Supplementary Material

Supplementary Methods

Synthesis of BMP-2–loaded PLGA particles

Aqueous solutions of 1% and 0.3% (w/v) poly(vinyl alcohol) were prepared by overnight stirring at 90 °C to permit complete dissolution. The former constituted the external aqueous phase, while the latter was combined with an equal volume of 2% (v/v) aqueous isopropanol to form the particle hardening solution. 500 mg of acid-terminated PLGA (Resomer® RG 503H, Evonik Industries; Birmingham, AL) with a 50:50 lactide:glycolide monomer ratio and an M_n of 26.3 kDa (M_w of 39.5 kDa) measured by APC (averaged values for three injections) with reference to polystyrene standards was dissolved in dichloromethane to form a 400 mg/mL solution as the oil phase. 248 µg of lyophilized BMP-2 (PeproTech®; Cranbury, NJ) were reconstituted in 120 µL of Milli-Q water to form the internal aqueous phase. For the first emulsion, this internal aqueous phase was emulsified with 1.25 mL of the oil phase using probe ultrasonication (Qsonica Q125, Qsonica LLC; Newtown, CT) for 60 sec at 50% amplitude, to form an emulsion containing 0.5 µg of BMP-2 per mg of PLGA. 2 mL of external aqueous phase were then added to this emulsion, and the second emulsion was prepared through 30 sec of probe ultrasonication at 50% amplitude. This double emulsion was then immediately poured into the hardening solution, and the dichloromethane was allowed to evaporate for 2 hr. The resulting particles were subsequently washed twice with 50 mL of Milli-Q water via centrifugation at 10,000 x g for 30 min at 4 °C. The washed particles were filtered using a 40 µm cell strainer (Fisher Scientific; Waltham, MA), flash-frozen in liquid nitrogen, and lyophilized. The final mass of particles resulting from this

procedure was used to determine the theoretical loading of growth factor in the particles. The same procedure was also employed to synthesize unloaded PLGA particles by using an internal aqueous phase of only Milli-Q water.

Degradation of 3D printed fibers in aqueous medium

Fibers printed with or without unloaded PLGA particles were separated into n = 3 samples and sterilized by ethylene oxide gas (Anprolene AN74i gas sterilizer, Andersen Sterilizers; Haw River, NC). The fibers were then immersed in a HEPES buffer solution (150 mg fibers per mL HEPES solution) consisting of 10 mM HEPES acid and 10 mM HEPES-sodium salt (Sigma-Aldrich Corporation; St. Louis, MO) adjusted to a 1.1:0.9 acid:salt volume ratio to reach physiologic pH (7.4). Unloaded PLGA particles (3 mg per mL buffer) were similarly prepared as a control. The samples were then placed under mild agitation at 37 °C. At 1, 4, 7, 10, 14, 18, 21, 24, and 28 days, particles and fiber samples were centrifuged at 1600 x g for 5 min or 400 x g for 2 min, respectively, to enable maximum removal of the buffer solution. Subsequently, the supernatant was removed, its pH was measured using an Accumet AP110 pH/ORP Meter (Fisher Scientific; Waltham, MA), and fresh buffer solution was added to each tube. After the final timepoint (28 days), the samples were rinsed three times with Milli-Q water and air-dried at 37 °C for 24 hr, followed by vacuum-drying for 48 hr to remove any remaining liquid. The final dry mass was measured and subtracted from the initial sample mass to determine mass loss during degradation. Ten fibers for each printing temperature were then selected spatially at random for optical imaging to determine their post-degradation diameters, which were manually quantified using ImageJ.

