increase in loaded BMP-2 concentration from load25 to load100, there were no statistically significant differences in calcium content at any time point between any of the groups with loaded BMP-2. This suggests that for the range studied, the effects of BMP-2 may not be concentration-dependent, and it may be possible to use the lower amounts of growth factor. Over four weeks of culture, the amount of growth factor used in the culture media with 100 ng/mL of exogenous BMP-2 is approximately equivalent to the amount loaded in a hydrogel with 25 µg/mL BMP-2. Therefore, while 300 ng/mL of exogenous BMP-2 did not lead to enhanced osteogenic differentiation compared to the control group, smaller amounts loaded in the hydrogels (load50) did show significant increases in ALP activity and/or calcium content compared to both the control and groups with exogenous BMP-2 presentation. Future work could examine the response of multiple donors to a wider range of BMP-2 loading amounts to identify a range where concentration dependence may occur, or a minimum threshold necessary to stimulate osteogenesis.

BMP-2 that is electrostatically immobilized in the GelMA may exhibit enhanced bioactivity because it mimics *in vivo* growth factor sequestration by affinity interactions in the ECM.<sup>40</sup> Here, scaffold may protect the BMP-2 from degradation prior to its being ultimately



released and acting as soluble growth factor. BMP-2 has exhibited instability in serum containing culture medium. For instance, when BMP-2 (100ng/mL) was placed in DMEM with 5% FBS, after only two hours 50% of the growth factor could not be detected by an ELISA assay, indicating enzymatic degradation or inactivation by BMP inhibitor molecules such as noggin.<sup>13</sup> This could also provide a potential mechanism for improved BMP-2 osteogenic effects when delivered from a GelMA hydrogel that can shield the growth factor. This additional benefit to the scaffold-based delivery is especially valuable *in vivo*, where biomaterial scaffolds can protect a loaded growth factor from enzymes in the body, preserving its bioactivity until it is presented to cells.<sup>41</sup> Future work could examine the bioactivity of covalently bound BMP-2 to the GelMA hydrogel, and compare it to the BMP-2 sequestered in the hydrogel by affinity interactions in this work. Such tethering in other systems has been reported to increase its bioactivity compared to the same amount of free BMP-2; the growth factor is not internalized and instead can continue to activate its receptor.<sup>42,43</sup>

Importantly, in addition to the increases in osteogenic differentiation seen by encapsulated cells in this system *in vitro*, sustained, localized BMP-2 presentation from the GelMA hydrogel can also deliver this growth factor to host cells. Therefore, the growth factor can act not only on the incorporated hASCs, but also on osteoprogenitor cells that are present at or recruited to the site of a bone defect during the normal healing process.<sup>44</sup> These cells are known to be BMP-2 responsive,<sup>45</sup> and delivery of BMP-2 without transplantation of cells has shown promise in a variety of systems by targeting host cells for repairing *in vivo* bone defects.<sup>20,32,46</sup> However, when a mineral-coated poly(lactic-co-glycolic acid) scaffold delivering BMP-2 was compared to a scaffold seeded with hASCs for its ability to repair mouse critical size

cranial defects, the scaffold with hASCs performed dramatically better, demonstrating the benefits of cell transplantation in addition to growth factor delivery *in vivo*.<sup>10</sup>

While not explored here, the modularity of this GelMA system allows for exploration of the role of a number of interesting microenvironmental variables, in addition to growth factor delivery, on osteogenesis. For example, it is possible to examine the combined signaling interactions hydrogel stiffness, a variable that has been extensively shown to affect cell behaviors including osteogenic differentiation,<sup>47,48</sup> and growth factors effects on cell function examined here. The hydrogels used here have a shear storage modulus,  $G' \approx 20$  kPa (measured without encapsulated cells, data not shown), which is in a range of stiffnesses shown previously to be conducive to osteogenesis of encapsulated stem cells or preosteoblasts.<sup>49</sup> However, GelMA offers a number of simple ways to tune hydrogel stiffness as well as nanoscale porosity independent of changing gelatin density, and thereby insoluble biochemical signals such as adhesion ligand density. This can be accomplished, for example, by varying the concentration of photoinitiator,<sup>50</sup> or changing the level of gelatin methacrylation<sup>27,50-51</sup> to achieve a range of stiffnesses from less than 1 to greater than 30 kPa.<sup>50</sup> Further, the addition of sacrificial porogens<sup>52,53</sup> could allow for investigation of the impact of changes in hydrogel porosity along with growth factor presentation on osteogenesis. Because these hydrogel modifications may also change BMP-2 release rate, future work could also use the method described earlier, incorporating methacrylated heparin to limit the growth factor burst release independent of hydrogel stiffness or porosity, to study the potential interacting effects of substrate stiffness and BMP-2 release kinetics on hASC osteogenesis.

