Published in final edited form as:

Acta Biomater. 2015 February ; 13: 101–110. doi:10.1016/j.actbio.2014.11.028.

Gelatin Methacrylate Microspheres for Growth Factor Controlled Release

Anh H. Nguyen¹, Jay McKinney¹, Tobias Miller¹, Tom Bongiorno¹, and Todd C. McDevitt^{1,2,*}

¹The Wallace H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology and Emory University, Atlanta, Georgia, USA

²The Parker H. Petit Institute of Bioengineering and Bioscience, Georgia Institute of Technology, Atlanta, Georgia, USA

Abstract

Gelatin has been commonly used as a delivery vehicle for various biomolecules for tissue engineering and regenerative medicine applications due to its simple fabrication methods, inherent electrostatic binding properties, and proteolytic degradability. Compared to traditional chemical cross-linking methods, such as the use of glutaraldehyde (GA), methacrylate modification of gelatin offers an alternative method to better control the extent of hydrogel cross-linking. Here we examined the physical properties and growth factor delivery of gelatin methacrylate (GMA) microparticles formulated with a wide range of different cross-linking densities (15–90%). Less methacrylated MPs had decreased elastic moduli and larger mesh sizes compared to GA MPs, with increasing methacrylation correlating to greater moduli and smaller mesh sizes. As expected, an inverse correlation between microparticle cross-linking density and degradation was observed, with the lowest cross-linked GMA MPs degrading at the fastest rate, comparable to GA MPs. Interestingly, GMA MPs at lower cross-linking densities could be loaded with up to a 10-fold higher relative amount of growth factor over conventional GA cross-linked MPs, despite an order of magnitude greater gelatin content of GA MPs. Moreover, a reduced GMA cross-linking density resulted in more complete release of bone morphogenic protein 4 (BMP4) and basic fibroblast growth factor (bFGF) and accelerated release rate with collagenase treatment. These studies demonstrate that GMA MPs provide a more flexible platform for growth factor delivery by enhancing the relative binding capacity and permitting proteolytic degradation tunability, thereby offering a more potent controlled release system for growth factor delivery.

1. Introduction

Gelatin has been used as a delivery vehicle for the controlled release of biomolecules due to its ability to form polyion complexes with charged therapeutic compounds such as proteins,

© 2014 Elsevier Ltd. All rights reserved.

*Address for Correspondence: Todd C. McDevitt, Ph.D., 313 Ferst Drive, Suite 2102, Atlanta, GA 30332-0535, Phone: 404-385-6647, Fax: 404-894-4243, todd.mcdevitt@bme.gatech.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

nucleotides, and polysaccharides [1,2]. Gelatin is obtained from denaturation of collagen via alkaline or acid treatment to yield gelatin with either a net negative (isoelectric point (IEP) = 5) or net positive (IEP = 9) charge, respectively, at pH 7.4. Modulating the net charge of gelatin allows for sequestering of growth factors of the opposite charge while maintaining their bioactivity. While molecules may be released from the gelatin via diffusion, gelatin's proteolytic degradability offers an additional mechanism to facilitate release of growth factors [2,3].

Gelatin microparticles (MPs) have been extensively studied for their ability to deliver growth factors for diverse applications such as therapeutic angiogenesis [3,4], cartilage tissue engineering [5–7], and post-myocardial infarction therapy [8], as well as stem cell differentiation within scaffolds, [9] embedded within a self assembling cell sheet, [10] or within aggregates [11–14]. Gelatin MPs are typically formed via a water-in-oil emulsion and subsequent cross-linking of gelatin microspheres with reagents such as glutaraldehyde (GA) [7,15], genipin [10,13], or carbodiimides [16].

The most common method for cross-linking gelatin MPs is via GA cross-linking, which occurs primarily through the reaction of GA aldehyde groups with the ϵ -amine groups of lysine or hydroxylysine residues, resulting in a Schiff base intermediate that cross-links gelatin through an aldol condensation reaction [17]. However, the Schiff base intermediates are unstable, and have been reported to react further to form products such as secondary amines and 6-membered dihydropyridines, which can form other types of cross-links, such as aliphatic crosslinks and quaternary pyridinium-type cross-links, among other classes of molecules [17–19]. Despite the use of a wide range of GA concentrations (from 0.05 to 2.5 wt%), cross-linking below 60% is rarely attained, with GA above 0.5 wt% typically yielding 100% cross-linking within 24 hours [20]. Furthermore, GA cross-linking yields similar MPs despite employing reaction times from 1 to 24 hours and different temperatures (4–37°C) [10,16,17,21]. Since a fifty-fold difference in GA concentration, as well as vast variations in temperature and reaction time, yield only a small range of cross-linking, very little correlation can be drawn between input GA and cross-linking density. These inherent caveats in GA cross-linking procedures make it difficult to fabricate MPs with varying levels of cross-linking density or accurately predict cross-linking *a priori*. Furthermore, GA cross-linking significantly reduces the degradability of collagen-based materials, and GA itself is cytotoxic and can lead to calcification and inflammation *in vivo* [20,22,23].

Methacrylation of gelatin has been reported to be less cytotoxic and enable a broader range of cross-linking densities [24]. Amine groups on gelatin can be substituted with glycidyl methacrylate (GyMA), methacryloyl chloride (MC) or methacrylic anhydride (MA) [24–27]. However, GyMA contains a hydrolytically degradable ester group, producing less stable hydrogels, and MC less efficiently facilitates methacrylate substitution compared to MA [24]. Methacrylate groups can be reproducibly introduced into gelatin with MA to achieve a wide range of methacrylation values. MA substitution affords greater control over hydrogel cross-linking density than other systems such as glutaraldehyde, genipin, or dehydrothermal cross-linking because the methacrylate groups on GMA restrict the maximum cross-linking density achievable, independent of soluble factors that are difficult to control, such as the

amount of radical initiator added, or experimental conditions, such as time and temperature of cross-linking.

Although GMA bulk hydrogels have been characterized extensively [24,26–28], our study is the first to our knowledge to characterize GMA MP physical properties and growth factor binding and release. In this study we present the synthesis of GMA MPs, which can be fabricated from gelatin with a wide range of methacrylate substitution. Resulting MPs were analyzed for size range, degradability, and their ability to bind and release growth factor compared to the conventional GA cross-linked MPs. These studies demonstrate that modulation of MP cross-linking density via gelatin methacrylation facilitates greater control over critical MP properties compared to conventional glutaraldehyde cross-linked MPs, and this enhanced control enables tailoring of particles for a variety of tissue engineering purposes.

