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Tunable Hydrogel-Microsphere Composites that Modulate Local Inflammation and Collagen Bulking

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Abstract

Injectable biomaterials alone may alter local tissue responses, including inflammatory cascades and matrix production (e.g., stimulatory dermal fillers are used as volumizing agents that induce collagen production). To expand upon the available material compositions and timing of presentation, a tunable hyaluronic acid (HA) and poly(lactide-co-glycolide) (PLGA) microsphere composite system was formulated and assessed in subcutaneous and cardiac tissues. HA functionalized with hydroxyethyl methacrylate (HeMA) was used as a precursor to injectable and degradable hydrogels that carry PLGA microspheres (~50 μm diameter) to tissues, where the HA hydrogel degradation (~20 or 70 days) and quantity of PLGA microspheres (0–300 mg/ml) are readily varied. When implanted subcutaneously, faster hydrogel degradation and more microspheres (e.g., 75mg/mL) generally induced more rapid tissue and cellular interactions and a greater macrophage response. In cardiac applications, tissue bulking may be useful to alter stress profiles and to stabilize the tissue after infarction, limiting left ventricular (LV) remodeling. When fast degrading HeMA-HA hydrogels containing 75 mg/mL microspheres were injected into infarcted tissue in sheep, LV dilation was limited and the thickness of the myocardial wall and the presence of vessels in the apical infarct region were increased ~35% and ~60%, respectively, compared to empty hydrogels. Both groups decreased volume changes and infarct areas at 8 weeks, compared to untreated controls. This work illustrates the importance of material design in expanding the application of tissue bulking composites to a range of biomedical applications.

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Keywords

hyaluronic acid; hydrogel; microspheres; remodeling; myocardial infarction

1. Introduction

Various biomedical and cosmetic applications benefit from the ability to bulk tissue with biomaterials (e.g., dermal fillers) that act as void fillers or to stimulate tissue production. Such approaches have evolved beyond simple volumizing replacements towards stimulatory composites that promote a transient tissue filling effect by inducing a foreign body response (FBR). This promotes collagen production and a gradual increase in tissue bulking, resulting in a natural filling effect over time [1–5]. Most stimulatory fillers are comprised of two main components: 1) a particle that determines the type of FBR and bulking that is endured and 2) a carrier material that delivers the particle and is subsequently resorbed [1–5]. The mechanism behind this response relies on the activation and “crosstalk” between macrophages and fibroblasts, which is mediated by particle and carrier design.

When a foreign object is implanted into the body, proteins immediately adsorb to its surface and activate an inflammatory cascade that determines the cellular response and extent of tissue remodeling [6–10]. This entails an initial innate response comprised of neutrophil infiltration and macrophage phagocytosis, followed by a more specialized response that relies on macrophage plasticity [11, 12]. Macrophages have recently been loosely characterized as having M1 (pro-inflammatory) and M2 (pro-healing) phenotypes [13–17]. Classically activated (M1) macrophages are induced by IFN-, LPS, and TNF- and play a dominant role in the initial inflammation, and are characterized as inducing a chronic inflammatory response [14, 15]. Alternatively activated (M2) macrophages are stimulated by IL-4, IL-10, and IL-13 cytokines and are anti-inflammatory because of their ability to facilitate tissue remodeling [13–15]. It is thought that these macrophages, in particular, play a large role in secreting TGF- β s to stimulate fibroblasts to produce collagen for constructive tissue repair [8, 14, 18–22]. One approach to mediate a specific biomaterial response is to control the initial protein adsorption to the biomaterial [23–25]; however, there is overlap and switching between pathways, making it difficult to clearly distinguish and activate one or the other [13]. Regardless, advances have been made in understanding material responses and have led to the improved design of dermal fillers for constructive remodeling.

Towards particle design, specific focus has been placed on particle chemistry, geometry, and size. Hydrophilicity [4, 13], crosslinking [26], and biodegradability [4] all influence the degree of inflammation, and have impacted the material selection for particle stimuli. Particle geometry and size have also been extensively studied and generally smooth surfaces and a smaller area for a given volume induce a macrophage mediated constructive collagen bulking and although irregularly shaped particles do result in inflammation, they elicit a macrophage mediated chronic healing response leading to inconsistent collagen distribution [4, 27, 28]. Microsphere size should be small enough for injectable delivery and to prevent fibrous capsule isolation (typically < ~100 nm), but large enough to avoid macrophage phagocytosis (> ~10 nm), which can amplify an undesired chronic inflammatory response [3, 6, 7, 27, 29].

Dermal fillers have been applied beyond cosmetic application, and have been studied as bulking agents to prevent maladaptive LV remodeling post myocardial infarction (MI) [30, 31]. Specifically, Radiesse is an FDA approved stimulatory dermal filler comprised of a suspension of 30% calcium hydroxyapatite (CaHA) microspheres (25–45 nm) in a 70% aqueous gel carrier of water, glycerin, and carboxymethylcellulose [1–5]. As a stimulatory

filler, cells migrate to the foreign implant and infiltrate the carrier and induce fibroblasts to produce collagen around the microspheres. Morita et al. recently demonstrated that Radiesse injection into the infarcted myocardium thickened the myocardium and resulted in geometric and functional improvements [30]. Here, we strive to further understand and improve on work such as this by investigating how material design parameters within a composite system influences tissue outcomes.

In this investigation, we developed a tunable and biodegradable composite of a crosslinked HA hydrogel carrier containing PLGA microspheres. Previous work by Kang et al. demonstrated successful bulking via a suspension of PLGA microspheres in high MW HA [32]; motivated by their work, this crosslinkable system will provide the addition of tunable control over microsphere presentation through an actual HA hydrogel with controlled degradation. HA is a naturally occurring glycosaminoglycan found in the extracellular matrix (ECM) [33] that is commonly used in dermal fillers [1–5] and can be functionalized to form hydrogels with controlled properties [34, 35]. As in previous work [35], HA was modified with a HeMA group to tailor hydrogel gelation, degradation, and mechanics, as well as microsphere temporal presentation via crosslinking. PLGA particles have been used extensively [36–38] and, as briefly discussed, even to promote bulking [32]. Here, smooth PLGA microspheres (20–60 μ m) were fabricated and encapsulated at varying concentrations (0, 10, 75, 300 mg/mL) in two formulations of the HeMA-HA (low and high modified) carrier to examine material design considerations on local and temporal bulking. Composites were evaluated *in vivo* in a subcutaneous model and similarly to the work of Morita et al. in an MI model to determine how controlled microsphere presentation influences bulking.

