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ARTICLE · AUGUST 2015

DOI: 10.1021/acsbiomaterials.5b00077

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Coacervate Delivery of Growth Factors Combined with a Degradable Hydrogel Preserves Heart Function after Myocardial Infarction

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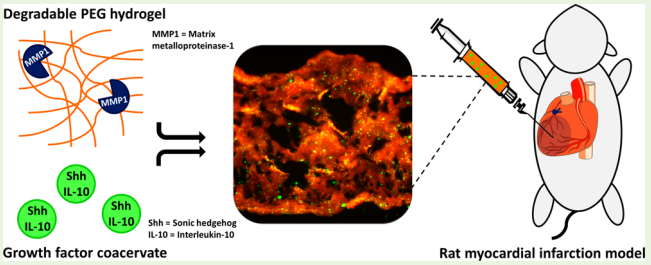
S *Supporting Information*

ABSTRACT: Regenerative therapies to improve prognosis after heart attack and mitigate the onset of heart failure are urgently needed. To this end, we developed a bioactive therapy of sustained release of the morphogen Sonic hedgehog (Shh) and the anti-inflammatory cytokine interleukin-10 (IL-10) from a coacervate delivery vehicle. This is combined with a structural therapy consisting of a biodegradable polyethylene glycol (PEG) hydrogel, harnessing the benefits of both components. Upon injection into the hearts of rats after heart attack, we found that each component synergistically improved the benefit of the other. Furthermore, their combination was critical to preserve heart function. These findings indicate that, when combined, growth factor delivery and an injectable hydrogel represent a promising therapeutic approach for treatment after heart attack.



KEYWORDS: *coacervate, controlled release, hydrogel, interleukin-10, myocardial infarction, sonic hedgehog*

INTRODUCTION

Ischemic heart disease has been the leading cause of death in the U.S. and worldwide for years.[1] Myocardial infarction (MI) is the most prevalent and life-threatening manifestation of progressed heart disease and commonly leads to congestive heart failure in those who survive.[2] Current treatments patients receive after MI aim to slow the progression of heart failure, with total heart transplantation being the only cure; still, there is insufficient availability and transplantation is not without complications.[3] Thus, new therapies seeking to preserve the function of the patient's own heart after MI are urgently needed.

Numerous technologies have been evaluated for alleviating or reversing the damage caused by cardiac ischemia.[4−6] These technologies fall into two general categories: bioactive therapies and structural "bulking" therapies. The former aims to provide the resources and signals necessary for tissue preservation and regeneration, and several therapeutic approaches including stem cells,[7−10] growth factors,[11] and gene therapy[12] have been evaluated in U.S. controlled clinical trials. On the other hand, the primary aim of structural therapies is to reinforce the damaged heart wall and thereby alleviate wall stress to prevent wall thinning and ventricular dilation.[5,6] Clinical trials have been initiated to evaluate intramyocardial injections of alginate and cell-seeded collagen.[13,14] However, no biological or structural therapy has shown enough promise to receive USFDA approval for treatment of MI. Therefore, new approaches need to be developed and those that combine both bioactive and structural components are particularly appealing.[15]

Sonic hedgehog (Shh) is a potent morphogen that plays a principal role in the developmental patterning of the embryonic heart.[16] Shh signaling is a promising therapeutic target for heart repair because it functions through three distinct mechanisms: angiogenesis, cardioprotection, and progenitor cell recruitment, which together may result in a strong functional benefit.[17] Its therapeutic potential has been previously evaluated through gene transfection,[18,19] transduced cell injection,[20] and bolus protein injection.[21] We previously evaluated a heparin-based coacervate delivery system for the controlled release of Shh protein.[22] Complex coacervates are self-assembled liquid droplets held together by ionic interactions between molecules of opposite charge.[23] In our delivery system, these molecules are heparin and a synthetic polycation. Growth factors are prebound to heparin, then the polycation is added to form micrometer-sized coacervates within which the growth factors

Received: February 13, 2015
Accepted: July 30, 2015

DOI: 10.1021/acsbiomaterials.5b00077
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are protected from degradation.[24] Shh is released slowly from the coacervate over 3 weeks in vitro and is highly bioactive, capable of stimulating cardiac fibroblasts to upregulate expression of multiple pro-angiogenic and pro-survival factors.[22] Shh coacervate also protected cardiomyocytes under oxidative stress.[22] These results warranted the investigation of Shh coacervate in an animal model of MI. However, the possibility of the liquid coacervate being suboptimally retained in the heart wall after injection posed a significant concern. We were therefore motivated to utilize an injectable hydrogel as an entrapment medium to improve the retention of the coacervate.