## Conclusions

In this work, GelMA hydrogels were shown to provide sustained, localized presentation of BMP-2 loaded at concentrations of 25 – 100  $\mu\text{g/mL}$ ; 27.0% - 35.9% of loaded growth factor was retained in the hydrogels due to electrostatic interactions for at least seven weeks. This loaded BMP-2 was shown to have a statistically significant effect on osteogenic differentiation of encapsulated hASCs, as compared to cells cultured in the absence of growth factor or with exogenous BMP-2. ALP activity was accelerated and there were significant increases in calcium content after one, two and four weeks of culture in the growth factor loaded groups compared with the control group or groups with BMP-2 presented exogenously, with little concentration dependence shown. This suggests that the GelMA system is protecting or enhancing the bioactivity of BMP-2 as compared to growth factor delivered in media. This system for sustained BMP-2 presentation also has the potential to deliver the growth factor to host osteoprogenitor cells in *in vivo* bone defects, further increasing its utility for bone tissue engineering.

**Disclosure Statement**

The author(s) declare that they have no competing interests

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**Table 1.** BMP-2 presentation experimental conditions

| Group   | Exogenous BMP-2<br>(ng BMP / mL culture media) | Loaded BMP-2<br>( $\mu\text{g BMP / mL GelMA}$ ) |
|---------|--|--|
| Control | None   | None   |
| Exo100  | 100  | None   |

|         |      |      |
|---------|------|------|
| Exo300  | 300  | None |
| Load25  | None | 25   |
| Load50  | None | 50   |
| Load100 | None | 100  |

### Figure Captions

Figure 1. Cumulative release of BMP-2 as a (a) percentage or (b) amount from GelMA hydrogels loaded with 100  $\mu\text{g/mL}$  (2  $\mu\text{g/hydrogel}$ ), 50  $\mu\text{g/mL}$  (1  $\mu\text{g/hydrogel}$ ) or 25  $\mu\text{g/mL}$  (0.5  $\mu\text{g/hydrogel}$ ). Release at each time point as a (c) percentage or (d) amount, and an inset showing release after two days as a (e) percentage or (f) amount.

Figure 2. (a,b) DNA content, (c,d) ALP activity and (e,f) ALP normalized to DNA for hydrogels from Donors (a,c,e) A and (b,d,f) B at 1, 2 and 4 weeks. Within each time point, individual comparisons are significant at  $p < 0.05$  for groups that do not share a letter.

Figure 3. (a) Calcium content and (b) calcium content normalized to DNA for hydrogels from Donors A and B at 1, 2 and 4 weeks. Within each time point, individual comparisons are significant at  $p < 0.05$  for groups that do not share a letter.

Figure 4. Alizarin Red S staining of hydrogel sections on day zero or after 4 weeks. Scale bars indicate 200  $\mu\text{m}$ .

Figure S1. (a) DNA content, (b) ALP activity, c) ALP normalized to DNA, (d) calcium content and (e) calcium content normalized to DNA, pooled across both donors at 1, 2, and 4 weeks. Within each time point, individual comparisons are significant at  $p < 0.05$  for groups that do not share a letter.

Figure S2. Hematoxylin and eosin staining of hydrogel sections after 4 weeks. Scale bars indicate 200  $\mu\text{m}$ .