W-20-17 cellular bioactivity assay

BMP-2 stock stored with 0.1% bovine serum albumin in phosphate-buffered saline at -80 °C was thawed to serve as growth factor bioactivity controls of known concentration (0, 10, 25, 50, and 100 ng/mL). These control samples were prepared with culture medium (DMEM, 22 mM sodium bicarbonate, 10 mM HEPES, 10% (v/v) FBS, and 1% (v/v) penicillin-streptomycin) for consistency with release samples, and frozen at -80 °C. W-20-17 mouse bone marrow stromal cells (passage 2; ATCC; Manassas, VA), which display upregulation of alkaline phosphatase (ALP) expression upon exposure to bioactive BMP-2 in a dose-dependent manner, were thawed and allowed to expand for three days. The cells were then re-plated in 96-well plates at a density of 10,000 cells/well, while four samples of cells were frozen in Milli-Q water at -20 °C to serve as DNA assay controls. After allowing cells to adhere for one day, prepared control samples of known BMP-2 concentration were thawed, and 200 µL of each were introduced to cell-containing wells. Release supernatants were also thawed and diluted 100-fold in culture medium prior to introducing 200 µL to cell-containing wells, as cytotoxicity was observed at lower (5-fold and 10-fold) dilutions of the release supernatants (Supplementary Fig. S4). After three days of exposure to these growth factor-containing samples, the cells were harvested for analysis of their ALP and DNA content by removing the media, performing a wash with 200 µL of phosphate-buffered saline, and adding 250 µL of Milli-Q water prior to freezing the well plates at -80 °C.

ALP activity assay

Harvested cells were lysed through three cycles of freeze-thawing in liquid nitrogen, and the lysed samples were analyzed for ALP activity using a colorimetric ALP assay[47]. All

reagents for the assay were purchased from Sigma-Aldrich Corporation (St. Louis, MO). Briefly, 80 µL of each cell lysate were added to wells of a transparent 96-well plate in duplicate. For the 100 ng/mL BMP-2 control sample, the lysate volume was first diluted four times in Milli-Q water to ensure the measured absorbance signal would not exceed detectable limits. Subsequently, 20 µL of 1.5 M alkaline buffer solution and 100 µL of substrate solution (prepared by dissolving phosphatase substrate tablets in alkaline buffer solution) were added to each well. The mixtures were then incubated at 37 °C for 1 hr, after which 100 µL of 0.3 M NaOH stop solution were added to each well. Absorbance at 405 nm was measured by a microplate spectrophotometer (PowerWave X340, BioTek Instruments; Winooski, VT), and these readings were analyzed relative to a standard curve of known quantities (ranging from 0 to 25 nmol) of the enzymatic reaction product, 4-nitrophenol (4-NP), upon subtracting the mean absorbance value of the blank standard.

DNA assay

Each of the cell lysate samples was also characterized for DNA content, in order to normalize the measured ALP activity values by the cellular DNA content. Each well of an opaque black 96-well plate was filled with 10 µL of cell lysate (from the bioactivity study or from the stored cell controls) in duplicate or with 10 µL of a DNA standard from an Invitrogen Quant-iT^{TM} 1X dsDNA HS Assay Kit (Thermo Fisher Scientific; Waltham, MA) in duplicate, followed by the addition of 200 µL of working solution from the kit. The plate was then placed in an SpectraMax M2 microplate reader (Molecular Devices; San Jose, CA), and plate mixing was performed for 10 sec followed by 2 min of incubation at room temperature. Subsequently, fluorescence signal was measured using excitation

and emission wavelengths of 480 and 530 nm, respectively. The DNA concentration and number of cells present in the experimental cell lysates were determined by comparing the measured fluorescence intensities to the DNA standard curve with its y-intercept set to zero, after subtracting the mean fluorescence signal of the blank standard. Cell number was determined from DNA measurements for samples of known cell concentration.

Calculation of normalized ALP activity and bioactive BMP-2 concentration

Normalized ALP activity was calculated by dividing the 4-NP concentration of each cell lysate by its DNA content. A calibration curve was then constructed for the average normalized ALP activity generated by the known concentrations of BMP-2 (0, 10, 25, 50, and 100 ng/mL) introduced to W-20-17 cells. A sigmoidal curve was chosen in order to reflect the monotone increasing relationship between the variables, and to account for the detection limit of the biological assay, below which the presence of bioactive BMP-2 was not statistically distinguishable from the absence of bioactive BMP-2. To enable fitting of a sigmoidal curve to these data, which display a skewed distribution prior to mathematical transformation, logarithmic transformation was performed on BMP-2 concentration (the independent variable) to yield a normal (symmetric) distribution of the data. A horizontal translation was also enacted by adding a constant within the logarithmic argument, to permit logarithmic transformation of the value corresponding to zero BMP-2 concentration. Four-parameter logistic regression of the calibration curve (Eqn. 1) was then employed in GraphPad Prism (version 8.0.2, Graphpad Software; San Diego, CA) to yield an equation for the fitted sigmoidal curve (Supplementary Fig. S2).