Poly(ethylene glycol) (PEG) is a suitable hydrogel material because it has variable stiffness based on polymer concentration and cross-linking and it is biologically inert.[25] We have previously described the structural benefit of a nondegradable PEG hydrogel, which prevented early ventricular wall thinning and dilation. However, it was unable to mitigate long-term pathological remodeling, likely due to chronic inflammation.[26] We therefore assessed the utility of a PEG hydrogel cross-linked by peptides with incorporated matrix metalloproteinase-1 (MMP1)-specific degradation sequences. The degradable nature of this hydrogel appeared to pacify the issue of chronic inflammation. However, functional improvement was only observed if injection was delayed until 1 week after infarction, in part due to the rapid hydrogel degradation if injected immediately after MI.[27] Yet cardioprotective therapies such as Shh will be most effective if applied as soon as possible after ischemic event. We therefore elected to include interleukin (IL)-10, an anti-inflammatory cytokine we hypothesized would inhibit rapid hydrogel degradation by reducing the inflammatory response to MI.

The anti-inflammatory effects of IL-10 are in part due to a downregulation of IL-1β, IL-6, and tumor necrosis factor-α signaling.[28] IL-10 thereby restricts macrophage activation and phagocytic capacity,[29] and promotes their transition from the M1 to the M2 phenotype, which is also critical to enable tissue regeneration.[30] Of particular relevance here, IL-10 has additionally been shown to inhibit MMP production and stimulate biosynthesis of tissue inhibitor of metalloproteinases (TIMP).[31] IL-10 expression in the infarcted human myocardium has been positively correlated with improved ventricular function,[32] and direct injection of IL-10 attenuated ventricular remodeling in mice.[33] Furthermore, heparin binds IL-10 with high affinity (K_d = 54 nM) and modulates its bioactivity,[34] permitting its delivery by the heparin-based coacervate alongside Shh.

Here we describe a combined treatment of a controlled release coacervate of Shh and IL-10 distributed within an MMP-degradable PEG hydrogel. The combination of regenerative molecules and a structural bulking agent has significant potential for synergistic effects. While the hydrogel may improve coacervate retention within the heart wall after injection, the regenerative environment engendered by Shh and IL-10 may improve the integration of the hydrogel into the native tissue and promote its structural benefit. We evaluated this combined treatment using an established in vivo MI model of permanent coronary ligation in rats.[35]

MATERIALS AND METHODS

Coacervate and PEG Hydrogel Preparation. To prepare the coacervate, we synthesized poly(ethylene argininylaspartate digylceride) (PEAD) as previously described.[36] PEAD and clinical-grade heparin (Scientific Protein Laboratories, Waunakee, WI) were each dissolved in DI water and 0.22 μm filter-sterilized. Heparin (0.33 mg) was combined with 1.5 μg of recombinant human Shh (Peprotech, Rocky Hill, NJ) and 150 ng of recombinant human IL-10 (Peprotech), then 1.65 mg PEAD was added to form the coacervate. The coacervate was pelleted by centrifugation and the supernatant removed and discarded. PEG hydrogels were polymerized by Michael-type addition reaction between vinyl sulfone groups on derivatized PEG and thiols of bis-cysteine MMP1-sensitive peptides. Vinyl sulfone-derivatized 8-arm PEG (MW = 20 kDa) was synthesized as previously described[26] and dissolved in PBS. A bis-cysteine peptide sensitive to MMP1 of the following sequence was used: CREGPQGIWGQERCG (GenScript, Puyyiscataway, NJ). The MMP1-sensitive peptide was dissolved in PBS and then used to resuspend the coacervate thoroughly. PEG and MMP1-sensitive peptide solutions were combined to form 10% w/v PEG hydrogels of 100 μL total volume with the Shh+IL-10 coacervate homogeneously dispersed throughout.

