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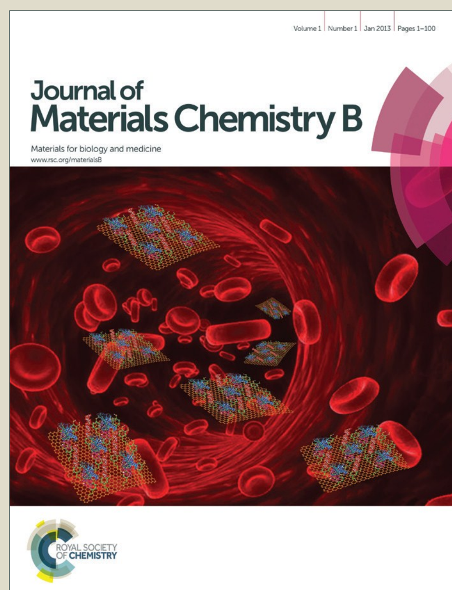

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Modulating *In Vivo* Degradation Rate of Injectable Extracellular Matrix Hydrogels

Jean W. Wassenaar[1], Rebecca L. Braden[1], Kent G. Osborn[2], Karen L. Christman[1*]

[1] Department of Bioengineering and Sanford Consortium for Regenerative Medicine, [2]Office of Animal Research, University of California, San Diego

* corresponding author

Karen L. Christman, PhD

Department of Bioengineering

Sanford Consortium of Regenerative Medicine

University of California, San Diego, 92093 (USA)

*Email: christman@eng.ucsd.edu

Abstract

Extracellular matrix (ECM) derived hydrogels are increasingly used as scaffolds to stimulate endogenous repair. However, few studies have examined how altering the degradation rates of these materials affect cellular interaction *in vivo*. This study sought to examine how crosslinking or matrix metalloproteinase (MMP) inhibition by doxycycline could be employed to modulate the degradation rate of an injectable hydrogel derived from decellularized porcine ventricular myocardium. While both approaches were effective in reducing degradation *in vitro*, only doxycycline significantly prolonged hydrogel degradation *in vivo* without affecting material biocompatibility. In addition, unlike crosslinking, incorporation of doxycycline into the hydrogel did not affect mechanical properties. Lastly, the results of this study highlighted the need for development of novel crosslinkers for *in situ* modification of injectable ECM-derived hydrogels, as none of the crosslinking agents investigated in this study were both biocompatible and effective.

Keywords: extracellular matrix, hydrogel, degradation, crosslinking, MMP inhibition

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Introduction

The use of ECM derived biomaterials as scaffolds for tissue engineering applications has increased dramatically in the past decade.[1] While originally thought of as an inert scaffold that primarily provides structural support to tissue, the ECM is now known to be an important modulator of cell behavior. Since ECM composition can vary widely across tissues, decellularization is a strategy that is increasingly employed since the resulting ECM scaffold retains the native mixture of macromolecules.[2,3] Further processing of decellularized ECM into a hydrogel form also allows them to be cast in a variety of 3D shapes for cell culture or injected into host tissue for *in situ* tissue engineering.[4-8] Stem and progenitor cells seeded on decellularized ECM scaffolds,[9-11] ECM-based coatings,[12,13] and ECM-derived hydrogels [14,15] have been shown to have improved survival, increased proliferation, and differentiate in a tissue-specific manner. Moreover, decellularized ECM and ECM derived hydrogels have been shown to recruit progenitors cells *in vivo* [16-18].

Our lab previously developed a myocardial matrix hydrogel derived from decellularized porcine myocardial extracellular matrix (ECM)[19] and showed that it improved cardiac function and attenuated negative LV remodeling when injected into both rat[17] and pig[20] models of MI. Recent studies have supported the conclusion that the bioactivity or cell response to injectable hydrogels plays a key role in their ability to improve cardiac function post-MI.[21,22] While the porous and nanofibrous structure of the myocardial matrix hydrogel provides a new physical scaffold to facilitate cell infiltration,[19] release of degradation products as cells infiltrate also likely influences the bioactivity of the material. Degradation products of decellularized ECMs are known to be chemoattractant[23] and angiogenic[24], and we have likewise shown that the myocardial matrix hydrogel promoted infiltration of vascular cells and progenitors *in vivo,*[17,19] and that the peptide cues in the material acted as a chemoattractant for smooth muscle cells and endothelial cells *in vitro.*[19] Since cell migration, proliferation, and angiogenesis are all dependent on degradation of scaffold proteins, it follows that slowing degradation could prolong the release of chemoattractant peptides, thereby increasing cell influx over time and potentially further enhancing the bioactivity of the material. This study therefore sought to examine methods to modulate the degradation rate of the injectable myocardial matrix hydrogel *in vivo.* We evaluated several crosslinkers frequently used for collagen-based biomaterials (glutaraldehyde, genipin, and transglutaminase). Currently, the most

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common technique for modifying naturally derived polymers is through crosslinking, however it also results in modifications in other material properties, such as stiffness and pore size.[25] For protein-based scaffolds such as collagen, fibrin, Matrigel, and the myocardial matrix, the primary mechanism for scaffold breakdown is the matrix metalloproteinase (MMP) family of proteases secreted by infiltrating cells.[26] We therefore also examined a MMP inhibitor (doxycycline) for its ability to modulate the degradation rate of the injectable ECM-derived hydrogel.