2. Materials and Methods

All animals studied in this work received care in compliance with protocols from the University of Pennsylvania that were approved by the Institutional Animal Care and Use Committee according to the guidelines for humane care. Materials and reagents were purchased from Sigma Aldrich and used without further purification unless otherwise specified.

2.1. HeMA-HA synthesis

Two formulations of HeMA-HA (low and high modified) were synthesized as previously described [35]. Briefly, HA-sodium salt (HA-Na, Lifecore, 66 kDa) was converted to HA-tetrabutylammonium salt (HA-TBA) and HeMA-COOH was synthesized by reacting hydroxyethylmethacrylate (HeMA) with succinic anhydride via a ring opening reaction catalyzed by N-methylimidazole (NMI) in dichloroethane (DCE). HA-TBA was coupled to HeMA-COOH by the catalyst dimethylaminopyridine (DMAP) and coupling agent di-*t*-butyldicarbonate (BOC₂O) in dimethyl sulfoxide (DMSO) at 45°C for 20 hours. Products were dialyzed, purified by acetone precipitation, and dialyzed again. Methacrylation was adjusted by varying the ratio of HeMA-COOH and BOC₂O and assessed with ¹H NMR (Bruker).

2.2. PLGA microsphere fabrication

PLGA microspheres between 20–60 μ m were fabricated by forming oil-in-water (O/W) single emulsions in a glass microfluidic device [39]. The inner phase (O) was 4 wt% PLGA (Lactel Absorbable Polymers, 75:25, 0.71 dL/g) in dichloromethane (DCM) and the outer phase (W) was a 2 wt% polyvinyl alcohol (PVA, 13,000–23,000 g/mol) aqueous solution. The outer phase hydrodynamically focused the inner fluid stream into the collection channel, leading to the formation of single emulsions. DCM was removed by rotovap at 37°C and 398 mbar, microspheres were washed 3 $\times$ in DI-H₂O and then lyophilized for storage. Emulsion

formation was monitored on a Nikon Diaphot 300 and a Nikon TMD/TME automated stage microscope with Vision Research Phantom V7.1.

Microsphere size was quantified using light micrographs and ImageJ software. For morphological evaluation with degradation, microspheres (75 mg/mL) were incubated in PBS at 37°C, and samples were removed at pre-defined time points and imaged under scanning electron microscopy (SEM, JEOL 7500F HRSEM, Penn Regional Nanotechnology Facility). Degradation was also assessed by monitoring the pH of microsphere/PBS solutions (75 mg/mL) incubated at 37°C at the same time points as morphology studies.

2.3. In vitro composite formation and characterization

Microspheres were mixed (0, 10, 75, 300 mg/mL) and crosslinked within the HA hydrogels (either low or high HeMA-HA, 4wt%) by redox reactions with ammonium persulfate (APS) and tetramethylethylenediamine (TEMED) to form hydrogel composites. For *in vitro* characterization and *in vivo* subcutaneous evaluation, hydrogel composites were formed between two glass slides within a Teflon mold sealed with vacuum grease at 37°C for 30 minutes.

Gelation was evaluated by monitoring the storage (G') and loss (G'') modulus after mixing using an AR2000ex Rheometer (TA Instruments) at 37°C under 1% strain and a frequency of 1 Hz in a parallel plate geometry (20 mm diameter). Degradation of the hydrogel portion of the composites was examined at 37°C in PBS with a uronic acid assay [40]. Hydrogels and composites were assessed initially and with degradation (PBS at 37°C) via compressive testing using a Dynamic Mechanical Analyzer (DMA) (Q800 TA Instruments) at a strain rate of 10%/min and moduli were calculated between 10–20% strain.

2.4. In vivo subcutaneous evaluation

Microsphere/PBS solutions were sterilized under germicidal ultraviolet (UV) light for 1 hour. HeMA-HA macromer was similarly sterilized for 30 minutes on each side, and all solutions were filter sterilized. Hydrogel composites (~ 50 L, n=3 per group) were formed *ex vivo*, sterilized under germicidal UV light for 30 minutes on each side, and then implanted subcutaneously into dorsal pockets of rats. Explants were collected at 1, 2, and 4 weeks, fixed in 4% formalin overnight and paraffin embedded using standard histological techniques. Samples were processed into 7 μ m sections and stained with hematoxylin and eosin (H&E) and Masson's Trichrome (MT) to evaluate the extent of tissue (primarily collagen) integration.

For immunohistochemical analysis, all PBS was filtered and all washes were 2 minutes. Immunohistochemical staining was performed on low HeMA-HA composites at the 4 week time point to evaluate the phenotypic macrophage response by targeting surface markers: CD68 (general), CCR7 (pro-inflammatory, M1), and CD163 (pro-healing, M2). Primary antibodies used were mouse anti-rat CD68 (Serotec, MCA341R) at 1:100, rabbit anti-CCR7 (Cell Applications Inc., CA0710) at 1:200, and mouse anti-rat CD163 (Serotec, MCA342R) at 1:50; all were incubated with 1% normal horse serum (Vector, S-2000) (CD68 and CD163) or 1% goat serum (Vector, S-1000) (CCR7). Secondary antibodies used were biotinylated horse anti-mouse IgG (CD68 and CD163, Vector, BA-2000) at 1:100 and goat anti-rabbit IgG (CCR7, BA-1000) at 1:200, all incubated with 1.5% normal horse serum (CD68 and CD163) or 1.5% goat serum (CCR7).