Fluorescence Labeling and Imaging. The coacervate was prepared as described above, except fluorescein-labeled heparin (Creative PEGWorks, Winston Salem, NC) was used. The PEG hydrogel was labeled with Alexa Fluor 660 nm dye (Life Technologies, Carlsbad, CA) as previously described.[27] The fluorescent hydrogel loaded with coacervate was polymerized in a Petri dish, then immersed in optimal cutting temperature (OCT) compound (Sakura Finetek, Torrance, CA) and 12 μm frozen sections were cut by cryomicrotome. Images were captured using an Eclipse Ti inverted fluorescence microscope (Nikon, Tokyo, Japan).

Rat MI Model. Animals were cared for in compliance with NIH guidelines and all experiments were approved by the University of Cape Town's Animal Research Ethics Committee. MI and intramyocardial injections were performed as we previously described.[27] Briefly, male Wistar rats (180−200 g) underwent permanent left anterior descending (LAD) coronary artery ligation using 6−0 Prolene suture (Ethicon, Somerville, NJ). Treatments were applied immediately after ligation as 100 μL of unpolymerized hydrogel divided into 3 injections in evenly spaced around the infarct center thru a 31G needle. The PEG and MMP1-sensitive peptide were combined and the injections timed such that the hydrogel polymerized within 5 min after injection into the myocardium. Treatment groups included saline (n = 8), coacervate alone (n = 10), PEG hydrogel alone (n = 9), or the hydrogel and coacervate combined (n = 10), and animals were randomized to the treatment groups.

Coacervate Retention Study. Fluorescence-labeled coacervate was prepared as described above and was injected into infarcts alone (n = 4 rats) or in the PEG hydrogel (n = 5 rats). After 24 h, the animals were sacrificed and their hearts explanted and frozen in OCT. Sections 10 μm thick were taken every 500 μm beginning at the apex and continuing through the entire heart. Fluorescence images of the infarct zone were acquired and ImageJ 1.47v was used quantify the percent area positive for the coacervate as percent positive area (%) = coacervate positive area (mm^2)/infarct zone area (mm^2) * 100%. The result for each animal was the average of all sections taken for that animal.

Hydrogel Integration Study. Fluorescence-labeled hydrogel was prepared as described above with or without the coacervate and injected into the infarct zone (n = 3 rats). The animals were sacrificed after 14 days and the hearts were frozen in OCT and sequentially sectioned at 6 μm thickness. Sections were observed by fluorescence microscopy and the area positive for hydrogel (mm^2) was multiplied by the spacing thickness (500 μm) and summed to estimate the hydrogel volume (mm^3).

Echocardiography. Echocardiography was performed prior to surgery and on days 14 and 28 by blinded investigator using a Vivid i Ultrasound imaging system (GE Healthcare, Little Chalfont, United Kingdom). Short-axis M-mode measurements of the LV were performed and fractional shortening (FS %) was calculated as (EDD - ESD)/EDD * 100% (EDD = end-diastolic diameter, ESD = end-systolic diameter).

Tissue Processing and Histology. Rats were sacrificed on day 28 and their hearts removed, rinsed in PBS, and fixed in 10% formalin for 24 h. Hearts were processed through graded alcohol and xylene, paraffin-embedded, and 3 μm sections were cut and dewaxed. Heart

DOI: 10.1021/acsbiomaterials.5b00077
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sections were stained with Masson's trichrome as previously described.[27] For detection of mural cells, sections were incubated with a 1:25 dilution of mouse antirat α-smooth muscle cell actin (Fitzgerald Industries Intl, Acton, MA) in PBS. The primary antibody was then detected with a 1:250 dilution of an AlexaFluor 488 nm conjugated goat antimouse secondary antibody (Jackson Immunoresearch, West Grove, CA).