2. Experimental Methods

2.1. Gelatin methacrylate (GMA) synthesis

Gelatin B (pI = 5.0) (Bovine skin, Sigma Aldrich, St. Louis, MO) was fully dissolved in water (10% w/v) at 60°C. Gelatin methacrylate (GMA) was produced by reacting the amine groups on gelatin type B with methacrylic anhydride (MA), similar to previously described methods [24–29]. Briefly, MA (Sigma Aldrich) was added dropwise to the gelatin solution at a 1:3, 2:3, or 1:1 ratio (mol MA: mol unsubstituted amines on gelatin). The pH was continuously monitored and adjusted to ~pH 7.4 to promote efficient substitution of amines to methacrylate groups. The GMA solution (15 mL) was added to 60 mL PBS and dialyzed (SpectraPor, MW cutoff 12–14 kDA, Spectrum Labs, Santo Dominguez, CA), for 5 days against 2 L deionized water, with water changed twice daily, followed by lyophilization and storage at –20°C until further use.

2.2. Gelatin methacrylate characterization

A fluorescamine assay was performed to determine the degree of substitution of free amine groups via methacrylation. Lyophilized GMA at molar concentrations of 1:3, 2:3, and 1:1 MA to unsubstituted amine groups on gelatin, as well as completely unsubstituted gelatin type B, were solubilized in 500 µL of deionized water and reacted with 1 mL of fluorescamine solution (7 mg fluorescamine (Sigma Aldrich) in 25 mL dimethylsulfoxide (DMSO)). Samples were read on a Biotek plate reader at excitation 390 nm, emission 465 nm. The number of free amine groups in each GMA formulation was calculated using a glycine standard curve and compared to unsubstituted gelatin type B to determine the final degree of substitution.

¹H NMR was also performed to determine the degree of substitution of gelatin. Each lyophilized gelatin derivative (5–10 mg) was dissolved in deuterium oxide (D₂O, Cambridge Isotope Laboratories, Inc., Andover, MA, USA). ¹H NMR spectra were recorded on a Bruker Avance III 400 MHz spectrometer and each resulting spectrum was phase corrected, baseline subtracted and integrated with ACD NMR processor 12.0 software. The degree of substitution was determined according to the method of Hoch et al [24]. In brief, the signal of protons resulting from aromatic amino acids in the polymer at δ=7.0 ppm to δ=7.5 ppm

were used as a reference in each spectrum. The signal of methylene protons ($\delta=2.7 - 2.9$ ppm) neighboring the lysine amino acid was used for quantification of the integrated signal areas. The integrated area of methacrylated gelatin relative to unmodified gelatin was used to determine the degree of substitution. The degree of methacrylate substitution for ^1H NMR was determined by the following equation: $[1 - (\text{lysine integration signal of methacrylated substituted gelatin} / \text{lysine integration signal of unsubstituted gelatin})]$.

2.3. Gelatin methacrylate microparticle fabrication

Lyophilized GMA was fully dissolved in water (10% w/v) at 37°C. Sixty mL of corn oil (Mazola) was heated to 37°C prior to the addition of 1 mL of polysorbate 20 (Promega, Fitchberg, WI) and homogenized at 1500 rpm (Polytron PT-3100 homogenizer) for 3 minutes. The 10% GMA solution was mixed with 3 μL of 0.3 M ammonium persulfate (APS) (Bio-Rad, Hercules, CA) and added dropwise to the corn oil phase. The oil and water emulsions were homogenized for 5 minutes at 1800 rpm for 15% and 50% GMA, and 1500 rpm for 90% GMA (to obtain MPs of similar size as 15% and 50% GMA MPs), and placed on a hotplate set to 45°C with agitation via stir bar. N_2 gas was bubbled through the emulsion for 20 minutes to purge oxygen. Under constant stirring, the hotplate was increased to 100°C to initiate the thermal cross-linking reaction and allowed to proceed for 40 minutes. The particle/corn oil mixture was centrifuged at 2500 rpm at 4°C to harvest the microparticles, and excess corn oil was removed with four successive washes with deionized water. In order to visualize the hydrogel microparticles, fluorescent labeling was performed by incubation with Alexa Fluor® succinimidyl ester 594 (Invitrogen, Carlsbad, CA) in 0.1 M sodium bicarbonate buffer followed by four deionized water washes. All particles were stored at 4°C in dH_2O until further use.

2.4. Gelatin glutaraldehyde microparticle fabrication and determination of cross-linking

Glutaraldehyde cross-linked gelatin particles were synthesized as previously described [21]. A 10% gelatin solution in water (2 mL) was added dropwise to 60 mL of corn oil and homogenized at 2500 rpm at room temperature. The corn oil/gelatin mixture was cooled to 4°C and incubated for 10 minutes before 35 mL of acetone was added and the whole mixture was homogenized at 2500 rpm at room temperature. The mixture was re-cooled to 4°C and centrifuged at 2500 rpm to pellet the particles. Excess corn oil and acetone were removed via 3 water washes. The particles were separated into 1.5 mL tubes and 1 mL glutaraldehyde (10 mM) was added to each tube to crosslink the particles overnight. Glycine (25 mM) was added 15 hours later to quench any remaining reactive aldehyde groups. The particles were then washed with water 3 times, lyophilized, and stored at -20°C until further use. In order to determine GA cross-linking, an equal weight of lyophilized GA cross-linked MPs and unsubstituted gelatin type B were solubilized in deionized water and reacted with fluorescamine reagent in a manner analogous to determining GMA degree of substitution as described above. Free amine groups in GA MPs were compared to amine groups in 0% cross-linked unsubstituted gelatin type B to determine the degree of cross-linking.

2.5. Scanning electron microscopy

MPs were fixed in 2.5% glutaraldehyde in sodium cacodylate buffer (Electron Microscopy Sciences (EMS), Hatfield, PA). The MPs were then thoroughly rinsed and incubated in 1%

osmium tetroxide (EMS) followed by graded ethanol dehydrations and critical point dried using a Polaron E3000 critical point dryer (Quorum Technologies Inc., Guelph, ON, Canada). Samples were sputter coated for 2 minutes at 2.2 kV using a Polaron SC7640 sputter coater and imaged using a Hitachi S-800 scanning electron microscope (Hitachi High Technologies, Pleasanton, CA).

2.6. Microparticle size analysis

MPs were suspended in ISOTON II diluent (Beckman Coulter, Hialeah, FL), a phosphate-buffered saline solution, at an approximate concentration of 0.1 mg/ml. Volume size distribution was determined via Coulter Counter (Beckman Coulter Z2, Brea, CA) with a 70 μm aperture. Size bin, or category, distributions were defined by the percentage of MPs that fell within the ranges of less than 5 μm , between 5 and 15 μm , and greater than 15 μm .

2.7. Gelatin microparticle content

The amount of gelatin per MP was quantified using a bicinchoninic acid (BCA) assay kit (Pierce, Rockford, IL). For each of the different MP formulations, 1 million particles in 50 μL of PBS were reacted with 400 μL BCA working reagent for 30 min at 37°C. Absorbance readings were taken at 562 nm on a Synergy H4 Biotek plate reader and total gelatin content was calculated by comparing the absorbance readings against a standard curve generated using a range of gelatin type B concentrations (0–2000 $\mu\text{g/mL}$).