Sections were deparaffinized with citrisolv and hydrated with a graded series of ethanol solutions ranging from 100% to 50%. Slides were placed in boiling antigen retrieval buffer (10 mM citric acid monohydrate, pH 6) for 20 minutes and then allowed to cool in the buffer. They were washed 2 $\times$ in tris buffer (tris buffered saline/ tween 20, pH 7.4) and 3 $\times$ in

PBS. Samples were blocked in 1.5% normal horse serum (CD68 and CD163) or 1.5% normal goat serum (CCR7) for 45 minutes at room temperature (RT). After removing blocking solution, samples were directly incubated with primary antibody (CD68, CCR7, or CD163) for 15 minutes at RT and overnight at 4°C in a humidified chamber. Following primary incubation, samples were washed 3× in PBS, quenched in 3% H₂O₂ in methanol for 30 minutes, and washed again 3× in PBS. Secondary antibody incubation was performed for 30 minutes at either RT (CD68) or at 37°C (CCR7 and CD163). Samples were washed 3× with PBS (CD68 and CD163) or 7× (CCR7) and incubated with Vectastain ABC reagent (Vector) for 1 hr (CD68 and CD163) or 30 min (CCR7) in a humidified chamber at 37°C. Samples were washed 3× with PBS and incubated with diaminobenzadine substrate (Dab, Vector, SK-4100) at RT for 1 minute. The reaction was stopped by washing 3× with DI-H₂O. Slides were counterstained with hematoxylin (Gill No. 2) for two minutes, dehydrated and cover slipped.

The phenotypic macrophage response due to microsphere concentration was analyzed in low HeMA-HA composites at week 4 by counting positively stained cells for each marker (CD68, CCR7, CD163) in each explant of each group (0, 10, 75, and 300 mg/mL, n=3). Six fields of view (FOV) at 400× magnification were analyzed; 3 FOV were counted at the biomaterial/tissue interface and 3 FOV in the surrounding tissue, which was defined as tissue more than 25 m away from the biomaterial. In samples where the composite was no longer present, 6 FOV of the tissue representing the area where the material was implanted were counted. Tissue area in each FOV was calculated by excluding microsphere and hydrogel area using ImageJ software, and cell number in each FOV was normalized by tissue area (mm²).

2.5. In vivo MI model

Microspheres, macromer, and all solutions were sterilized as in the subcutaneous studies. An established MI ovine model was used to evaluate the efficacy of the selected composite in attenuating LV remodeling [31]. Briefly, 6 adult male Dorset sheep (30–50 kg) were anesthetized, underwent a left thoracotomy to expose the heart, and were monitored for arterial, ventricular, and pulmonary artery pressure and electrocardiogram throughout the surgery. Baseline echocardiographic and hemodynamic data were obtained and followed by infarction, induced by ligation of the left anterior descending (LAD) and second diagonal coronary artery to create an infarct that was ~40% of the distance from the apex to the base of the heart. Thirty minutes post-MI, the composite (macromer/ microsphere) pre-polymer solution was mixed for 3 minutes and injected into the infarct (20, 0.3 cc injections).

Hemodynamic data and real time 3D echocardiographs were obtained before MI, 30 minutes post-MI, 30 minutes post-injection, and at 2 and 8 weeks after composite treatment. All animals were sacrificed at 8 weeks and a histological evaluation was performed (H&E and MT staining). Composite treated sheep were compared to infarct and low HeMA-HA controls from a previous study [35].

Vessel density was evaluated by staining for anti- α -smooth muscle actin (α -SMA, mouse anti-human, Dako, MO851). The apical, middle, and basilar regions of the infarct were examined in 5 sections from each group. In each section, vessel density was calculated in 3 FOV at 200× magnification at the apical and basilar regions and in 9 views in the middle region. Vessels were defined by the following criteria; all vessels were identified by positive α -SMA stain and were quantified in 3 ways, 1) all vessels greater than 10 μ m, 2) all vessels with visible lumen greater than 10 μ m, and 3) all thick vessels (vessels staining for more than 1 cell comprising the wall) greater than 10 μ m. The inflammatory response was examined by staining for major histocompatibility complex (MHC) class II (mouse anti-sheep, Serotec, MCA901).

Briefly, sections were deparaffinized, hydrated and quenched for endogenous peroxidase activity for 5 minutes in 4% H₂O₂ in DI-H₂O. After quenching, samples were washed 3× in Dako 1× wash buffer and incubated with primary antibody (SMA: 1:500, MHC Class II: 1:10) in Dako diluent at RT for 30 minutes in a humidified chamber. After incubation, samples were washed 3× in wash buffer and incubated with HRP labeled polymer (Dako, K4000) for 30 min at RT in a humidified chamber. After washing 3× in wash buffer, sections were incubated in Dab substrate (Vector, SK-4100) at RT. Samples were washed in DI-H₂O 3× to stop the reaction, counterstained in hematoxylin stain (Gill No. 2), dehydrated, and cover slipped.

2.6. Statistical analysis

Data is presented as either mean ± SD or mean ± SEM as indicated in the figure caption. All changes were assessed using a one-way ANOVA with Tukey's post hoc evaluation to account for differences between groups. $p < 0.05$ was considered statistically significant for comparisons.

3. Results and Discussion

3.1. HeMA-HA synthesis

HeMA-HA was synthesized (Fig. 1A) [35] and the extent of HeMA functionalization was varied by changing the ratio of HeMA to the coupling reagent BOC₂O during the synthesis to formulate macromers with a low (~10%) or high degree (~30%) of modification. HeMA-HA undergoes a radical polymerization through the methacrylate groups to form hydrogels that are able to undergo either enzymatic degradation through the HA backbone or hydrolytic degradation through the esters on the HeMA groups [35]. The extent of modification leads to changes in the crosslinking density, which alters the gelation, mechanics, and degradation behavior of this class of hydrogels. In this work, the rate of degradation also alters how fast the microspheres are released from the carrier hydrogel.