Image Analysis. Infarct size and scar thickness were determined by analysis of Masson's trichrome-stained slides using Visiopharm Integrator System (VIS; Visiopharm, Hørsholm, Denmark) as previously described.[27] Briefly, for infarct size measurement, the circumference of the left ventricular myocardial midline was measured and the section of the midline where more than 50% of the thickness of the myocardial wall was occupied by infarct was defined as midline infarct length. The midline infarct lengths were summed for four sections and given as a percentage of the sum of midline circumferences. Scar thickness was assessed at 1 mm intervals wherever scar occupied more than 50% of the thickness of the myocardial wall. For blood vessel quantification, vessels were counted using VIS in four fluorescence micrographs per data point, captured at 10× magnification. All image analysis was performed by an investigator blinded to the group design.

Statistical Analysis. Statistical analysis was performed using Minitab 16 software (Minitab Inc., State College, PA). Statistical differences were calculated using analysis of variance (ANOVA) followed by Tukey posthoc testing, and differences were considered significant at $p < 0.05$.

RESULTS

Fluorescence Imaging of the Combined Treatment. To ensure that the coacervate and PEG hydrogel were compatible, we fluorescence-labeled each and encapsulated the coacervate in the hydrogel. Fluorescence microscopy indicated that the coacervate was homogeneously embedded throughout the hydrogel and consisted of spherical droplets with diameters ranging from approximately 0.5−10 μm (Figure 1), consistent with our previous reports.[22]

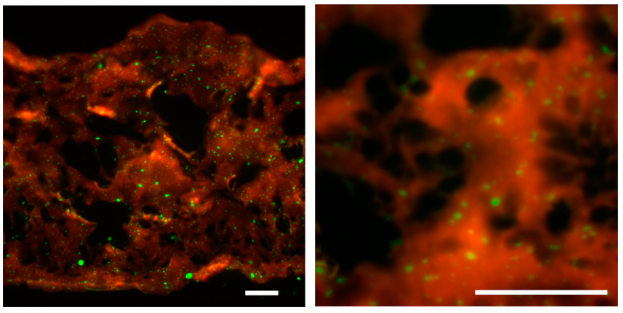

Figure 1. Fluorescence-labeled PEG hydrogel and coacervate. PEG hydrogel was labeled with AlexaFluor 660 dye (red) and the coacervate was labeled with fluorescein (green). Scale bars = 100 μm.

Hydrogel Improves Coacervate Retention after Myocardial Injection. We next tested the effect of the PEG hydrogel on the retention of the coacervate in the heart wall. MI was induced by permanent LAD coronary artery ligation and injections performed at 3 locations within the infarct zone. One day later we observed a much lower fluorescence signal intensity and signal area in animals receiving fluorescent coacervate alone (Figure 2a) rather than dispersed in the PEG hydrogel (Figure 2b). The appearance of the coacervate was also very different; when injected alone it had a punctate morphology and when in the hydrogel it had a fibrillar appearance. Coacervate droplets congealed and followed the contours of the hydrogel which was forced into interstices between myocytes, resembling the hydrogel morphology we described previously.[27] We quantified the area of the infarct region that was positive for fluorescent coacervate signal and found it to be 3-fold higher when the hydrogel was used (Figure 2c). These data demonstrate that the hydrogel increases coacervate retention and spreading within the myocardium.