2.8. MP degradation analysis

Collagenase 1A (Sigma Aldrich) was adsorbed to magnetic polystyrene beads (4–5 μm in diameter, Spherotech, Lake Forest, IL), as previously described [30]. Briefly, a 25 μL aliquot of Spherotech bead solution was incubated in 1 mL of 2 mg/mL of collagenase for 2 hours at room temperature in a low retention 1.5 mL tube (Axygen, Pittsburgh, PA). Collagenase-coated beads were separated from solution via a magnet, the supernatant removed, and unbound collagenase was removed via 3 PBS washes. A BCA assay was used to determine the amount of beads necessary to obtain 1 μg collagenase. Beads with 1 μg of collagenase were added to 0.5 mg of each MP formulation in 1 mL PBS with 0.036 mM CaCl_2 . The collagenase beads and MPs were rotated continuously at 37°C, and the collagenase coated-beads were replaced with freshly coated beads under sterile conditions every 48 hours. At specified time points, collagenase-coated magnetic beads were removed and the MP suspension was transferred to a new tube with 0.5 mL of 0.03% fluorescamine in DMSO. Increasing values, indicating the generation of new amino terminus groups by peptide bond cleavage by collagenase, were determined on a Biotek plate reader with an excitation of 390 nm and emission of 465 nm, and compared to non-degraded MPs and completely degraded MPs (50 μg collagenase for 48 hours).

2.9. MP swelling studies

An equal volume of each MP formulation (100 μL) was deposited in a 1.5 mL microcentrifuge tube, and all excess water was aspirated and blotted from the MPs before the swelling weight was recorded. MPs were lyophilized for 48 hours before recording the dry weight of the MPs. Swelling ratio (q) = (swollen MP weight)/(dry MP weight).

Equilibrium swelling ratio (Q_m) = $1 + (\rho_{\text{gelatin}}/\rho_{\text{water}})^*(q+1)$, with ρ = density. Water content was determined as $(W_s - W_d)/W_s \times 100$, with W_s = swollen weight of MPs and W_d = dry weight of MPs.

2.10. MP elastic moduli measurement

The elastic moduli of MPs were measured via atomic force microscopy (AFM). MPs were conjugated to glass coverslips prior to AFM measurements. Coverslips were functionalized with amine groups via incubation with 1% (3-aminopropyl)-trimethoxysilane (APTMS) in absolute ethanol for 2 hours at room temperature under gentle agitation and then washed with dH_2O to remove excess APTMS. MPs were incubated with 2 mM 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) and 5 mM N-hydroxysulfosuccinimide (sNHS) in 0.1 M 2-(N-morpholino)ethanesulfonic acid (MES) activation buffer for 15 minutes, and subsequently quenched with 1.4 μL of 2-mercaptoethanol. Each MP sample was centrifuged to remove activation buffer, resuspended in dPBS, incubated on a coverslip for 2 hours at room temperature (23°C), and washed with dPBS and placed in a 50 mm plastic dish (BD, Franklin Lakes, NJ) in dPBS before mechanical characterization was performed with an atomic force microscope (Asylum Research, Santa Barbara, CA) stationed on a vibration isolation table (Herzan, Laguna Hills, CA). A brightfield microscope (Eclipse Ti, Nikon, Melville, NY) was used to locate and position a tipless silicon nitride cantilever (MLCT-O10, Bruker, Camarillo, CA, Cantilever E, $k=50\text{--}200$ pN/nm) with a 5.5 μm polystyrene bead (Bangs Labs, Fishers, IN) over the center of each MP. The cantilever spring constant ($k=110.56$ pN/nm) was determined by thermal calibration, and a probe velocity of 2 $\mu\text{m/s}$ was used. Indentations of approximately 25 nm for typical particles were obtained using a 5 nN force trigger. The Young's modulus of each particle was determined using IGOR software (Wavemetrics, Portland, OR), which applies the Hertzian contact model to the extension force-displacement curves from 60–95% of the maximum indentation, over which range the Young's modulus was largely independent of indentation variability during the early contact of the cantilever bead with the MP due to softness of the sample. Each particle was represented as the average Young's modulus of two measurements, assuming cellular Poisson's ratio, $\nu=0.5$ and using indentation offset as a free variable.

2.11. Mesh size calculation

Mesh size of the MPs was determined from MP elastic moduli measurements via AFM and swelling ratios as previously described [29]. Briefly, molecular weight between crosslinks (M_c) was determined as equivalent to $3\rho T/E$, where E = the elastic modulus as determined via AFM, ρ = gelatin density, R = gas constant (8.3145 J/Kmol), and T = absolute temperature (K). The mesh size (ξ) was calculated via the formulation: $\xi = 2\alpha(M_c/M_r)^{1/2}(2.21\text{\AA})(Q_m)^{1/3}$, with $M_r = 100$ g/mol, Q_m as the equilibrium swelling ratio, and α , the expansion factor, as 2 for gelatin.

2.12. Microparticle growth factor loading

MPs (0.5 mg) were added to 1 mL PBS with 1% BSA and incubated overnight with 10, 50, 100, 150, 200, or 300 ng/mL sterile solutions of recombinant human BMP-4 or recombinant human FGF-2 (R&D) under rotational agitation at 4°C . After 15 hours, the tubes were

centrifuged to separate out the MPs with loaded growth factor, and the supernatant was removed and assayed for BMP-4 or FGF-2 content via ELISA (R&D Duoset). Growth factor loading was determined by subtracting the amount of growth factor in the supernatant from the input amount. Loading efficiency at each concentration was determined via total amount loaded divided by total growth factor initially added to the MPs.

2.13. Growth factor release from microparticles

GMA MPs of varying degrees of methacrylation, as well as GA MPs (0.5 mg) were incubated for 15 hours at 4°C with equal concentrations of growth factor (10 ng/mg MP for BMP-4 and 50 ng/mg MP for bFGF). After the incubation period, the MPs were centrifuged, and the supernatant was replaced with fresh buffer consisting of 1% BSA solution in PBS with or without collagenase 1 under sterile conditions. At each time point thereafter, 300 μ L of the supernatant was collected for analysis and replaced with an equal volume of fresh buffer. Enzymatically treated samples were either treated with 100 ng/mL or 1 μ g/mL collagenase 1A. Collagenase buffer was changed every 3 to 4 days as previously reported [6,7]. The amount of growth factor collected in the supernatant was determined via BMP4 and bFGF ELISA (R&D duoset). The percent of cumulative growth factor release was determined via normalization of total growth factor released at each time period with the total growth factor initially loaded onto the MPs.