3.2. PLGA microsphere fabrication and characterization

PLGA microspheres were fabricated with a glass capillary microfluidic device to guarantee a smooth surface and high uniformity in size (Fig. 1B) [39], as opposed to standard homogenization techniques that resulted in variations in size and topography (data not shown). To form microspheres, the dispersed phase (4 wt% PLGA) and continuous phase (2% PVA) were delivered from opposite sides of the square outer capillary at adjusted flow rates until solutions formed a cone at the orifice of the collection tube and pinched off to form microspheres. The majority of microspheres obtained after solvent removal were smooth and between 20–60 μm, meeting the criteria for bulking agents (Fig. 1C) [3, 27–29]. In addition to being FDA approved and used for numerous TE applications [36–38], PLGA was selected because of its biodegradable nature. While it is desirable for microspheres to eventually degrade, it is also important that they remain stable and maintain a smooth geometry to promote constructive collagen remodeling. PLGA microsphere surfaces and diameters remained unchanged after 8 weeks (Fig. 1D) and there were minimal changes in pH (~7.4 to 7.2) during degradation (data not shown) suggesting insignificant degradation within 8 weeks. In addition to this work, others have shown that while the molecular weight (MW) of PLGA decreases in 8 weeks, the mass of the microspheres changes very little [41]. Thus, there is likely little release of acidic byproducts during the span of 8 weeks.

3.3. In vitro composite characterization

Composites were formed by crosslinking HeMA-HA formulations (low or high) in the presence of 0, 10, 75, or 300 mg/mL of PLGA microspheres (Fig. S1). Microspheres had insignificant effects on gel onset in most composites compared to empty HeMA-HA

hydrogels, where only the gelation of low HeMA-HA at 300 mg/mL was significantly increased (~40% slower than empty hydrogels, data not shown). Composite hydrogel mass loss in both low and high HeMA-HA hydrogels were influenced slightly by microsphere concentration; however, degradation trends remained generally comparable (Fig. 2A and 2B). In low HeMA-HA hydrogels, concentrations of 10 and 75 mg/mL significantly decreased the amount of mass loss compared to empty hydrogels, yet, their degradation rate was similar until the final week when they accelerated to compensate for initial lower mass losses (Fig. 2A). While results depict that a concentration of 300 mg/mL did not affect mass loss, this is likely not due to degradation and, instead, to incomplete gelation as suggested by slower gel onset. High HeMA-HA hydrogel mass loss was predominantly influenced at 10 and 300 mg/mL, being significantly lower at all time points compared to empty hydrogels except for at week 8 where only 10 mg/mL was affected (Fig. 2B). Significant differences in degradation rate were only observed at 7 days (300 mg/mL) and at 8 and 10 weeks (10mg/mL), as in low hydrogels, to make up for mass losses.

The presence of microspheres only increased the compressive modulus of hydrogels at the initial time points (Fig 2C and 2D). Specifically, significant increases in moduli were observed at 75 and 300 mg/mL in low HeMA-HA composites at day 0 compared to empty hydrogels (Fig. 2C) and at days 0 and 1 for 300 mg/mL in high HeMA-HA composites (Fig. 2D). Thus, *in vitro* characterization demonstrated that microspheres had minimal influences on composite material properties (particularly degradation and mechanics) and that differences are likely due to initial changes in the gelation around the microspheres. Nonetheless, composite material properties should be characterized when selecting a formulation for implantation.

3.4. In vivo subcutaneous evaluation

3.4.1. Macroscopic and histological evaluation—Macroscopic evaluation of subcutaneous explants demonstrated a temporal correlation in bulking with hydrogel formulation (low HeMA-HA vs. high HeMA-HA composites, Fig. 3). Specifically, the low HeMA-HA hydrogels were explanted as a mass of tissue at 4 weeks, whereas the more slowly degrading high HeMA-HA hydrogels remained primarily as hydrogel disks, surrounded by tissue. This is likely due to the slower hydrogel degradation behavior in the high formulations, limiting initial interactions of the material and microspheres with local tissue; whereas, the low formulation degrades within a few weeks and cells and tissue are able to interact more quickly with the microspheres. While gross evaluation revealed distinct differences in bulking with polymer modification (low vs. high), there did not appear to be a macroscopic response based on the microsphere introduction or concentration.

MT staining was performed to assess the influence of both macromer concentration and microspheres on bulking by imaging collagen infiltration within and surrounding each composite (Fig. 4). Collagen integrated at the edge of low HeMA-HA composites by 1 week and into composites by 2 weeks; whereas there was minimal collagen within the slowly degrading high HeMA-HA composites and was limited to the edge of the hydrogel. In contrast, to low HeMA-HA, which showed tissue integration within the composite, slower degrading high HeMA-HA composites resulted in the formation of a fibrous capsule at 4 weeks, limiting microsphere presentation. Due to negligible microsphere exposure in high HeMA-HA, microsphere effects on collagen bulking could only be assessed in low HeMA-HA composites. This highlights the importance of the design of the carrier material, when designing bulking formulations. At week 4 in the low formulations, it was evident that microsphere concentration influenced the tissue response with increasing collagen integration from 0 to 75 mg/mL in low HeMA-HA composites (Fig. 4) with primarily adipose tissue (determined by pathological assessment) in 0 and 10 mg/mL explants. The

high concentration of 300 mg/mL microsphere composites presented primarily aggregates of microspheres throughout the gel, limiting tissue and cellular interactions in some areas.

Interestingly, in contrast to *in vitro* degradation results, while empty hydrogels and 10 mg/mL composites appeared completely degraded by week 4, 75 and 300 mg/mL composites still had remnants of hydrogel composite present between infiltrated tissue. This observation may be due to a combination of the influence of the local tissue response on degradation and the suspected microsphere hydrophobic interactions. Staining showed that as tissue infiltrated the edge of the hydrogel, the hydrogel broke into smaller pieces. Composites with more microspheres may further decrease the rate of tissue infiltration due to additional hydrophobic interactions.

3.4.2. Immunohistochemical evaluation—Immunohistochemical staining for general, M1 (pro-inflammatory), and M2 (pro-healing) macrophages was performed to determine composite influences on the type of inflammatory response (Fig. 5). As an overall trend, for all markers, macrophage presence was highest at 75 and 300 mg/mL, suggesting inflammatory macrophage dependence on microsphere concentration. Composites at 75 mg/mL elicited the largest general macrophage response, significantly higher than with empty hydrogels (641 ± 113 vs. 93 ± 28 macrophages/ mm^2) (Fig. 6A). Lower numbers in 300 mg/mL composites could be due to observed microsphere aggregation limiting their cellular interactions. In contrast, in 75 mg/mL composites, cells and tissue were able to penetrate the hydrogel and readily access microspheres (Fig. 5).