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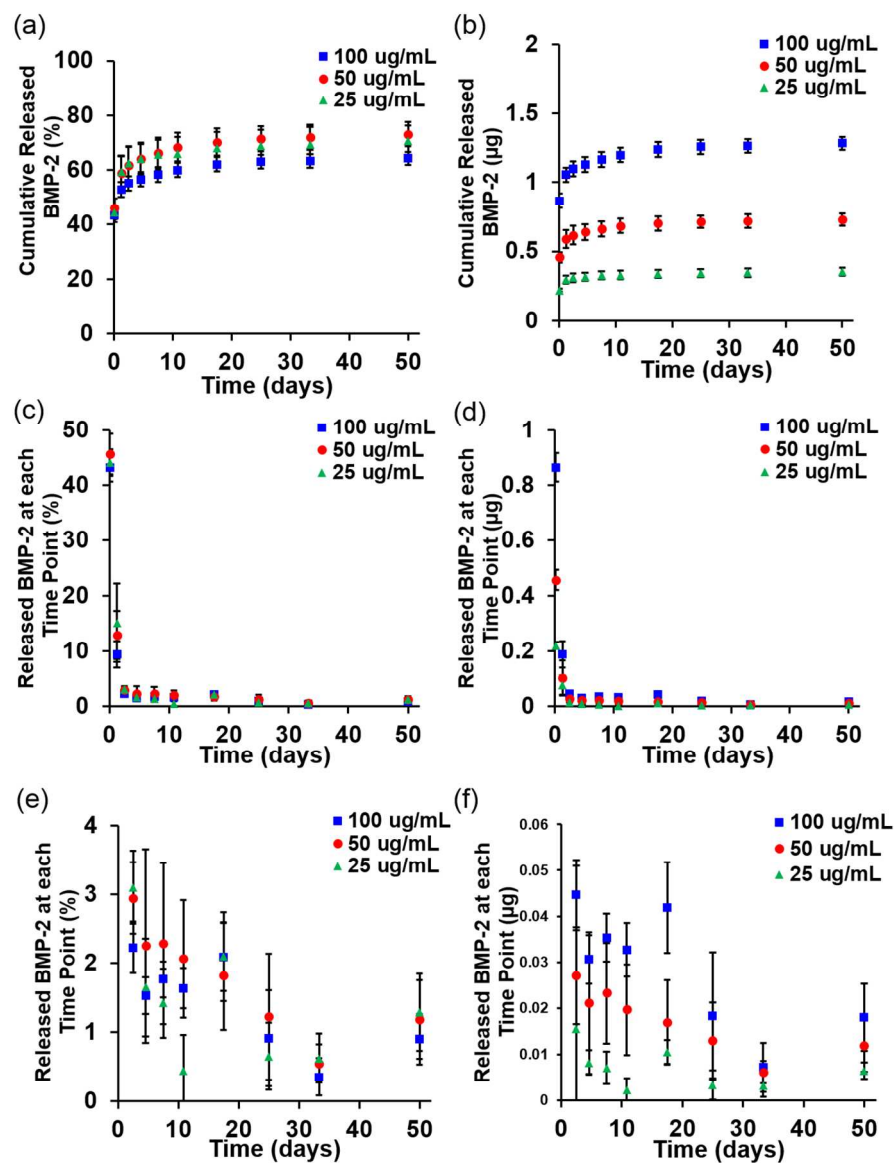
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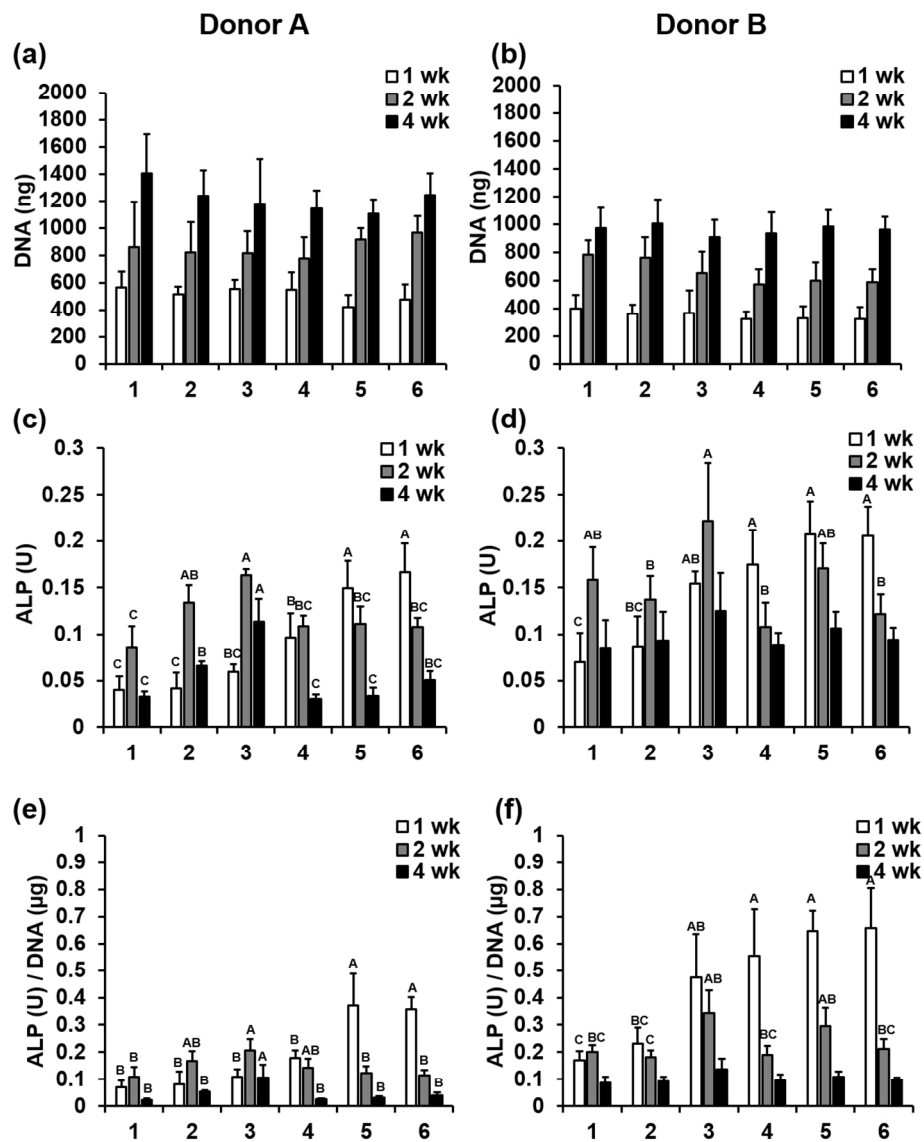
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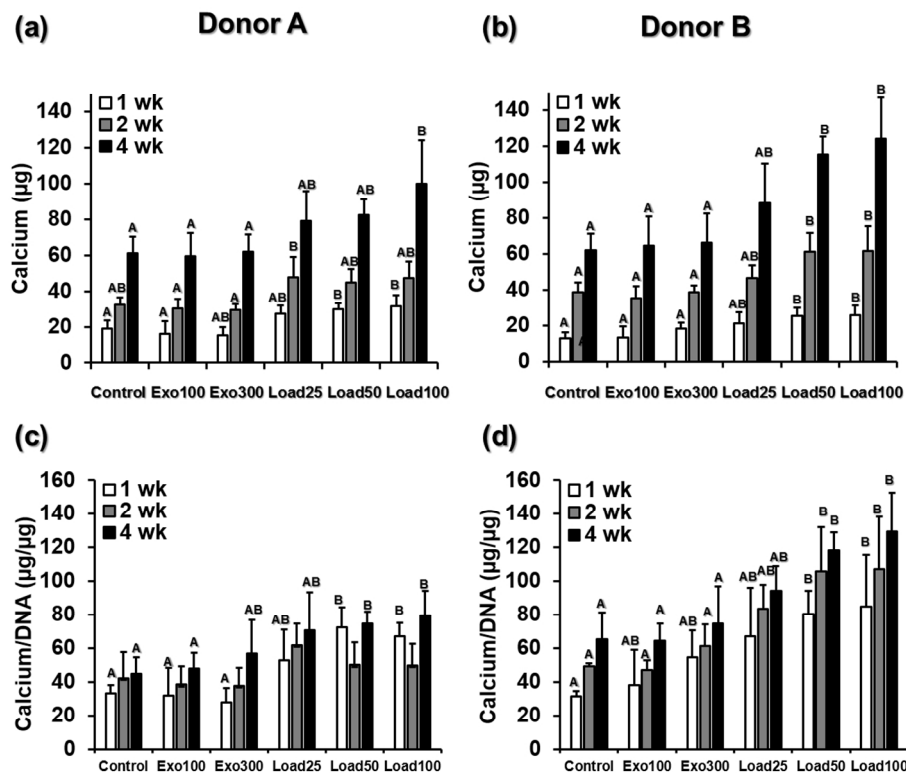
Cumulative release of BMP-2 as a (a) percentage or (b) amount from GelMA hydrogels loaded with 100  $\mu\text{g/mL}$  (2  $\mu\text{g/hydrogel}$ ), 50  $\mu\text{g/mL}$  (1  $\mu\text{g/hydrogel}$ ) or 25  $\mu\text{g/mL}$  (0.5  $\mu\text{g/hydrogel}$ ). Release at each time point as a (c) percentage or (d) amount, and an inset showing release after two days as a (e) percentage or (f) amount.