2.14. Statistical analysis

All values are reported as mean $\pm$ standard error, with a minimum of triplicate experimental samples. Before statistical analysis, a Box-Cox power transform was used to process all non-normal data to a Gaussian distribution. Statistical significance was determined using one-way ANOVA with Tukey's post hoc analysis with 95% confidence intervals after performing Levene's equality of variances test. P-values < 0.05 were determined to be statistically significant.

3. Results

3.1. Gelatin methacrylate modification

GMA degree of substitution was determined via both ^1H NMR and a fluorescamine assay. Based on ^1H NMR analysis, GMA synthesized with low, medium, and high degrees of methacrylation (molar ratios of 1:3, 2:3, and 1:1 of MA to unsubstituted groups on gelatin), corresponded to approximately 7%, 39%, and 100% methacrylate substitution, respectively (Figure 1B,D). Similarly, fluorescamine analysis indicated that the three GMA formulations were approximately 15%, 50% and 90% substituted (Figure 1C,D). The two substitution analysis methods were highly correlated, and the GMA formulations were referred to thereafter by the fluorescamine-determined substitution values. In comparison to the GMA MPs, GA MPs were $93.4 \pm 4.5\%$ cross-linked as determined by assaying the remaining unsubstituted amines after GA treatment via the fluorescamine assay.

3.2. Gelatin methacrylate particle characterization

The average diameters of hydrated 15%, 50% and 90% GMA MPs were $4.9 \pm 3.6 \mu\text{m}$, $5.5 \pm 5.2 \mu\text{m}$, and $5.0 \pm 6.9 \mu\text{m}$, respectively, while GA MPs were determined to be $5.1 \pm 7.7 \mu\text{m}$

(Supplemental figure 1). All MPs had a smooth, round morphology as illustrated via phase microscopy of the MPs (Figure 2A). SEM analysis confirmed this morphology, however, the 15% substituted MPs appeared to fuse together during preparation for SEM and thus their individual morphology could not be assessed by SEM (Figure 2A v). MP sizes were analyzed via Coulter Counter and no significant differences were found in the size distribution of MPs across methacrylated groups. Overall, the methacrylated MPs could be reliably produced with higher proportions of monodisperse particles ($<5\ \mu\text{m}$) compared to GA MPs, which were the most polydisperse (Figure 2B, Supplemental figure 1).

The GA MPs contained significantly more gelatin per particle ($2.5\ \mu\text{g}/\mu\text{m}^3$) than any of the GMA formulations ($<1\ \mu\text{g}/\mu\text{m}^3$). However, increasing the methacrylation decreased the gelatin content of the fabricated MPs, as the 15% and 50% MA MPs had two-fold greater gelatin content than the 90% MA formulation (Figure 3A). Degradation studies of the MPs upon exposure to $1\ \mu\text{g}/\text{mL}$ of collagenase 1A exhibited an inverse correlation between degradation and methacrylation levels (Figure 3B). By 6 hours, almost half of the GA formulation had degraded compared to about one fourth of the higher methacrylated MPs (50% and 90%). Furthermore, the GA MPs were completely degraded by 48 hours, whereas the higher methacrylated MPs took over 96 hours to reach 100% degradation.

Elastic moduli of the MPs were determined via AFM on surface-immobilized MPs. The lower methacrylated MPs (15% MA and 50% MA) were significantly less stiff (39.5 kPa and 54.0 kPa, respectively) and almost an order of magnitude smaller modulus than the 90% MA MPs, which had an equivalent stiffness to GA MPs with moduli of 222.0 and 243.6 kPa, respectively (Figure 4a). Moreover, the ability of all methacrylated MPs to swell in a hydrated environment was significantly greater than that of the GA MPs with the lowest methacrylated MPs (15% MA) exhibiting an increased swelling volume compared to the other two methacrylated MPs (Figure 4b). Thus, gelatin particle stiffness and swelling potential could be modulated via methacrylate modification.

Mesh size was calculated for all of the MPs based on their elastic moduli and swelling ratio values. The lower methacrylated MPs had a significantly larger molecular weight between crosslinks (M_c) (Supplemental Table 1), and therefore greater mesh size than the GA MPs, with mesh sizes of $157.1 \pm 7.1\ \text{nm}$ and $124.2 \pm 9.6\ \text{nm}$ for 15% MA and 50% MA, respectively, compared to $54.5 \pm 5.4\ \text{nm}$ for GA MPs. In addition, 90% MPs had a mesh size of $57.9 \pm 4.1\ \text{nm}$, which was comparable to those of GA MPs (Figure 4c). A decrease in mesh size was observed with increased methacrylation, thereby enabling control of particle mesh size through modulation of the degree of methacrylate substitution.

3.3. Gelatin microparticle growth factor loading

The capacity of the different gelatin MPs to bind BMP4 ($pI = 9.0$) and bFGF ($pI = 9.6$) was examined via an overnight loading assay. Even at the highest loading concentration of 300 ng/mg, GA MPs bound low quantities of BMP4, whereas the lower (15% and 50%) GMA MPs, had peak loading efficiencies at 99.6% and 63.1%, with average efficiencies across all loading concentrations of 69.2% and 43.9%, respectively (Figure 5A,B). At the lower loading concentrations, 50 and 100 ng/mg, the GA MPs bound significantly less BMP4 than all of the GMA formulations. Similarly, at the two highest loading concentrations, GA

bound less BMP4 than the two lower methacrylated MPs, 15% ($p < 0.01$) and 50% MA ($p < 0.038$). Decreasing the degree of methacrylation resulted in a significant increase in BMP4 binding capacity, with the 15% MPs binding more of the growth factor than the 90% MPs at the highest loading concentrations (150-300 ng/mL). The GA MPs also bound significantly less bFGF than all GMA MPs at the lowest (10 and 50 ng/mg) and highest (200 and 300 ng/mg) loading concentrations (Figure 5C,D). Similar to BMP4 results, reduced methacrylation levels enabled an increase in bFGF binding capacity, with greater binding of the two lowest methacrylated MPs compared to the 90% MPs at all of the loading concentrations.

3.4. Gelatin MP growth factor release

Each MP formulation was loaded with 10 ng of BMP4 and 50 ng of bFGF per mg of MP. No significant differences were observed in passive release of BMP4 between any of the MP formulations (Figure 6A). However, low collagenase treatment (100 ng/mL) enabled a greater release of BMP4 from the GA MPs compared to the 50% MPs at 50 hours (Figure 6B), whereas high collagenase treatment (1 μ g/mL) enabled greater BMP4 release at the earliest time point (3 hours) of the GA MPs compared to both the 50% and 90% MA MPs (Figure 6C). Furthermore, GA MPs did not release any more BMP4 in response to protease treatment compared to passive release, except for the initial release at 3 hours (Figure 7A). However, the release of BMP4 from the higher methacrylated MPs, 50% MA and 90% MA, was more sustained, as it increased with collagenase treatment at all time points assayed, even up to 170 hours (Figure 7, Supplemental Figure 2). Treatment with 1 μ g/mL collagenase facilitated the release of 49.5% and 54.7% BMP4 by the 50% and 90% MA MPs, respectively, compared to 79.2% released by the GA MPs by 3 hours. In this case, while the GA MPs released nearly all (91.1%) of bound BMP4 by 11 hours, the 50% and 90% MA MPs had only released 75.4% and 72.1% BMP4, respectively, by 170 hours (Figure 7, Supplemental Figure 2). Thus, the higher methacrylated MPs afford a more sustained release of BMP4 compared to the GA MPs, and varying the degree of methacrylation enables modulation of BMP4 release kinetics.