Composites with 75 mg/mL microspheres resulted in a greater M1 (0 mg/mL: 41 ± 10 , 10 mg/mL: 61 ± 6 , 75 mg/mL: 171 ± 55 macrophages/ mm^2) and M2 (0 mg/mL: 25 ± 14 , 10 mg/mL: 24 ± 14 , 75 mg/mL: 80 ± 16 macrophages/ mm^2) response compared to less concentrated composites (Fig. 6B and 6C). The presence of fewer macrophages in empty and 10 mg/mL composites is likely due to complete hydrogel degradation by week 4 implying that they do not stimulate a sufficient inflammatory response to bulk. Low HeMA-HA 300 mg/mL composites, however, elicited the largest M2 response (300 mg/mL: 130 ± 25 macrophages/ mm^2) with significantly higher numbers than empty hydrogels and resulted in slightly greater M2 responses compared to their respective M1 (300 mg/mL: 99 ± 20 macrophages/ mm^2) response. This observation is not completely understood; however, it could be attributed to the plasticity of M2 macrophages [13], the time point investigated, and the nature of collagen distribution at the implant. As a general observation, more M2 macrophages were observed in the surrounding tissue, while M1 macrophages were more prominent in areas with tissue and biomaterial contact. It is speculated that this may be because M1 macrophages initiate a response with the composite as it is degrading; HA fragments have also been shown to induce a strong pro-inflammatory response [42]. After material degradation and acute inflammatory activity, M2 macrophages may also play a role in remodeling [20]. As discussed, in 300 mg/mL composites, there was a tendency to wall off composites and limit tissue penetration. Consequently, this restricted analysis in most samples to tissue interaction at the edge as opposed to within composites of infiltrated tissue regions. Prolonging this study may provide more information on long-term macrophage polarization with time after complete hydrogel degradation. However, this analysis supports a phenotypic dependence on composite design and the presence of microspheres. Due to the above results, it was determined to pursue the low HeMA-HA containing 75 mg/mL formulation for application as a bulking agent in cardiac repair.

3.5. In vivo MI evaluation

Subcutaneous work validated the ability of composites to temporally and locally control remodeling by varying hydrogel crosslinking and microsphere concentration, respectively.

Exploiting these tunable properties can be beneficial in injuries, such as MI, where bulking may improve outcomes, but where more control is necessary to effectively attenuate maladaptive geometric changes that occur [43]. Timing of treatment is critical due to the time dependent remodeling observed in MI where necrosis dominates for the first week and the overall process can extend for eight weeks and beyond [44]. Previous work with empty low HeMA-HA hydrogel revealed that compared to empty high HeMA-HA hydrogels it did not provide sufficient stability to attenuate long-term negative remodeling, which was attributed to its accelerated degradation and decrease in mechanics with time [35]. Other agents that have been investigated range from synthetic and natural materials; however, most of their bulking capacity relies on their integrity to support the damaged myocardium, and few directly stimulate and control natural thickening [43]. As discussed, Mortita et al. applied the already FDA approved dermal filler, Radiesse, to induce bulking in the myocardium post-MI and demonstrated its efficacy in limiting geometric changes [30]. This study and temporal MI remodeling provide motivation to improve on our previous approach by implementing low HeMA-HA composites where hydrogels carry microsphere stimuli to control delivery (avoiding prime necrosis 1 week post-MI) and potentially induce long lasting natural bulking and stability after hydrogel degradation.

Low HeMA-HA, 75 mg/mL was selected for this work because of its similar material properties (gelation, degradation and compressive moduli trends) to empty hydrogels and ability to induce a macrophage response in subcutaneous studies. At this formulation, material properties can for the most part be decoupled from microsphere effects and the contribution from microsphere stimuli can be directly compared to empty low HeMA-HA in attenuating LV remodeling.

LV remodeling leads to a series of adverse changes (thinning of the myocardial wall, dilation and infarct expansion) that can negatively affect function and lead to heart failure [45–48]. When analyzing normalized thickness, both composite and hydrogel alone treatments significantly thickened the apical infarct region compared to infarct controls (composite: 0.40, empty: 0.29, infarct control: 0.18, normal: 0.52) (Fig. 7A); however, the composite also significantly thickened the apical region further compared to empty hydrogels. Increased thicknesses were also observed within the basilar infarct region for both hydrogel groups (composite: 0.51, empty: 0.51, infarct control: 0.38, normal: 0.72), whereas all groups were similar in the borderzone (BZ) region. MT staining in the apical, middle, and basilar infarct regions demonstrated cellular infiltration around microspheres surrounded by a collagen network (Fig. 7B). In assessing dilation, composite hydrogels showed trends in decreasing normalized end diastolic volume (NEDV) and normalized end systolic volume (NESV) changes at 2 and 8 weeks compared to infarct controls and empty hydrogels, particularly at 2 weeks (composite 2W NEDV: 1.63, composite 2W NESV: 1.94, composite 8W NEDV: 1.98, composite 8W NESV: 2.50 vs. empty 2W NEDV: 1.85, empty 2W NESV: 2.22, empty 8W NEDV: 2.15, empty 8W NESV: 2.61 vs. infarct 2W NEDV: 1.75, infarct 2W NESV: 2.21, infarct 8W NEDV: 2.31, infarct 8W NESV: 3.02) (Fig. 8A). This suggests that compared to empty hydrogels, composites were more effective in attenuating geometric changes associated with MI, specifically by bulking and stabilizing the myocardial wall. Post-MI the area of non-viable myocardium expands as remodeling progresses; consequently, several groups have assessed infarct area to evaluate the efficacy of their treatment [42]. Infarct area was significantly decreased by both composite and empty low HeMA-HA treatments compared to infarct controls (composite: 22.9%, empty: 22.5%, infarct control: 29.0%) (Fig. 8B), yet there was no statistical difference between hydrogel groups.