Equation 1:

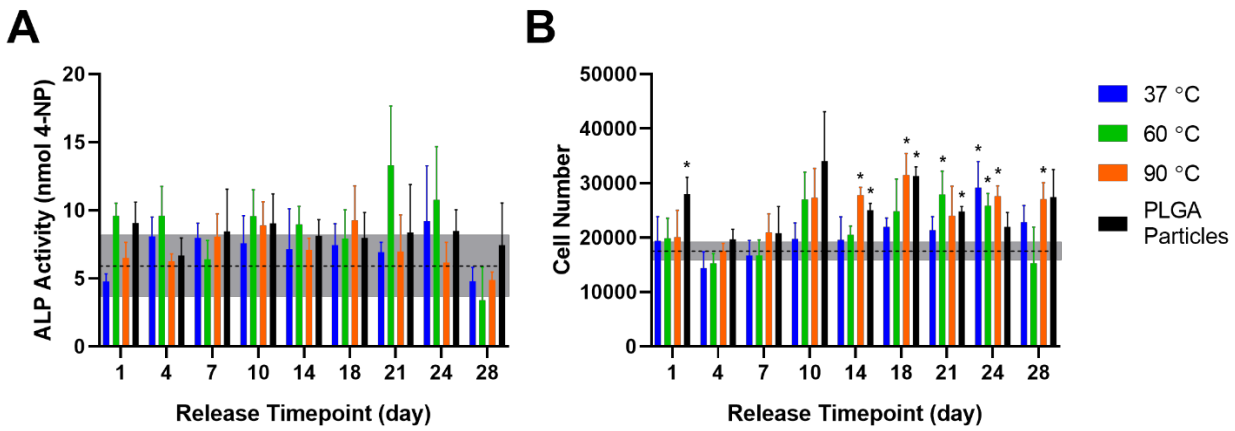
$$\frac{Y}{A-D}$$

for which X = Log_{10}(1+[BMP-2 concentration in ng/mL]), Y = normalized ALP activity in nmol 4-NP/ng DNA, A = maximum asymptote of normalized ALP activity, B = slope at inflection point of sigmoidal curve, C = X-value at inflection point of sigmoidal curve (for which Y = (A+D)/2), and D = minimum asymptote of normalized ALP activity.

MATLAB software (version R2020a, MathWorks; Natick, MA) was then used to calculate bioactive BMP-2 present in each release sample from the measured normalized ALP activity based on the derived equation. Samples whose normalized ALP activity was less than the minimum asymptote value were first corrected to this minimum value to account for the inability for samples to contain less than zero BMP-2 concentration. The resulting normalized ALP values were inputted along with the four parameters determined from the regression in order to calculate the BMP-2 concentration present in each sample. These calculated values of BMP-2 concentration were then summated to quantify cumulative release of bioactive growth factor from 3D printed fibers and particulate delivery vehicles. Cumulative release is expressed as the average percentage of total growth factor loaded in each sample, as quantified from the growth factor added during PLGA particle synthesis (assuming 100% loading efficiency)