Passive release of bFGF from 50% MPs was less than release from all other MP formulations after 25 hours and remained less than GA MPs throughout the assayed time period (Figure 6D). Compared to passive release, collagenase treatment resulted in an increase in bFGF released by the 50% MA MPs until 25 hours and the 90% MA MPs until 170 hours, further illustrating the tunability of the methacrylated MPs (Figure 7, Supplemental Figure 2). While low collagenase treatment enabled greater release of bFGF from the 15% MPs compared to the 50% MA formulation after 11 hours, and the 90% MA MPs released more bFGF from 11 hours to 170 hours (Figure 6E), no differences were found in growth factor release between MP formulations under high collagenase treatment (1 μ g/mL) (Figure 6F). Importantly, there were no differences between bFGF passive release and release after proteolytic treatment from GA MPs. Moreover, the 50% MP formulation offered a more sustained release of bFGF compared to all other MPs, releasing only 72.4% by 170 hours with higher collagenase treatment (1 μ g/mL) compared to 94.0%, 97.6%, and 92.3% from the 15% MA, 90% MA, and GA MPs respectively (Figure 6F). Therefore, modulation of MP methacrylation levels enables greater sustained release of growth factors

compared to the GA formulation and demonstrates that modification of cross-linking density enables control over growth factor release upon protease treatment.

4. Discussion

GMA MPs can be synthesized from a wide range (15–90%) of methacrylate substituted gelatin species with methacrylation enabling highly reproducible fabrication of relatively monodisperse MPs while allowing control over a range of critical parameters. Compared to conventional glutaraldehyde cross-linking, varying the degree of methacrylation directly influences the amount of gelatin/particle, thereby allowing greater control over degradation kinetics. Additionally, the degree of methacrylation positively correlates to MP elastic moduli and inversely correlates with swelling potential and mesh size, with GA MPs having comparable mechanical properties to the methacrylated MPs with similar cross-linking densities (>90%). Furthermore, decreasing the degree of methacrylation increases growth factor binding and promotes more complete growth factor release, with GA MPs behaving similarly to the lowest methacrylated MPs.

GMA MPs offer greater control over cross-linking density compared to glutaraldehyde MPs. Methacrylation of gelatin can be controlled *a priori* by introducing the vinyl moiety responsible for the cross-linking, thus offering a predetermined number of reactive sites for cross-linking. Conversely, GA MPs have all amine sites available for cross-linking, and soluble addition of glutaraldehyde for modification of cross-linking density results in varying intermediate reaction compounds, making the overall reaction difficult to control [17]. Additionally, although bulk GMA hydrogels at lower substitution percentages (20%) lack sufficient physical integrity to be handled [31], stable GMA MPs can be formed with cross-linking densities as low as 15% MA substitution. In contrast, despite manipulation of GA molecule concentration as well as reaction time and temperature, the lowest cross-linked GA MPs still have greater than 60% cross-linking [10,16,17,20,21]. Also of note, no significant differences in size distribution between GMA MPs were observed. A previous study has reported that increasing the degree of methacrylation resulted in a decrease in gelatin viscosity [24]. Therefore, despite differences in substitution, methacrylated MPs can be synthesized with similar size distributions by raising the emulsification speed for lower substituted, or more viscous GMA. In contrast to glutaraldehyde cross-linking methods, which typically result in more polydisperse MPs, GMA MPs can be reliably fabricated in a more monodisperse manner. Highly uniform GMA microdroplets have recently been synthesized using a T-junction microfluidic device, although the minimum size of reported droplets was greater than 30 μm , despite tuning GMA concentration and flow rate [32]. Since bulk properties and growth factor interactions of the microdroplets have not yet been characterized, our study represents the first report on these critical properties of GMA microspheres [32].

GA MPs degraded more than the 50% and 90% methacrylated MPs over the first 48 hours of collagenase treatment. Previous studies have reported GA molecule diffusion limitations through chitosan gel beads during GA cross-linking, resulting in a more heavily cross-linked MP exterior [33]. Thus, non-homogeneous GA cross-linking of gelatin microspheres could likely occur, since gelatin sphere formation via a water-in-oil emulsion is required before the

addition of any cross-linking reagents. Despite lower cytotoxicity and a greater range of cross-linking attainable [10], genipin cross-linked MPs may also be non-homogenously cross-linked, especially since genipin has a higher molecular weight than GA. Once proteases degrade the exterior surface of GA MPs, the remainder of the MPs can easily degrade, explaining the rapid degradation rate of the GA MPs after the first 6 hours of collagenase incubation. Conversely, increasing the methacrylation level above 15% results in a more sustained degradation of GMA MPs over 96 hours and prevents the rapid degradation observed with the GA MPs. Moreover, since thermal initiators are mixed in with the gelatin during the water-in-oil emulsion of GMA MP synthesis, free radicals can be distributed more uniformly within the bulk of the material, possibly promoting more homogenous cross-linking throughout the MPs [34,35]. Although dehydrothermal cross-linking may also be more uniform than GA cross-linking, dehydrothermally modified MPs also degrade rapidly in all reported studies, persisting no more than 24 hours in a variety of environments [36,37]. Despite some differences in experimental conditions, higher methacrylated GMA can persist for up to 96 hours in the presence of collagenase. Thus, increasing the methacrylation of GMA MPs can enable slower and more sustained MP degradation than compared to other gelatin MP cross-linking methods.