231x283mm (150 x 150 DPI)



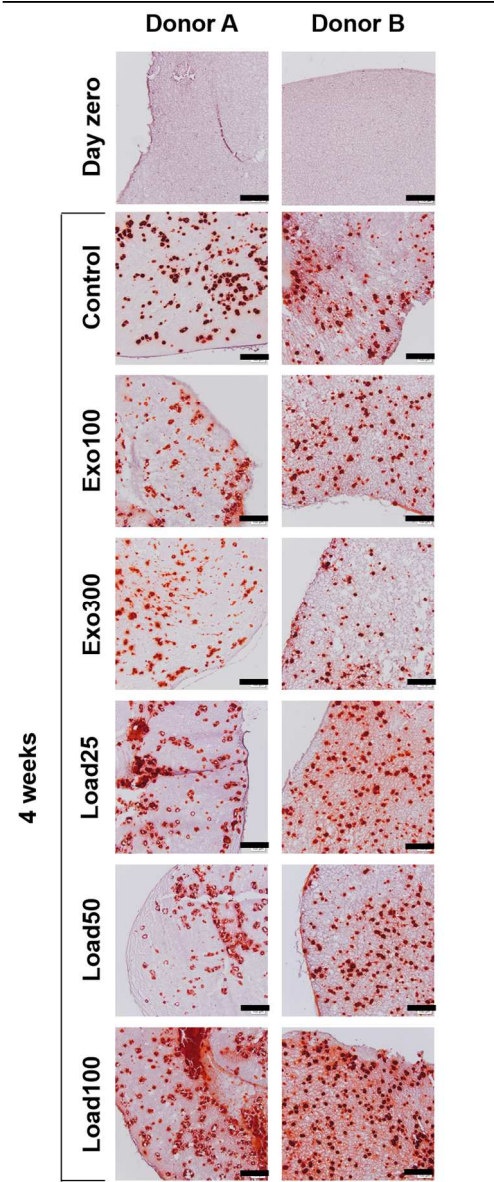
(a,b) DNA content, (c,d) ALP activity and (e,f) ALP normalized to DNA for hydrogels from Donors (a,c,e) A and (b,d,f) B at 1, 2 and 4 weeks. Within each time point, individual comparisons are significant at  $p < 0.05$  for groups that do not share a letter. (b,d,f) B at 1, 2 and 4 weeks. Individual comparisons within time points are significant at  $p < 0.05$  for groups that do not share a letter.