Another important parameter in infarct remodeling and repair is the supply of nutrients to myocardium through vessel growth in ischemic tissue [49–55]. In this study, infarct control

(Fig. 9A), empty hydrogel (Fig. 9B) and composite (Fig. 9C) treated infarct tissues were stained for -SMA to identify vessels. Quantification revealed increases in all vessels over 10 μ m (Fig. 9D), with lumen (Fig. 9E), and thick vessels (Fig. 9F) in both composite and empty cohorts. Overall, composites tended to result in the highest vessel number, with significant increases in thicker vessels compared to infarct controls; however, there were no significant differences compared to empty hydrogels. Interestingly, composite stimulated vessel development was prominent at the apex region, agreeing with thickness data where composite therapy significantly bulked this region. Vessel assessment suggests that biological and mechanical factors come into play in attenuating LV remodeling. Additionally, HA may influence vessel development and is thought to be a contributing stimulator in this system [32, 33, 56]; however, there is no difference in the rate of HA release between the gel only and composite groups.

MHC class II was performed to assess inflammation at 8 weeks in infarct controls and in empty hydrogel and composite treatments to assess inflammation after hydrogel degradation. MHC class II proteins are expressed on antigen presenting cells such as macrophages, dendritic cells, and B lymphocytes, which later present foreign digested fragments to stimulate an adaptive immune response [57, 58]. Investigating MHC Class II expressing cells, thus, provides a general idea on the degree of inflammation induced by each group. Both treatments resulted in more positive staining for MHC Class II cells compared to infarct controls. While a few samples displayed positive staining around microspheres, in general, empty and composite hydrogels induced a similar inflammatory response (Fig. S2).

MI work agrees with Radesse studies by Morita et al., confirming similar geometric trends [30]. While composite design enhanced low HeMA-HA treatment, it is important to place this work in perspective with the rest of the field, and note that other stable agents with proven high mechanics have provided higher degrees of prolonged support in MI [34]. As previously discussed, composite benefits could potentially be improved upon by tailoring composite design (microsphere concentration and HA crosslinking) and material properties.

4. Conclusion

A composite hydrogel microsphere system was developed with tunable material properties (gelation, degradation, and mechanics) to adjust the extent of inflammation and collagen bulking. Subcutaneous *in vivo* work demonstrated a temporal dependence on macromer functionalization and a local dependence on microsphere concentration. This system can potentially benefit applications that require controlled collagen remodeling, such as MI treatment. Composites improved on the design of low HeMA-HA for MI repair by stimulating myocardial bulking and providing stability to attenuate maladaptive geometric changes. This investigation with tunable HeMA-HA/PLGA microsphere composites shows promise to broaden the application of stimulatory fillers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- [1]. Carruthers, J. a continueing medical education program. Medical Insight Inc; Aliso, Viejo, CA: 2006. Review of long-lasting dermal fillers; p. 1-27.
- [2]. Goldberg DJ. Breakthroughs in US dermal fillers for facial soft-tissue augmentation. *J Cosmet Laser Ther.* 2009; 11:240–247. [PubMed: 19951196]
- [3]. Kusin S, Lippitz J. Skin fillers. *Dis Mon.* 2009; 55:236–256. [PubMed: 19286057]
- [4]. Nicolau PJ. Long-lasting and permanent fillers: biomaterial influence over host tissue response. *Plast Reconstr Surg.* 2007; 119:2271–2286. [PubMed: 17519731]
- [5]. Jones DH. Semipermanent and permanent injectable fillers. *Dermatol Clin.* 2009; 27:433–444. [PubMed: 19850193]
- [6]. Anderson JM. Biological responses to materials. *Annu. Rev. Mater. Res.* 2001; 31:81–101.
- [7]. Anderson JM, Rodriguez A, Chang DT. Foreign body reaction to biomaterials. *Semin Immunol.* 2008; 20:86–100. [PubMed: 18162407]
- [8]. Badylak SF, Gilbert TW. Immune response to biologic scaffold materials. *Semin Immunol.* 2008; 20:109–116. [PubMed: 18083531]
- [9]. Madden LR, et al. Proangiogenic scaffolds as functional templates for cardiac tissue engineering. *Proc Natl Acad Sci U S A.* 2010; 107:15211–15216. [PubMed: 20696917]
- [10]. Mikos AG, McIntire LV, Anderson JM, Babensee JE. Host response to tissue engineered devices. *Adv Drug Deliv Rev.* 1998; 33:111–139. [PubMed: 10837656]
- [11]. Mills CD, Kincaid K, Alt JM, Heilman MJ, Hill AM. M-1/M-2 macrophages and the Th1/Th2 paradigm. *J Immunol.* 2000; 164:6166–6173. [PubMed: 10843666]
- [12]. Stout RD, Jiang C, Matta B, Tietzel I, Watkins SK, Suttles J. Macrophages sequentially change their functional phenotype in response to changes in microenvironmental influences. *J Immunol.* 2005; 175:342–349. [PubMed: 15972667]
- [13]. Kou PM, Babensee JE. Macrophage and dendritic cell phenotypic diversity in the context of biomaterials. *J Biomed Mater Res A.* 2011; 96:239–260. [PubMed: 21105173]
- [14]. Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol.* 2004; 25:677–686. [PubMed: 15530839]
- [15]. Martinez FO, Sica A, Mantovani A, Locati M. Macrophage activation and polarization. *Front Biosci.* 2008; 13:453–461. [PubMed: 17981560]
- [16]. Wang W, et al. Immunomodulatory activity of andrographolide on macrophage activation and specific antibody response. *Acta Pharmacol Sin.* 2010; 31:191–201. [PubMed: 20139902]
- [17]. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol.* 2008; 8:958–969. [PubMed: 19029990]
- [18]. Badylak SF, Valentin JE, Ravindra AK, McCabe GP, Stewart-Akers AM. Macrophage phenotype as a determinant of biologic scaffold remodeling. *Tissue Eng Part A.* 2008; 14:1835–1842. [PubMed: 18950271]
- [19]. Brown BN, Valentin JE, Stewart-Akers AM, McCabe GP, Badylak SF. Macrophage phenotype and remodeling outcomes in response to biologic scaffolds with and without a cellular component. *Biomaterials.* 2009; 30:1482–1491. [PubMed: 19121538]
- [20]. Duffield JS. The inflammatory macrophage: a story of Jekyll and Hyde. *Clin Sci (Lond).* 2003; 104:27–38. [PubMed: 12519085]
- [21]. Mosser DM. The many faces of macrophage activation. *J Leukoc Biol.* 2003; 73:209–212. [PubMed: 12554797]
- [22]. Song E, Ouyang N, Horbelt M, Antus B, Wang M, Exton MS. Influence of alternatively and classically activated macrophages on fibrogenic activities of human fibroblasts. *Cell Immunol.* 2000; 204:19–28. [PubMed: 11006014]
- [23]. Kao WJ, Hubbell JA, Anderson JM. Protein-mediated macrophage adhesion and activation on biomaterials: a model for modulating cell behavior. *J Mater Sci Mater Med.* 1999; 10:601–605. [PubMed: 15347973]