Our GMA MP results corroborate with previous studies, which demonstrate that in GMA bulk hydrogels, enhanced methacrylation increases particle stiffness but decreases the molecular weight between crosslinks, therefore decreasing hydrogel pore/mesh size [24–26,29,38]. We found that less cross-linked MPs bound greater concentrations of oppositely charged growth factor from solution, suggesting that reducing the methacrylation of MPs increases the distance between cross-links, facilitating greater swelling and greater mesh size, so that growth factors can more freely enter the hydrogel. The increase in mesh size could explain why lower methacrylated GMA MPs bind higher amounts of growth factor than the GA cross-linked MPs, despite the significantly lower gelatin content per volume of GMA MPs. As previously mentioned, since GA MPs are synthesized by adding GA to pre-formed gelatin spheres, the GA MPs may have a dense shell of cross-linking at the surface [33]. The presence of a highly cross-linked outer shell region may restrict growth factor binding primarily to the MP surface and sterically hinder molecules from entering the interior of the microsphere during growth factor loading [39,40]. More homogenous gelatin network formation, as seen during GMA synthesis where the initiator is added to the gelatin solution before spheres are formed, would facilitate greater access of the growth factors to the charged sites within the particle interior, and thus enhance biomolecule loading. Modulation of physical factors, such as mesh sizes and swelling potential, via methacrylation appears to play a larger role in growth factor binding than the reduction in charge inherent to methacrylation. Increasing methacrylation reduces the net positive charge on the MP, and although the two growth factors assessed in the study are both positively charged at physiological pH, the increasingly methacrylated MPs did not have enhanced binding as would be expected with reduced electrostatic repulsion. Since we did not see enhanced binding with increased methacrylation, it is postulated that physical factors modulated by the degree of methacrylation may play a more significant role in growth factor binding than simply a change in the net charge.

Decreased growth factor release from GMA MPs was also observed with increasing gelatin methacrylation. In agreement with earlier reports, our studies indicate that increasing methacrylation reduces hydrogel swelling [31], physically limiting the solvent and growth factor interaction that enables release [41]. Reduced swelling results in smaller hydrogel mesh sizes [38], within which the biomolecules encounter more steric hindrance contributing to the more sustained release from the higher methacrylated GMA MPs. Increasing the collagenase treatment concentration accelerated the release rate from the MPs, with the majority of release occurring 24 hours before the release in MPs without collagenase treatment. Furthermore, in the higher (90%) methacrylated MPs, increased growth factor release occurred only after exposure to higher collagenase concentrations, thus illustrating the protease-mediated tunability of the delivery system. Modulating the degree of gelatin methacrylation also resulted in varying degradation and release profiles, both passive and active. Overall, the sustained release profiles of two positively charged growth factors from the negatively charged GMA MPs presented herein, suggest that modulation of GMA cross-linking density can provide a platform for controlled release of different growth factors. Thus, GMA MP technology can be engineered for a large variety of *in vivo* and *in vitro* applications where smaller sized biomolecule delivery vehicles are desired, from wound bed packing for release of anti-inflammatory agents [42,43] or within stem cell aggregates to direct differentiation with morphogenic factors [44,45]. Hence, GMA MPs can be easily customized for the controlled delivery of charged biomolecules for a variety of tissue engineering and regenerative medicine applications.

5. Conclusions

We have demonstrated the synthesis and characterization of gelatin methacrylate MPs, which offer greater control over cross-linking density than traditional glutaraldehyde cross-linked gelatin MPs. Modification of GMA MP cross-linking density modulates gelatin content, proteolytic degradation kinetics, mesh sizes, and the ability to bind and release growth factors. GMA MPs therefore provide a robust platform for controlled release of electrostatically coupled growth factors, particularly within a proteolytic environment, for emerging tissue engineering technologies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial support was provided by funding from the National Institutes of Health RO1 (GM088291). AHN was supported by an NIH training grant (GM008433) as well as an NSF Graduate Research Fellowship. We are grateful to Radu Reit for assistance with SEM analysis, Dr. Todd Sulchek for use of his AFM, as well as Marissa Cooke for critical review of the manuscript.

References

1. Yamamoto M, Ikada Y, Tabata Y. Controlled release of growth factors based on biodegradation of gelatin hydrogel. *J Biomater Sci Polym Ed.* 2001;77–88. [PubMed: 11334191]
2. Young S, Wong M, Tabata Y, Mikos AG. Gelatin as a delivery vehicle for the controlled release of bioactive molecules. *J Control Release.* 2005; 109:256–74. [PubMed: 16266768]

3. Patel ZS, Ueda H, Yamamoto M, Tabata Y, Mikos AG. In vitro and in vivo release of vascular endothelial growth factor from gelatin microparticles and biodegradable composite scaffolds. *Pharm Res.* 2008; 25:2370–8. [PubMed: 18663411]
4. Zhao Y, Liu Z, Pan C, Li Z, Zhou J, Wang J, et al. Preparation of gelatin microspheres encapsulated with bFGF for therapeutic angiogenesis in a canine ischemic hind limb. *J Biomater Sci Polym Ed.* 2011:665–82. [PubMed: 20566051]
5. Tabata Y, Nagano A, Ikada Y. Biodegradation of hydrogel carrier incorporating fibroblast growth factor. *Tissue Eng.* 1999; 5:127–38. [PubMed: 10358220]
6. Holland TA, Tessmar JKV, Tabata Y, Mikos AG. Transforming growth factor- β 1 release from oligo(poly(ethylene glycol) fumarate) hydrogels in conditions that model the cartilage wound healing environment. *J Control Release.* 2004; 94:101–14. [PubMed: 14684275]
7. Holland TA, Tabata Y, Mikos AG. Dual growth factor delivery from degradable oligo(poly(ethylene glycol) fumarate) hydrogel scaffolds for cartilage tissue engineering. *J Control Release.* 2005; 101:111–25. [PubMed: 15588898]
8. Cittadini A, Monti MG, Petrillo V, Esposito G, Imparato G, Luciani A, et al. Complementary therapeutic effects of dual delivery of insulin-like growth factor-1 and vascular endothelial growth factor by gelatin microspheres in experimental heart failure. *Eur J Heart Fail.* 2011; 13:1264–74. [PubMed: 22045926]
9. Ogawa T, Akazawa T, Tabata Y. In vitro proliferation and chondrogenic differentiation of rat bone marrow stem cells cultured with gelatin hydrogel microspheres for TGF- β 1 release. *J Biomater Sci Polym Ed.* 2010; 21:609–21. [PubMed: 20338095]
10. Solorio LD, Vieregge EL, Dhimi CD, Dang PN, Alsberg E. Engineered cartilage via self-assembled hMSC sheets with incorporated biodegradable gelatin microspheres releasing transforming growth factor- β 1. *J Control Release.* 2012; 158:224–32. [PubMed: 22100386]
11. Hayashi K, Tabata Y. Preparation of stem cell aggregates with gelatin microspheres to enhance biological functions. *Acta Biomater.* 2011; 7:2797–803. [PubMed: 21549223]
12. Baraniak PR, Cooke MT, Saeed R, Kinney MA, Fridley KM, McDevitt TC. Stiffening of human mesenchymal stem cell spheroid microenvironments induced by incorporation of gelatin microparticles. *J Mech Behav Biomed Mater.* 2012; 11:63–71. [PubMed: 22658155]
13. Solorio LD, Dhimi CD, Dang PN, Vieregge EL, Alsberg E. Spatiotemporal regulation of chondrogenic differentiation with controlled delivery of transforming growth factor- β 1 from gelatin microspheres in mesenchymal stem cell aggregates. *Stem Cells Transl Med.* 2012; 1:632–9. [PubMed: 23197869]
14. Bratt-Leal AM, Carpenedo RL, Ungrin MD, Zandstra PW, McDevitt TC. Incorporation of biomaterials in multicellular aggregates modulates pluripotent stem cell differentiation. *Biomaterials.* 2011; 32:48–56. [PubMed: 20864164]
15. Patel ZS, Yamamoto M, Ueda H, Tabata Y, Mikos AG. Biodegradable gelatin microparticles as delivery systems for the controlled release of bone morphogenetic protein-2. *Acta Biomater.* 2008; 4:1126–38. [PubMed: 18474452]
16. Liang H, Chang W. Crosslinking structures of gelatin hydrogels crosslinked with genipin or a water soluble carbodiimide. *J Appl Polym Sci.* 2004; 91:4017–26.
17. Damink LO, Dijkstra P. Glutaraldehyde as a crosslinking agent for collagen-based biomaterials. *J Mater Sci Mater Med.* 1995:4530.
18. Hardy P, Nicholls A, Rydon H. The Nature of the cross-linking of proteins by glutaraldehyde. Part 1. Interaction of glutaraldehyde with the amino-groups of 6-aminohexanoic acid and of α -N-acetyl-lysine. *JCS Perkin I.* 1969:958–62.
19. Woodroof E. Use of glutaraldehyde and formaldehyde to process tissue heart valves. *J Bioeng.* 1978; 2:1–9. [PubMed: 98522]
20. Bigi A, Cojazzi G, Panzavolta S, Rubini K, Roveri N. Mechanical and thermal properties of gelatin films at different degrees of glutaraldehyde crosslinking. *Biomaterials.* 2001; 22:763–8. [PubMed: 11246944]
21. Iwanaga K, Yabuta T, Kakemi M, Morimoto K, Tabata Y, Ikada Y. Usefulness of microspheres composed of gelatin with various cross-linking density. *J Microencapsul.* 2003; 20:767–76. [PubMed: 14594665]