Experimental

Myocardial Matrix Preparation and Modification

Porcine ventricular myocardium was decellularized with a solution of a 1% sodium dodecyl sulfate (SDS) as previously reported.[27] The resulting ECM was then processed into a liquid form through partial digestion with pepsin, adjusted to pH 7.4 and 8 mg/mL with sodium hydroxide and phosphate buffer saline (PBS). The liquid myocardial matrix was aliquoted and immediately frozen for lyophilization. Lyophilized matrix was rehydrated with water then mixed with the following to achieve a final concentration of 6 mg/mL: 1) 1x PBS for unmodified myocardial matrix, 2) 1% glutaraldehyde (Sigma-Aldrich) for 0.05% final concentration, 3) 4 mM genipin (Wako Chemicals) for 1 mM final concentration, 4) 480 mU/mL transglutaminase (from guinea pig liver, Sigma-Aldrich) for 120mU/mL and 5) 4 mg/mL doxycycline (Sigma-Aldrich) for 1 mg/mL final concentration. For in vivo degradation assessment, prepared liquid myocardial matrices were mixed with Alexa Fluor 568 Succinimidyl Ester (AF568, Life Technologies) dissolved in dimethyl sulfoxide (DMSO) at least 15 minutes prior to injection. Concentration of AF568 was 100 µg/mL, or approximately 21 nmol of the fluorophore per mg of matrix.

Cytotoxicity

The elution cytotoxicity assay was performed according to ISO 10993-5 standards for evaluating cytotoxicity of biomaterials. After hydrogels were formed by overnight incubation at 37 °C, culture media were added and the resulting elution media collected after a 24-hour extraction period. Per ISO 10993-5, a standard cell line, L929 mouse areolar fibroblasts, was chosen for this study and cultured in DMEM (Mediatech) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich) and 1%

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penicillin/streptomycin (P/S; Life Technologies). Cells were either split 1:3 using trypsin or plated at 20k/well in 24-well plates when 90% confluence was reached. After allowing cells to adhere overnight, culture media was replaced with elution media collected from various modified myocardial matrix hydrogels with media from unmodified hydrogels and 0.5% glutaraldehyde crosslinked hydrogels used as negative and positive controls, respectively (six conditions, n = 4 per condition, per assay). After a 48-hour culture period, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Cell Proliferation Assay Kit (Life Technologies) and PicoGreen dsDNA Assay Kit (Life Technologies) were performed to assess cell viability and survival per manufacturer's directions.

Doxycycline Release

Unmodified and doxycycline loaded hydrogels (n = 3) were formed in glass scintillation vials after overnight incubation at 37 °C. Hydrogels were unmolded and placed in free floating in 1 mL of PBS. The concentration of doxycycline diffused into solution was measured by a NanoDrop 2000c Spectrophotometer (Thermo Scientific) using the peak absorbance of 350 nm over 24 hours with periodic refreshment of PBS. To account for absorbance from components released by the myocardial matrix, readings taken at the same time from an unmodified hydrogel were used as the blank.

In vitro Degradation Assessment

Bacterial collagenase (Worthington Biochemical Corporation) was used as an *in vitro* mimic to assess the degree to which crosslinking and doxycycline loading was able to affect degradation, measured by ninhydrin reactivity as previously described.[28] Briefly, after overnight gelation at 37 °C incubation, myocardial matrix hydrogels, unmodified and modified, were digested with an equal volume of collagenase at 125 U/mL in 0.1 M Tris-base, 0.25 M CaCl2, pH 7.4 solution. Hydrogels in PBS, without addition of collagenase, were used as negative controls. After 4 and 24 hour incubation, samples were centrifuged for 10 minutes at 15000 rpm to pellet the undigested solid portion. The supernatant, containing degraded peptides, was reacted with 2% ninhydrin solution (Sigma-Aldrich) in a boiling water bath for 10 minutes. A standard curve made with acetyl-L-lysine (Tokyo Chemical Industry) was used to calculate the primary amine concentration from collagenase degradation. Samples were read on a BioTek

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Synergy 4 spectrophotometer (BioTek Instruments) at 570 nm. The same procedure was also used to determine whether the amount of doxycycline sequestered by the myocardial matrix hydrogel was sufficient to reduce degradation. Hydrogels, both unmodified and doxycycline loaded, after undergoing doxycycline release experiments described previously, were then digested with collagenase and assayed for ninhydrin reactivity.

Rheological Measurements

Liquid myocardial matrix (300 µL, n = 4), both unmodified and modified, was pipetted between two glass slides sandwiched by 1 mm spacers. Glass slides were treated with Rain-X to allow for easy removal of the hydrogels after overnight incubation at 37 °C. Rheological properties of the hydrogels were measured by a AR-2000 Rheometer (TA Instruments) using a 20 mm parallel plate geometry, set at 1 mm gap height and 37 °C. A frequency spread from 0.25 to 100 rad/s (0.5 to 10 is reported) was conducted at 2.5% strain, which was previously determined to be within the linear viscoelastic strain region.[28] Storage modulus (G') and loss modulus (G") were reported for the different hydrogels at 0.4 rad/s.