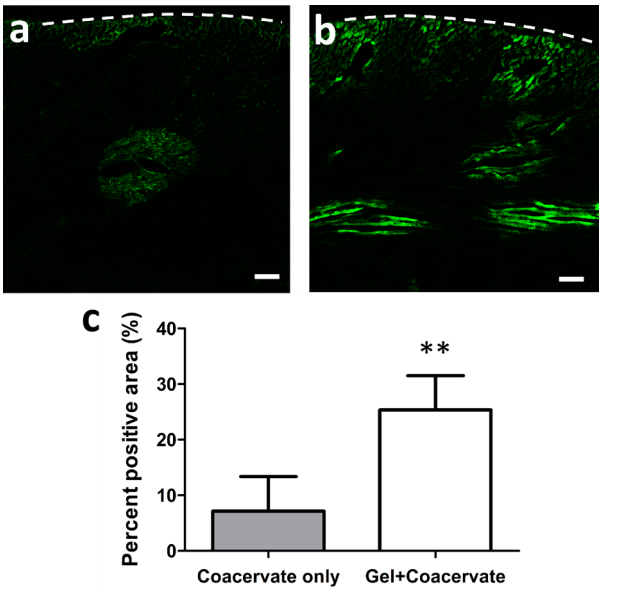

Figure 2. Coacervate retention 1 day postinjection. The coacervate was fluorescence-labeled (green) and infarcted rat hearts were injected with (a) coacervate only or (b) hydrogel+coacervate. Animals were sacrificed after 24 h and the coacervate was identified by fluorescence microscopy. Dotted lines indicate the outer boundary of the left ventricular wall. Scale bars = 100 μm. (c) Percent area of the heart positive for coacervate. Bars indicate mean ± SD of 4−5 rats per group. $^{**}p < 0.01$.

Coacervate Improves Hydrogel Integration after Myocardial Injection. We next evaluated the effect of the coacervate on the hydrogel integration and residence time in the ventricle wall. We induced MI and injected the fluorescence-labeled PEG hydrogel with or without the coacervate, then observed the hydrogel by fluorescence microscopy 2 weeks later. When the coacervate was not included we observed numerous regions of hydrogel remaining that were completely acellular, indicating incomplete acceptance of the material by the body (Figure 3a). In contrast, the hydrogel containing coacervate was evenly distributed throughout the wall and had much higher cell infiltration (Figure 3b). We estimated the total volume of hydrogel remaining at 2 weeks by quantifying the fluorescence area in sequential sections thru the entire infarct. These results revealed a trend toward less hydrogel remaining when it contained the coacervate, though this difference was not statistically significant ($p = 0.39$) (Figure 3c). Taken together, these data indicate that the coacervate does promote integration of the hydrogel into the myocardial wall, but does not retard its rate of degradation.

Combined Treatment Preserves Heart Function after MI. We next assessed the synergistic benefits of the combined hydrogel and coacervate toward improving heart function after MI. Echocardiography performed at 2 and 4 weeks revealed a higher fractional shortening with the combined treatment