- [24]. McNally AK, Anderson JM. Complement C3 participation in monocyte adhesion to different surfaces. *Proc Natl Acad Sci U S A*. 1994; 91:10119–10123. [PubMed: 7937848]
- [25]. Wilson CJ, Clegg RE, Leavesley DI, Percy MJ. Mediation of biomaterial-cell interactions by adsorbed proteins: a review. *Tissue Eng*. 2005; 11:1–18. [PubMed: 15738657]
- [26]. Brown BN, Barnes CA, Kasick RT, Michel R, Gilbert TW, Beer-Stolz D, Castner DG, Ratner BD, Badylak SF. Surface characterization of extracellular matrix scaffolds. *Biomaterials*. 31:428–437. [PubMed: 19828192]
- [27]. Gelb H, Schumacher HR, Cuckler J, Ducheyne P, Baker DG. In vivo inflammatory response to polymethylmethacrylate particulate debris: effect of size, morphology, and surface area. *J Orthop Res*. 1994; 12:83–92. [PubMed: 8113946]
- [28]. Ziats NP, Miller KM, Anderson JM. In vitro and in vivo interactions of cells with biomaterials. *Biomaterials*. 1988; 9:5–13. [PubMed: 3280039]
- [29]. Lemperle G, Morhenn VB, Pestonjamas V, Gallo RL. Migration studies and histology of injectable microspheres of different sizes in mice. *Plast Reconstr Surg*. 2004; 113:1380–1390. [PubMed: 15060350]
- [30]. Morita M, et al. Modification of infarct material properties limits adverse ventricular remodeling. *Ann Thorac Surg*. 92:617–624. [PubMed: 21801916]
- [31]. Ryan LP, et al. Dermal filler injection: a novel approach for limiting infarct expansion. *Ann Thorac Surg*. 2009; 87:148–155. [PubMed: 19101288]
- [32]. Kang S, CE, Kim B. PLGA microspheres in hyaluronic acid gel as a potential bulking agent for urologic and dermatologic injection therapies. *J Microbio and Biotech*. 2004; 15:510–519.
- [33]. Toole BP. Hyaluronan in morphogenesis. *Semin Cell Dev Biol*. 2001; 12:79–87. [PubMed: 11292373]
- [34]. Ifkovits JL, et al. Injectable hydrogel properties influence infarct expansion and extent of postinfarction left ventricular remodeling in an ovine model. *Proc Natl Acad Sci U S A*. 2010; 107:11507–11512. [PubMed: 20534527]
- [35]. Tous E, et al. Influence of injectable hyaluronic acid hydrogel degradation behavior on infarction-induced ventricular remodeling. *Biomacromolecules*. 2011; 12:4127–4135. [PubMed: 21967486]
- [36]. Cohen S, Yoshioka T, Lucarelli M, Hwang LH, Langer R. Controlled delivery systems for proteins based on poly(lactic/glycolic acid) microspheres. *Pharm Res*. 1991; 8:713–720. [PubMed: 2062800]
- [37]. Newman KD, McBurney MW. Poly(D,L lactic-co-glycolic acid) microspheres as biodegradable microcarriers for pluripotent stem cells. *Biomaterials*. 2004; 25:5763–5771. [PubMed: 15147822]
- [38]. Oldham JB, et al. Biological activity of rhBMP-2 released from PLGA microspheres. *J Biomech Eng*. 2000; 122:289–292. [PubMed: 10923299]
- [39]. Shah RK, et al. Designer emulsions using microfluidic. *Materialstoday*. 2008; 11:18–28.
- [40]. Platzer M, Ozegowski JH, Neubert RH. Quantification of hyaluronan in pharmaceutical formulations using high performance capillary electrophoresis and the modified uronic acid carbazole reaction. *J Pharm Biomed Anal*. 1999; 21:491–496. [PubMed: 10701415]
- [41]. Kang SW, Cho ER, Jeon O, Kim BS. The effect of microsphere degradation rate on the efficacy of polymeric microspheres as bulking agents: an 18-month follow-up study. *J Biomed Mater Res B Appl Biomater*. 2007; 80:253–259. [PubMed: 16850474]
- [42]. Zhang X, Mosser DM. Macrophage activation by endogenous danger signals. *J Pathol*. 2008; 214:161–178. [PubMed: 18161744]
- [43]. Tous E, Purcell B, Ifkovits JL, Burdick JA. Injectable acellular hydrogels for cardiac repair. *J Cardiovasc Transl Res*. 4:528–542. [PubMed: 21710332]
- [44]. Holmes JW, Borg TK, Covell JW. Structure and mechanics of healing myocardial infarcts. *Annu Rev Biomed Eng*. 2005; 7:223–253. [PubMed: 16004571]
- [45]. Jackson BM, et al. Border zone geometry increases wall stress after myocardial infarction: contrast echocardiographic assessment. *Am J Physiol Heart Circ Physiol*. 2003; 284:H475–479. [PubMed: 12414441]