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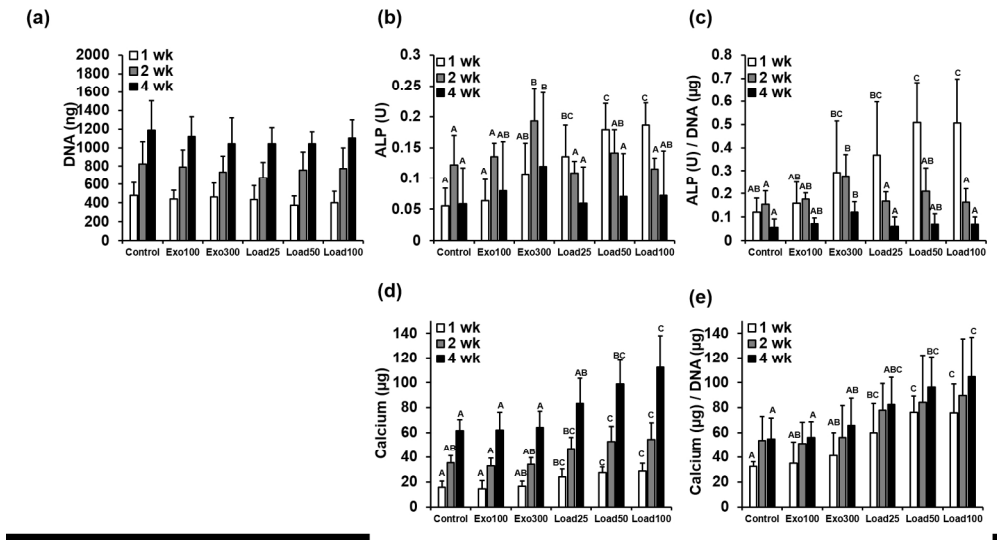


(a) Calcium content and (b) calcium content normalized to DNA for hydrogels from Donors A and B at 1, 2 and 4 weeks. Within each time point, individual comparisons are significant at  $p < 0.05$  for groups that do not share a letter.  
225x187mm (150 x 150 DPI)

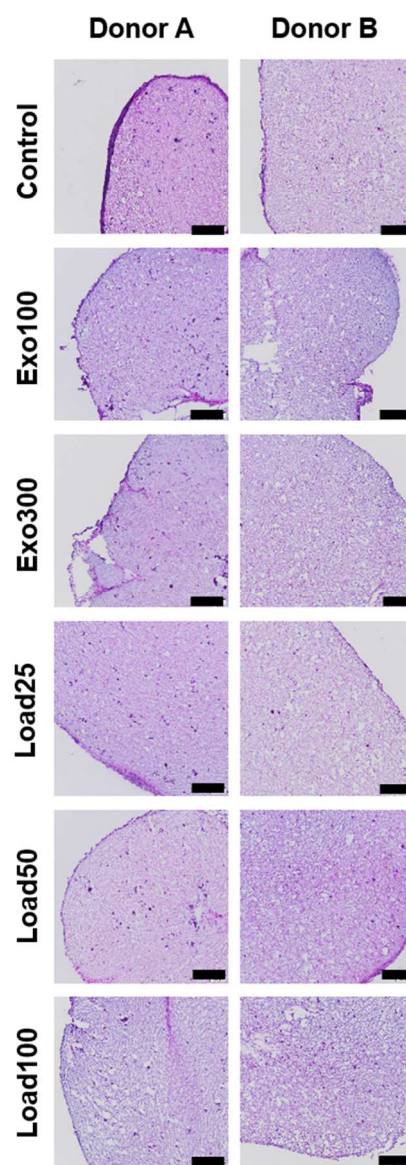
Accel



Alizarin Red S staining of hydrogel sections on day zero or after 4 weeks. Scale bars indicate 200 μm. 131x319mm (150 x 150 DPI)



(a) DNA content, (b) ALP activity, (c) ALP normalized to DNA, (d) calcium content and (e) calcium content normalized to DNA, pooled across both donors at 1, 2, and 4 weeks. Within each time point, individual comparisons are significant at  $p < 0.05$  for groups that do not share a letter.  
326x177mm (150 x 150 DPI)



Hematoxylin and eosin staining of hydrogel sections after 4 weeks. Scale bars indicate 200  $\mu\text{m}$  81x191mm (150 x 150 DPI)