DOI: 10.1021/acsbiomaterials.5b00077
ACS Biomater. Sci. Eng. XXXX, XXX, XXX−XXX

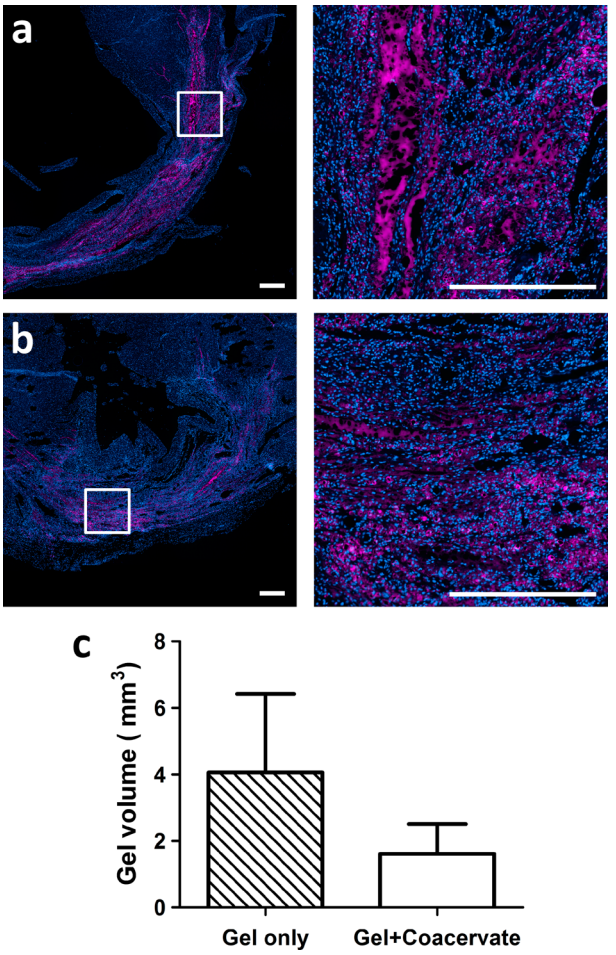

Figure 3. Hydrogel integration 2 weeks postinjection. The hydrogel was fluorescence-labeled (purple) and infarcted rat hearts were injected with (a) hydrogel only or (b) hydrogel+coacervate. After 2 weeks, the animals were sacrificed and the hydrogel was identified by fluorescence microscopy in sections stained with DAPI for cell nuclei (blue). Indicated regions of the left column are magnified in the right column. Scale bars = 500 μm. (c) Quantified hydrogel volume in the infarct region after 2 weeks. Bars represent mean ± SD of 3 rats per group.

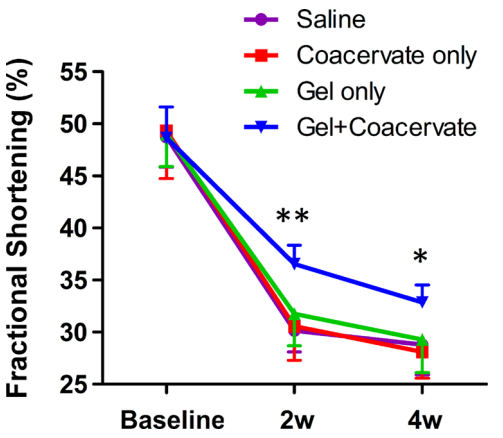

Figure 4. Functional analysis. Rat infarcts were injected with saline, coacervate alone, hydrogel alone, or the combined hydrogel and coacervate. Echocardiography was performed prior to MI (baseline) and 2 and 4 weeks after MI, and data presented as percent fractional shortening. Bars indicate mean ± SD of 8−10 rats per group. *p < 0.05, **p < 0.01 compared to all other groups.

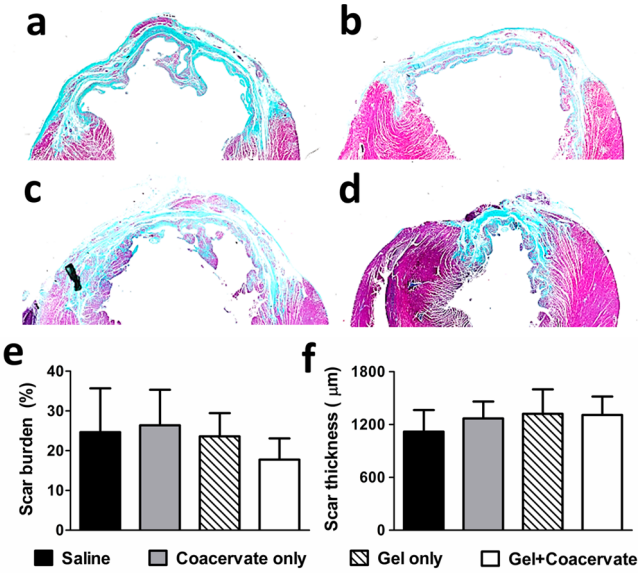

Figure 5. Scar burden and scar wall thickness. Masson's trichrome staining was performed 4 weeks after MI to observe fibrosis. Representative images are shown of hearts treated with (a) saline, (b) coacervate only, (c) hydrogel only, (d) hydrogel+coacervate. Collagenous scar tissue appears blue and healthy muscle appears purple. (e) Quantification of scar burden, the percent of the heart circumference containing scar. (f) Quantification of scar thickness, the average wall thickness of the scar region. Bars indicate mean ± SD of 8−10 rats per group.

compared to all other groups at both time points (Figure 4). Neither treatment alone showed any difference from the saline control group at either time point. We also observed a similar functional benefit of the combined treatment when it was applied 1 week after MI (Figure S1). These results demonstrate that the synergism between the hydrogel and coacervate is vital to preservation of heart function.