- [46]. Pfeffer MA, Braunwald E. Ventricular remodeling after myocardial infarction. Experimental observations and clinical implications. *Circulation*. 1990; 81:1161–1172. [PubMed: 2138525]
- [47]. Pfeffer MA, Pfeffer JM. Ventricular enlargement and reduced survival after myocardial infarction. *Circulation*. 1987; 75:IV93–97. [PubMed: 2952370]
- [48]. Pilla JJ, Gorman JH 3rd, Gorman RC. Theoretic impact of infarct compliance on left ventricular function. *Ann Thorac Surg*. 2009; 87:803–810. [PubMed: 19231393]
- [49]. Christman KL, Fang Q, Yee MS, Johnson KR, Sievers RE, Lee RJ. Enhanced neovasculture formation in ischemic myocardium following delivery of pleiotrophin plasmid in a biopolymer. *Biomaterials*. 2005; 26:1139–1144. [PubMed: 15451633]
- [50]. Fujita M, et al. Efficacy of photocrosslinkable chitosan hydrogel containing fibroblast growth factor-2 in a rabbit model of chronic myocardial infarction. *J Surg Res*. 2005; 126:27–33. [PubMed: 15916971]
- [51]. Hao X, et al. Angiogenic effects of sequential release of VEGF-A165 and PDGFBB with alginate hydrogels after myocardial infarction. *Cardiovasc Res*. 2007; 75:178–185. [PubMed: 17481597]
- [52]. Iwakura A, Fujita M, Kataoka K, Tambara K, Sakakibara Y, Komeda M, Tabata Y. Intramyocardial sustained delivery of basic fibroblast growth factor improves angiogenesis and ventricular function in a rat infarct model. *Heart Vessels*. 2003; 18:93–99. [PubMed: 12756606]
- [53]. Liu Y, Sun L, Huan Y, Zhao H, Deng J. Effects of basic fibroblast growth factor microspheres on angiogenesis in ischemic myocardium and cardiac function: analysis with dobutamine cardiovascular magnetic resonance tagging. *Eur J Cardiothorac Surg*. 2006; 30:103–107. [PubMed: 16730451]
- [54]. Wu J, Zeng F, Huang XP, Chung JC, Konecny F, Weisel RD, Li RK. Infarct stabilization and cardiac repair with a VEGF-conjugated, injectable hydrogel. *Biomaterials*. 2011; 32:579–586. [PubMed: 20932570]
- [55]. Yamamoto T, et al. Intramyocardial delivery of basic fibroblast growth factor-impregnated gelatin hydrogel microspheres enhances collateral circulation to infarcted canine myocardium. *Jpn Circ J*. 2001; 65:439–444. [PubMed: 11348050]
- [56]. Yoon SJ, Fang YH, Lim CH, Kim BS, Son HS, Park Y, Sun K. Regeneration of ischemic heart using hyaluronic acid-based injectable hydrogel. *Journal of Biomedical Materials Research Part B: Applied Biomaterials*. 2008:163–172.
- [57]. Alberts, B.; Johnson, A.; Lewis, J.; Raff, M.; Roberts, K.; Walter, P. *Molecular Biology of The Cell*. 4th ed. Garland Science; New York: 2002. T Cells and MHC proteins; p. 18
- [58]. Mitchell, RN. Innate and adaptive immunity: the immune response to foreign materials. In: Ratner, BD.; Hoffman, AS.; Schoen, FJ.; Lemons, JE., editors. *Biomaterials Science*. Vol. vol. 2. Elsevier Academic Press; San Diego: 2004. p. 304

Fig. 1. HeMA-HA chemical synthesis (A). PLGA microsphere fabrication in glass capillary microfluidic device (B) and resulting microsphere size distribution and SEM image (inset) (C). Microsphere SEM images with degradation up to 8 weeks (D). Scale bar = 250 m in B and scale bar = 40 m in C and D.

Fig. 2.

In vitro characterization of low HeMA-HA (A) and high HeMA-HA (B) hydrogel composite degradation and low HeMA-HA (C) and high HeMA-HA (D) hydrogel composite mechanics with degradation. Data presented as mean $\pm$ SD. * $p < 0.05$ for 10 mg/mL vs. 0 mg/mL, \$ $p < 0.05$ for 75 mg/mL vs. 0 mg/mL, + $p < 0.05$ for 300 mg/mL vs. 0 mg/mL.

Fig. 3. Macroscopic evaluation of bulking in low and high HeMA-HA hydrogel composites at 1 day (D1), 1 week (W1), 2 weeks (W2) and 4 weeks (W4) after subcutaneous implantation and containing 0, 10, 75, or 300 mg/mL of microspheres. Scale bar = 5 mm.

Fig. 4. Masson's Trichrome staining of explants 1 week (W1), 2 weeks (W2) and 4 weeks (W4) after subcutaneous implantation and containing 0, 10, 75, or 300 mg/mL of microspheres. Scale bar = 500 μ m. Red circles and red positive signs represent gel and adipose tissue, respectively.

Fig. 5. Immunohistochemical evaluation of macrophage phenotype in composites at week 4. Top row is Masson's Trichrome (MT) stain, scale bar=200 m. Other rows are general (CD68) and M1 (CCR7) and M2 (CD163) macrophage stains, scale bar=30 m. Red circles represent gel. Red stars and red arrows indicate representative microspheres and macrophages respectively.

Fig. 6. Quantification of general (A), M1 (B) and M2 (C) macrophage phenotype in low HeMA-HA composites at week 4 from immunohistochemistry. Data presented as mean $\pm$ SEM. * $p < 0.05$ vs. 0 mg/mL.

Fig. 7. Myocardial wall thickness in apical infarct, basilar infarct, and borderzone (BZ) regions normalized to remote healthy myocardium (A) and Masson's Trichrome staining in the basilar, middle, and apical infarct regions (B). Data are presented as mean $\pm$ SEM. All groups are significantly different from normal control in apical and basilar regions and from infarct control in the apical region. * $p < 0.05$ vs. empty Low HeMA-HA. Scale bar = 100 μ m.

Fig. 8.

Normalized end diastolic volume (NEDV) and normalized end systolic volume (NESV) at 2 and 8 weeks (2W NEDV and NESV and 8W NEDV and NESV) (A) and initial infarct length and infarct area at week 8 (B). Data presented as mean $\pm$ SEM. * $p < 0.05$ vs. infarct control.

Fig. 9.

Staining for -SMA to identify vessels in infarct controls (A) and empty (B) and composite hydrogels (C). Quantification of all vessels greater than 10 m (D), all vessels greater than 10 m with lumen (E), and all thick vessels greater than 10 m (F). Scale bar = 100 m. Data presented as mean $\pm$ SEM. * $p < 0.05$ vs. infarct control. Red arrows = representative thick-walled vessels. Black arrows = representative thin-walled vessels.