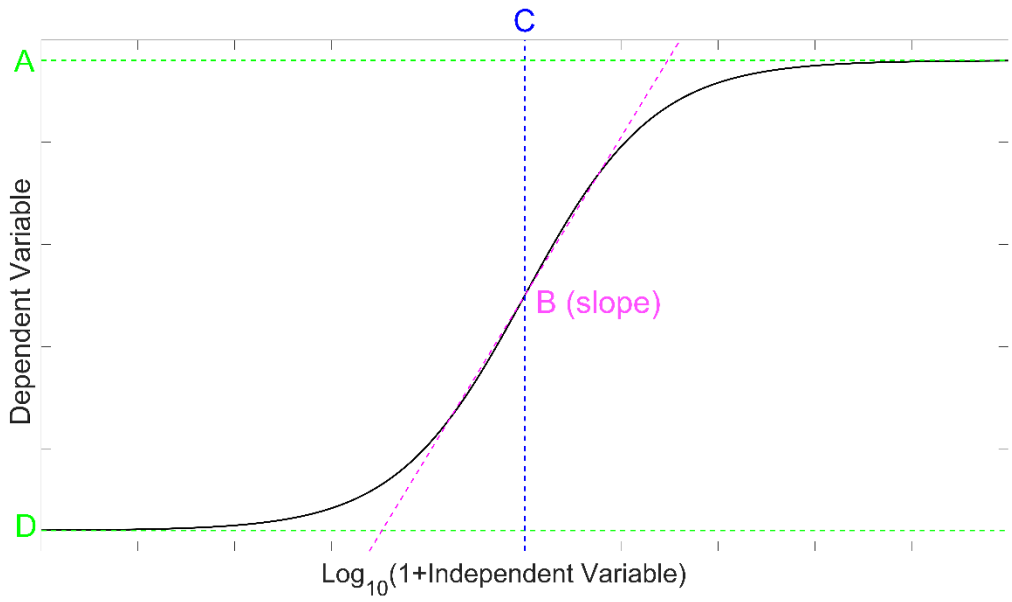
and the mass of PLGA particles present in each sample (considering 2 wt% mass concentration in the PPF-based mixture used for 3D printing).

Supplementary Figures and Tables



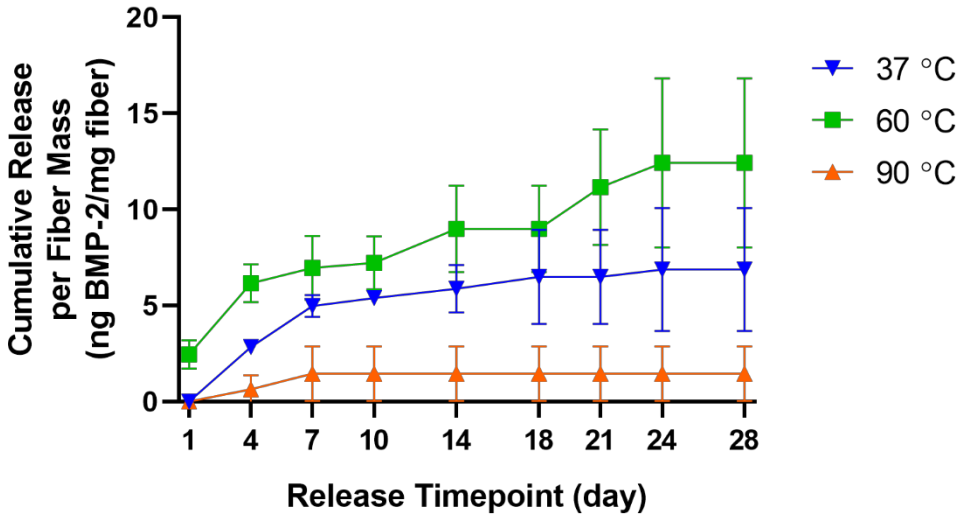
Supplementary Figure S1. Cellular bioactivity assay of ALP upregulation.

A) Measured ALP activity for cells exposed to release supernatants collected from BMP-2–loaded 3D printed fibers and PLGA particles. B) Cell number resulting from exposure of release samples. Data are reported as mean ± standard deviation for n = 3-4 samples. Horizontal dotted line indicates averaged value of each measurement for control cells incubated in culture medium without added growth factor; grey box represents ± standard deviation of these controls cells' values. * denotes significant upregulation ($p < 0.05$) from the averaged value for control cells as determined by unpaired t-tests. Legends refer to printing temperature or non-printed PLGA particle control.