22. Sung HW, Huang RN, Huang LLH, Tsai CC. In vitro evaluation of cytotoxicity of a naturally occurring cross-linking reagent for biological tissue fixation. *J Biomater Sci Polym Ed.* 1999; 10:63–78. [PubMed: 10091923]
23. Ruijgrok J, de Wijn J, Boon M. Glutaraldehyde crosslinking of collagen: effects of time, temperature, concentration and presoaking as measured by shrinkage temperature. *Clin Mater.* 1994; 17:23–7. [PubMed: 10150174]
24. Hoch E, Schuh C, Hirth T, Tovar GEM, Borchers K. Stiff gelatin hydrogels can be photo-chemically synthesized from low viscous gelatin solutions using molecularly functionalized gelatin with a high degree of methacrylation. *J Mater Sci Mater Med.* 2012; 23:2607–17. [PubMed: 22890515]
25. Van Den Bulcke A, Bogdanov B, De Rooze N, Schacht EH, Cornelissen M, Berghmans H. Structural and rheological properties of methacrylamide modified gelatin hydrogels. *Biomacromolecules.* 2000; 1:31–8. [PubMed: 11709840]
26. Sutter M, Siepmann J, Hennink WE, Jiskoot W. Recombinant gelatin hydrogels for the sustained release of proteins. *J Control Release.* 2007; 119:301–12. [PubMed: 17467099]
27. Nichol JW, Koshy ST, Bae H, Hwang CM, Yamanlar S, Khademhosseini A. Cell-laden microengineered gelatin methacrylate hydrogels. *Biomaterials.* 2010; 31:5536–44. [PubMed: 20417964]
28. Barbetta A, Dentini M, Zannoni EM, De Stefano ME. Tailoring the porosity and morphology of gelatin-methacrylate polyHIPE scaffolds for tissue engineering applications. *Langmuir.* 2005; 21:12333–41. [PubMed: 16343011]
29. Hu X, Ma L, Wang C, Gao C. Gelatin hydrogel prepared by photo-initiated polymerization and loaded with TGF-beta1 for cartilage tissue engineering. *Macromol Biosci.* 2009; 9:1194–201. [PubMed: 19890886]
30. Goodman TT, Olive PL, Pun SH. Increased nanoparticle penetration in collagenase-treated multicellular spheroids. *Int J Nanomedicine.* 2007; 2:265–74. [PubMed: 17722554]
31. Nichol JW, Khademhosseini A. Modular tissue engineering: engineering biological tissues from the bottom up. *Soft Matter.* 2009; 5:1312–9. [PubMed: 20179781]
32. Jung J, Oh J. Cell-induced flow-focusing instability in gelatin methacrylate microdroplet generation. *Biomicrofluidics.* 2014; 8:036503. [PubMed: 24932318]
33. Hsien T, Rorrer GL. Heterogeneous cross-linking of chitosan gel Beads : kinetics, modeling, and influence on cadmium ion adsorption capacity. *Ind Eng Chem Res.* 1997:3631–8.
34. Elbert DL, Hubbell JA. Conjugate addition reactions combined with free-radical cross-linking for the design of materials for tissue engineering. *Biomacromolecules.* 2001; 2:430–41. [PubMed: 11749203]
35. Bresler S, Kazbekov E, Shadrin V. Study of radical polymerization by means of ESR. *Die Makromol Chemie.* 1974:2875–80.
36. Pulieri E, Chiono V, Ciardelli G, Vozzi G, Ahluwalia A, Domenici C, et al. Chitosan/gelatin blends for biomedical applications. *J Biomed Mater Res Part A.* 2008; 86:311–22.
37. Tajima S, Tabata Y. Preparation and functional evaluation of cell aggregates incorporating gelatin microspheres with different degradabilities. *J Tissue Eng Regen Med.* 2012
38. Chen YC, Lin RZ, Qi H, Yang Y, Bae H, Melero-Martin JM, et al. Functional human vascular network generated in photocrosslinkable gelatin methacrylate hydrogels. *Adv Funct Mater.* 2012; 22:2027–39. [PubMed: 22907987]
39. Ho HO, Lin CW, Sheu MT. Diffusion characteristics of collagen film. *J Control Release.* 2001; 77:97–105. [PubMed: 11689263]
40. Dai W, Barbari T. Hydrogel membranes with mesh size asymmetry based on the gradient crosslinking of poly(vinyl alcohol). *J Memb Sci.* 1999; 156:67–79.
41. Gonçalves V, Laranjeira M, Fávere V, Pedrosa R. Effect of crosslinking agents on chitosan microspheres in controlled release of diclofenac sodium. *Polímeros.* 2005; 15:6–12.
42. Khan OF, Jean-Francois J, Sefton MV. MMP levels in the response to degradable implants in the presence of a hydroxamate-based matrix metalloproteinase sequestering biomaterial in vivo. *J Biomed Mater Res A.* 2010; 93:1368–79. [PubMed: 19911383]