Combined Treatment Reduces Scar Burden. Fibrotic scar formation is often the leading cause of reduced heart function and eventually congestive heart failure. We observed the dense, collagen-rich scar tissue in rat infarcts after 4 weeks using Masson's trichrome staining. Hearts receiving the combined hydrogel and coacervate treatment had a noticeably smaller scar region compared to other groups (Figure 5a−d). Quantification of scar burden, the percent of the heart circumference containing scar, revealed a trend toward reduced scar burden with the combined treatment, however it was not statistically significant (Figure 5e). There were also no significant differences in wall thickness of the scar region between groups (Figure 5f).

Coacervate Improves Myocardial Vascularization. We also investigated the vascularity of the infarct zone in order to elucidate the effect of our treatments on angiogenesis and survival of pre-existing vessels. At 4 weeks post-MI, we observed nearly 2-fold more vessels in hearts treated with coacervate alone compared to saline (Figure 6). There was no significant difference in vascularization between either treatment alone or the combined treatment. Similar trends were also observed in the border zone surrounding the infarct region, though the vessel numbers were proportionally lower (Figure S2). Representative images of blood vessels in the infarct center are provided in Figure S3. These data demonstrate that

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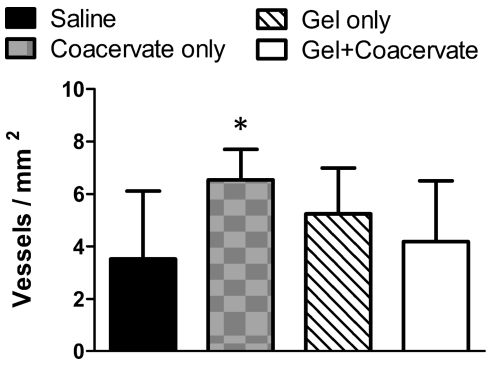

Figure 6. Blood vessel density. Blood vessels in the infarct zone were identified by α-smooth muscle actin immunohistochemical staining. Blood vessel density was quantified in the infarct zone 4 weeks after MI. Bars indicate mean ± SD of 8−10 rats per group. *$p < 0.05$ compared to saline group.

sustained release of Shh induces a significant improvement in vascular density compared to saline treatment.

DISCUSSION

Several interesting phenomena were revealed in these studies. The first was a trend toward less PEG hydrogel containing the Shh and IL-10 coacervate remaining at 2 weeks compared to the hydrogel alone. This trend was surprising because we had expected that IL-10 released from the coacervate would reduce the degradation rate of the hydrogel and prolong its residence time by suppressing the macrophage-driven inflammatory response. On the other hand, Shh has been shown to upregulate cardiac fibroblast expression of stromal-cell derived factor-1α, a progenitor cell homing chemokine.[18] Shh released from the coacervate may have therefore promoted a greater host cell presence in the infarct zone; these cells could have enhanced the regenerative environment but also could have secreted MMPs and contributed to hydrogel degradation.[37] We have yet to characterize the effect of the Shh and IL-10 coacervate on the temporal presence of these cell populations in the infarct zone after MI. Those studies will also elucidate the relative contributions of those cells and others toward hydrogel degradation and preservation of heart function.

In this study, we showed that a degradable PEG hydrogel alone has no significant benefit when injected immediately after MI, which is consistent with our previous report.[27] However, when combined with the coacervate, it effectively preserved heart function when injected immediately or 1 week post-MI with similar outcomes. These results suggest a therapeutic window lasting for at least 1 week, which is more clinically feasible than necessitating treatment immediately after heart attack.[38] It is possible that the beneficial effect of the combined treatment is transient and would be lost beyond 4 weeks after infarction. We believe this to be unlikely, however, as ventricular remodeling is nearly complete by 4 weeks after MI in rats.[39] We also found that the Shh and IL-10-releasing coacervate alone had no significant benefit, which is likely due to low retention after injection without the support of the hydrogel. We previously reported that sustained release of fibroblast growth factor-2 (FGF-2) from the coacervate alone reduced scar burden and improved heart function after MI in mice.[40] The discrepancy in outcomes is likely a result of the different growth factors evaluated and different dosages. However, the current study suggests that the FGF-2 coacervate may be more effective if combined with a hydrogel to improve retention. A future study directly comparing these growth factors and the benefit of the hydrogel for each in a large animal model of MI will be very informative.