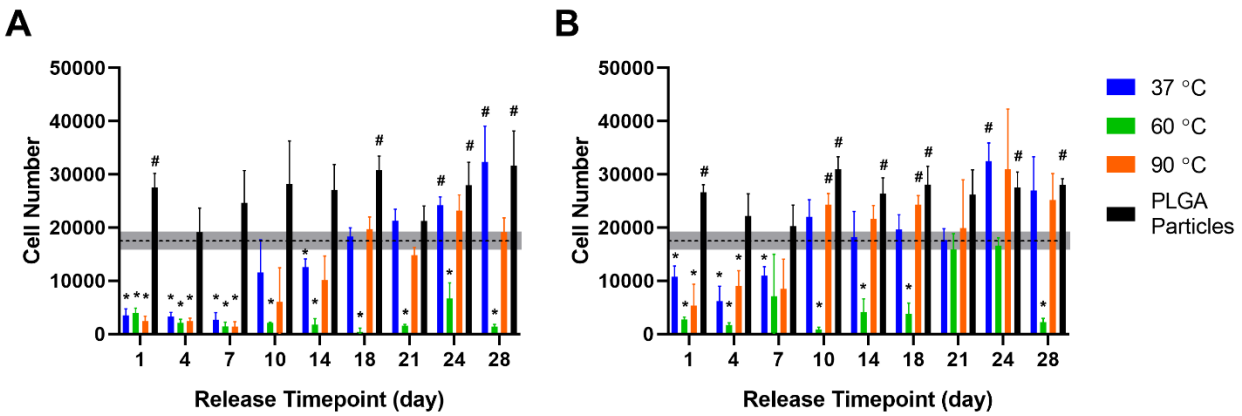


Supplementary Figure S2. Illustration of sigmoidal curve derived from four-parameter logistic regression.

A = Maximum asymptote of Y-value, B = slope at inflection point of sigmoidal curve, C = X-value at inflection point (for which Y = (A+D)/2), and D = minimum asymptote of Y-value.



Supplementary Figure S3. Cumulative release of BMP-2 per 3D printed PPF fiber mass. Cumulative release profiles of bioactive BMP-2 detected for each release interval relative to the mass of 3D printed PPF-based fibers comprising the sample. Legend refers to printing temperature.



Supplementary Figure S4. Cytotoxicity of 5-fold and 10-fold dilutions of release supernatants.

Cell number resulting from exposure to A) five-fold or B) ten-fold dilutions of release samples. Data are reported as mean ± standard deviation for *n* = 3-4 samples. Horizontal dotted line indicates averaged value of each measurement for control cells incubated in culture medium without added growth factor; grey box represents ± standard deviation of these controls cells' values. * denotes significant reduction in cell number ($p < 0.05$) from the averaged value for control cells as determined by unpaired t-tests; # denotes significant increase in cell number ($p < 0.05$) from the averaged value for control cells as determined by unpaired t-tests. Legends refer to printing temperature or non-printed PLGA particle control.

Supplementary Table S1. Statistical comparisons of pH measurements during degradation study.

Significant differences ($p < 0.05$) were determined by two-way ANOVA with repeated measures and *post hoc* Tukey's honestly significant difference test. For the multiple comparison tests within release timepoints, the PLGA particle group was designated as the control to which each 3D printed fiber group was compared.

Release Timepoint	Significant Comparisons
Day 1, Day 4, Day 7, Day 10, Day 14, Day 18	PLGA particles > 37 ºC (+) and (-) PLGA, 60 ºC (+) and (-) PLGA, 90 ºC (+) and (-) PLGA
Day 24	PLGA particles > 37 ºC (+) PLGA
Day 28	PLGA particles > 37 ºC (+) PLGA, 60 ºC (+) PLGA
Experimental Group	Significant Comparisons
37 ºC (+) PLGA	Day 0 > all subsequent timepoints Day 4 < Day 1, Day 7, Day 10 Day 18 < Day 1, Day 10, Day 14, Day 21 Day 28 < all preceding timepoints
60 ºC (+) PLGA	Day 0 > all subsequent timepoints Day 1 > Day 4, Day 14, Day 18, Day 24 Day 4 < Day 7, Day 10, Day 14, Day 21, Day 24 Day 10 > Day 7, Day 14, Day 18 Day 18 < Day 7, Day 24 Day 21 > Day 14, Day 18, Day 24 Day 28 < all preceding timepoints