43. Chen F, Zhang M, Wu Z. Toward delivery of multiple growth factors in tissue engineering. *Biomaterials*. 2010; 31:6279–308. [PubMed: 20493521]
44. Carpenedo RL, Bratt-Leal AM, Marklein RA, Seaman SA, Bowen NJ, McDonald JF, et al. Homogeneous and organized differentiation within embryoid bodies induced by microsphere-mediated delivery of small molecules. *Biomaterials*. 2009; 30:2507–15. [PubMed: 19162317]
45. Purpura KA, Bratt-Leal AM, Hammersmith KA, McDevitt TC, Zandstra PW. Systematic engineering of 3D pluripotent stem cell niches to guide blood development. *Biomaterials*. 2012; 33:1271–80. [PubMed: 22079776]

Figure 1. Gelatin methacrylate characterization

A) Schematic of methacrylate substitution of the primary amines of gelatin. B) ¹H NMR spectra was recorded for unsubstituted gelatin and GMA with 1:3, 2:3, and 1:1 mol methacrylic anhydride: mol unsubstituted amines on gelatin. The MA modification of lysine residues with increasing methacrylic anhydride addition can be confirmed by the continuous decrease in the lysine signal at $\delta = 2.9$ ppm (y), and increase in the methacrylate vinyl group signal at $\delta = 5.4$ ppm and 5.7 ppm (x) and methyl group signal at $\delta = 1.8$ ppm. C,D) Degree of methacrylate substitution was also determined for the GMA formulations via a Fluorescamine assay normalized to unmodified gelatin determined as 0% substitution.

Figure 2. Gelatin microparticle morphology and sizing

A) i–iv) Phase and v–viii) scanning electron microscopy images of GMA and GA cross-linked MPs indicate round morphology and smooth surfaces. Ai–iv) Scale bar: 100 μ m. Av–viii) Scale bar: 10 μ m. B) Coulter Counter size analysis of MPs indicate that no differences were found between MP sizes despite different extents of MA substitution. GMA MPs can be fabricated with a greater proportion of smaller sized MPs and lower quantities of large sized MPs than can be achieved through GA formulations. *: denotes statistical significance, n 3, p<0.05.

Figure 3. Microparticle gelatin composition and degradation

A) Gelatin content per volume of MPs was determined for each of the 4 gelatin MP formulations. The GA MPs had the highest gelatin content and for the GMA formulations, increasing the degree of methacrylation appeared to decrease the gelatin content. B) Degradation profiles were also determined via incubation of the MPs with collagenase. Increasing the methacrylation level resulted in an increase in MP degradation time, and GA MPs were comparable to the lowest methacrylated MP formulation in degradation profile. Degradation kinetics below 12 hours are expanded in C) for clearer visualization. The 15% MA and GA formulations were the most degraded by 12 hours, degrading completely by 48 hours. The higher methacrylated MPs were completely degraded by 96 hours. n 3, *: denotes statistical significance with $p < 0.05$ *: 90% MA vs. 15% MA and GA. ^: 15% MA vs. 50% MA. #: 50% MA vs. 15% MA and GA. +: 90% MA vs. 50% MA.

Figure 4. MP mechanical properties and mesh sizes

A) Elastic moduli were determined for MPs, which were conjugated to a glass coverslip and analyzed via AFM. The GA MPs and highest methacrylated MPs (90% MA) were significantly stiffer than the two lower methacrylated MPs (15% and 50%) by almost a magnitude. B) Swelling studies of MPs determined that all GMA MPs had a greater ability to swell in a hydrated environment than the GA MPs, with the 15% MA MPs swelling to a greater extent than all other MPs. C) Mesh sizes of the MPs were determined based on their elastic moduli and swelling ratios. The 15% and 50% MA MPs had larger mesh sizes than the GA MPs, as well as the highest methacrylated MPs (90% MA). E: elastic modulus. q: swelling ratio. ξ : mesh size. n = 15, *: denotes statistical significance with $p < 0.05$.

Figure 5. Microparticle growth factor loading capacity

Gelatin MPs were incubated with six concentrations of A) BMP4 and B) bFGF to determine the growth factor binding capacity. A) GA MPs bound less BMP4 than all GMA formulations at the lower loading concentrations (50 and 100 ng/mg) and bound less than the two lowest GMA formulations at the highest loading concentrations (200 and 300 ng/mg). B) While GA MPs bound minute BMP4, even at the highest loading concentration, the lowest GMA MPs had a peak binding efficiency at 99.6%. C) The GA MPs also bound less BMP4 than all GMA formulations at the lowest and highest loading concentrations (50,100 ng/mg and 200,300 ng/mg). D) bFGF loading efficiency peaked at 100 ng/mL for all MP formulations. n = 3, symbols denote statistical significance with p<0.05. *: GA vs. all GMA MPs, #: GA vs. 15% MA and 50% MA, \$: GA vs. 15% MA, &: 15% MA vs 90% MA, %: 50% MA vs 90% MA.

Figure 6. BMP4 and bFGF release from microparticles

The MPs were incubated with 10 ng/mg MPs of BMP4 (A–C) and 50 ng/mg MPs bFGF (D–F), and release kinetics was obtained over the course of 170 hours. A) Passive release of BMP4 was similar between all MP formulations. B) Greater release of BMP4 in 100 ng/mL collagenase was observed in the GA MPs compared to the 50% MA MPs at 60 hours. C) Larger BMP4 burst release at 3 hours was observed in the GA formulation compared to the higher methacrylated MPs with 1 µg/mL collagenase treatment. D) Less bFGF was released passively in the 50% MPs compared to the GA MPs after 25 hours. E) Greater bFGF was released from the GA MPs compared to the 50% MA MPs by 170 hours following treatment with 100 ng/mL collagenase. F) High collagenase treatment resulted in no observable differences in bFGF release between MP formulations. n = 3, symbols denote statistical significance with $p < 0.05$. *: GA vs. 50% MA, %: GA vs. 15% MA, \$: GA vs. 90% MA, #: 50% MA vs. 15% MA, &: 50% MA vs. 90% MA.

Figure 7. Time point specific collagenase-mediated BMP4 and bFGF release from MPs
A, C, E) BMP4 release at 3, 25, and 170 hours. Besides the initial release at 3 hours, GA MPs under protease treatment did not release any more BMP4 compared to passive release. On the other hand, the release of BMP4 from the higher methacrylated MPs, 50% MA and 90% MA, increased with collagenase treatment at all time points assayed, and this effect was sustained up to 170 hours. B, D, E) bFGF release at 3, 25, and 170 hours. No differences were observed between bFGF passive release and proteolytic-mediated release of GA MPs. However, an increase in bFGF released by the 50% MA MPs and 90% MA MPs were increased with collagenase treatment up to 25 hours and 170 hours, respectively. The three additional time points can be viewed in Supplemental Figure 2. *: denotes statistical significance, n = 3, p < 0.05.