Myocardial fibrosis occurs, in part, in response to myocyte cell death and thus these two pathological processes are correlated.[41] Shh can directly reduce oxidative stress on myocytes or it may act indirectly through upregulation of IGF-1.[18] Therefore, animals receiving the combined treatment may have experienced lower levels of myocyte apoptosis than those receiving the hydrogel alone. The trend toward lower scar burden when the hydrogel is included compared to coacervate alone can be attributed to greater coacervate retention. Therefore, a reduction in myocardial fibrosis and assumedly greater myocyte survival explain, in part, the observed preservation in cardiac function by the combined treatment. However, the precise effect of Shh and IL-10 coacervate on myocyte survival, proliferation, and apoptosis requires further evaluation.

Blood vessels play a key role in promoting a pro-healing environment by supplying oxygen, nutrients, and progenitor cells and by removing waste. MI blocks the primary blood supply and angiogenesis must occur from neighboring vessels to gradually reperfuse the affected tissue. Shh has been shown to play a role in angiogenesis by upregulating local expression of vascular endothelial growth factor and angiopoeitin-1,[21] and by enhancing the contribution of blood-derived endothelial progenitor cells.[42] The coacervate resulted in greater vascularization 4 weeks after MI compared to saline, but not compared to the hydrogel treatment. Injection of the hydrogel alone results in an inflammatory response;[26] we suspect that this inflammation may contribute to angiogenesis which may be pacified by the IL-10 released from the coacervate in the combined treatment. Indeed, IL-10 has been reported in cancer studies to dampen macrophage based angiogenesis,[43] and it may be that the more sustained release of IL-10 that would be achieved in the combined therapy may have resulted in the nonsignificant reduction relative to the coacervate alone. However, additional studies are needed to fully understand the interactions between the therapy components, inflammation, and angiogenesis.

As this was the first evaluation of this combination therapy, we did not perform extensive optimization of therapeutic parameters; however, this will be an interesting area of future investigation. The most important of these parameters include Shh and IL-10 dosages, MMP-labile sequence and cross-link density within the hydrogel, and coacervate release rate and concentration within the hydrogel. Regulating the coacervate residence time via the degradation characteristics of PEG cross-linkers is also possible.[44] Systematic variation of these parameters will allow for control of the spatiotemporal presence of the proteins and the degradation rate and mechanical properties of the hydrogel for optimal therapeutic efficacy. Following optimization, it will be important to evaluate how therapeutically improved cardiac function influences the long-term progression to heart failure.

CONCLUSIONS

We developed a combination therapy for post-MI cardiac repair consisting of sustained Shh and IL-10 release from a coacervate vehicle and a degradable hydrogel. We found the cotreatment to significantly preserve heart function after MI in rats, whereas either treatment alone did not. The hydrogel improved the

DOI: 10.1021/acsbiomaterials.5b00077
ACS Biomater. Sci. Eng. XXXX, XXX, XXX−XXX

retention of the coacervate after myocardial injection, which in turn enhanced the integration of the hydrogel into the heart wall. The coacervate stimulated greater vascularization within the infarct region after 4 weeks and the combined treatment showed a trend toward reduced fibrotic scar burden. Therefore, it would appear the improved functional outcome of the combined treatment results from several enhancements in the different factors governing tissue repair.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsbiomaterials.5b00077.

Figures S1−S3 (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by a fellowship to N.J. from the Whitaker Foundation, and grants from the South African Medical Research Council (415626), the South African National Research Foundation (78802), the National Institutes of Health (2T32HL076124), the National Science Foundation (DMR 1005766), and the American Heart Association (12EIA9020016).

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DOI: 10.1021/acsbiomaterials.5b00077
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