90 ºC (+) PLGA	Day 0 > all subsequent timepoints Day 1 > Day 4, Day 14 Day 10 > Day 21 Day 18 < Day 24 Day 28 < Day 10, Day 18, Day 24
37 ºC (-) PLGA	Day 0 > all subsequent timepoints except Day 10 Day 4 < Day 1, Day 10, Day 21, Day 24, Day 28 Day 7 < Day 1 Day 21 > Day 1, Day 7 Day 10 > 14
60 ºC (-) PLGA	Day 0 > all subsequent timepoints Day 4 < all other timepoints Day 10 > Day 1, Day 4, Day 7, Day 14, Day 18 Day 18 < Day 1, Day 7, Day 21, Day 24, Day 28 Day 21 > Day 1, Day 4, Day 7, Day 10, Day 14 Day 24 > Day 1, Day 4, Day 7, Day 10, Day 14
90 ºC (-) PLGA	Day 0 > all subsequent timepoints Day 4 < all other timepoints except Day 18 Day 10 > Day 1, Day 7, Day 14, Day 18, Day 28 Day 14 < Day 1, Day 7, Day 28 Day 18 < Day 1, Day 7, Day 28 Day 21 > Day 1, Day 4, Day 7, Day 14, Day 18, Day 28 Day 24 > Day 1, Day 4, Day 7, Day 14, Day 18, Day 28

PLGA Particles	Day 0 > Day 18, Day 24, Day 28 Day 1 > Day 10, Day 18 Day 4 > Day 24, Day 28 Day 7 > Day 14, Day 24 Day 18 < Day 1, Day 4, Day 7, Day 10

Supplementary Table S2. Statistical comparisons of bioactivity study measurements within each release timepoint.

Significant differences ($p < 0.05$) were determined by two-way ANOVA with repeated measures and *post hoc* Tukey's honestly significant difference test.

Release Timepoint	Significant Comparisons
a) ALP Activity	
Day 1	Fibers printed at 37 °C < fibers printed at 60 °C, PLGA particles Fibers printed at 60 °C > fibers printed at 90 °C
b) Cell Number	
Day 14	Fibers printed at 60 °C < fibers printed at 90 °C, PLGA particles
Day 18	Fibers printed at 37 °C < fibers printed at 90 °C, PLGA particles
c) Normalized ALP Activity	
Day 4	Fibers printed at 37 °C > fibers printed at 90 °C, PLGA particles Fibers printed at 60 °C > fibers printed at 90 °C, PLGA particles
Day 14	Fibers printed at 60 °C > fibers printed at 90 °C
d) Cumulative BMP-2 Released	
Day 1	Fibers printed at 37 °C < fibers printed at 60 °C Fibers printed at 60 °C > fibers printed at 90 °C, PLGA particles
Day 4	Fibers printed at 37 °C < fibers printed at 60 °C Fibers printed at 37 °C > fibers printed at 90 °C Fibers printed at 60 °C > fibers printed at 90 °C, PLGA particles
Day 7, Day 10, Day 14	Fibers printed at 37 °C > fibers printed at 90 °C Fibers printed at 60 °C > fibers printed at 90 °C, PLGA particles

Day 18, Day 21	Fibers printed at 60 °C > fibers printed at 90 °C, PLGA particles
Day 24, Day 28	Fibers printed at 60 °C > fibers printed at 90 °C

Supplementary Table S3. Statistical comparisons of bioactivity study measurements within each experimental group.

Significant differences ($p < 0.05$) were determined by two-way ANOVA with repeated measures and *post hoc* Tukey's honestly significant difference test.

Experimental Group	Significant Comparisons
a) ALP Activity	
Fibers printed at 37 °C	Day 4 > Day 28
Fibers printed at 60 °C	Day 4 > Day 18 Day 7 < Day 14
Fibers printed at 90 °C	Day 10 > Day 28
PLGA particles	Day 1 > Day 4
b) Cell Number	
Fibers printed at 37 °C	Day 4 < Day 10, Day 21, Day 24 Day 10 < Day 24
Fibers printed at 60 °C	Day 4 < Day 10, Day 21, Day 24 Day 7 < Day 21, Day 24
Fibers printed at 90 °C	Day 4 < Day 14, Day 18, Day 24 Day 18 > Day 1, Day 7
PLGA particles	Day 4 < Day 18, Day 21
c) Normalized ALP Activity	
Fibers printed at 37 °C	Day 1 < Day 4, Day 10 Day 4 > Day 21, Day 28 Day 28 < Day 7, Day 10

Fibers printed at 60 °C	Day 4 > Day 1, Day 7, Day 14, Day 18, Day 21, Day 24, Day 28
Fibers printed at 90 °C	Day 1 > Day 14, Day 24 Day 18 > Day 28 Day 24 < Day 4, Day 7