Cellular Migration through Hydrogels

The effects of crosslinking and doxycycline loading on the ability of cells to migrate through the myocardial matrix hydrogel was assessed using Fluoroblok 24 Well Plate Inserts with 8.0 µm pores (Corning Incorporated) as previously described.[28] L929 fibroblasts were cultured as described above. Prior to passaging with trypsin, cells were serum starved DMEM with 1% P/S for 24 hours. Liquid myocardial matrices – unmodified, crosslinked or loaded with doxycycline (100 µL, n = 3) – were pipetted onto the upper well of the transmembrane insert and incubated at 37 °C overnight for gelation. After trypsinization, serum starved cells were labeled with CFSE CellTrace (Life Technologies) diluted to 10 µM in PBS for 15 minutes at 37 °C. Cells were then washed and incubated with media alone for 37 °C for 30 minutes to remove excess CFSE and activate the fluorescent marker. Transwell inserts were placed into VisiPlate-24 Black Microplates (Perkin Elmer) with 1 mL of serum-supplemented media with FSB as the chemoattractant to induce migration. Fluorescently labeled L929s (150,000 cells/insert) were seeded on top of the hydrogels with 250 µL of serum-free media). Fluorescence was read on a Biotek Synergy 4

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Spectrophotometer immediately after cell seeding and then periodically for 24 hours. Cells that have migrated through the hydrogel and the opaque transmembrane were detectable by fluorescence. Experiments were conducted in triplicate with results from one representative test shown.

Surgical Procedures

All procedures in this study were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, San Diego and were conducted according to the guidelines established by UCSD IACUC and the American Association for Accreditation of Laboratory Animal Care. All in vivo experiments were performed on female adult Sprague-Dawley rats (225 to 250 g). Injections of 75 µL of myocardial matrix were made directly into healthy myocardium by diaphragmatic access as previously described.[17,19,29] For the biocompatibility study, myocardial matrix crosslinked with 0.05% glutaraldehyde, 1 mM genipin, 120 mU/mL transglutaminase, or loaded with 1 mg/mL doxycycline was injected (n = 4). For the *in vivo* degradation study, myocardial matrices – unmodified, crosslinked with genipin or transglutaminase, or loaded with doxycycline – were fluorescently labeled with AF568 for visualization and intramyocardially injected (n = 3-4).

Histology and Immunohistochemistry

Animals were euthanized two weeks post-injection for the *in vivo* biocompatibility study and one week post-injection for the *in vivo* degradation study by an intraperitoneal injection of sodium pentobaribal (200 mg/kg). Hearts were immediately removed, fresh frozen in Tissue Tek OCT freezing medium (Sakura Finetek) and cyrosectioned. Short axis sections of 10 µm spanned the ventricle at 16 locations, with approximately 350 µm between each location. One slide at each location was stained with H&E, mounted with Permount (Fisher Chemical), and scanned at 20x using an Aperio Scan Scope CS2 slide scanner (Leica Biosystems) to identify the injection site. The injection site for each modified hydrogel was evaluated by a histopathologist blinded to the treatment groups. To assess the amount of AF568-labeled hydrogel remaining at one week post-injection, neighboring slides at each location were stained with Hoescht 33342 (Life Technologies) to visualize nuclei and provide a counterstain for scanning. Fluorescently labeled slides were mounted with Fluoromount (Sigma) and imaged on an Ariol Platform

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with the DM6000 B microscope (Leica Biosystems). Slides from five locations with the largest area of red fluorescence were used for analysis. Number of red pixels within the entire heart section was quantified by ImageJ and summed for each heart.

Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM). For analysis of *in vitro* characterization and *in vivo* degradation assessment, single-factor ANOVA with Dunnett's test comparing to unmodified hydrogels was used. For analysis of degradation of doxycycline hydrogels after release experiments, a student t-test was used.

Results and Discussion

Cytotoxicity Assessment

Two methods were used to modulate the degradation rate of the myocardial matrix in this study – crosslinking and use of a MMP inhibitor. Glutaraldehyde was chosen because it is a commonly used chemical crosslinker for modifying collagen-based materials.[30,31] In addition, our lab had previously demonstrated that various concentrations of glutaraldehyde could be used to tailor the material properties of the myocardial matrix hydrogel *in vitro.*[28] Genipin, a small molecule crosslinker derived from gardenia fruit, and tissue transglutaminase, a mammalian enzyme that catalyzes the formation of γ-glutamyl-ε-lysine bonds between two peptide chains, were also investigated based on previous reports of their use in crosslinking collagen gels.[32-35] Transglutaminase, in particular, provides an additional advantage because it is an enzymatic crosslinker, so no chemical residues or by-products are generated from the reaction, reducing risk of cytotoxicity.[36] For MMP inhibition, doxycycline was selected because it is a widely used tetracycline antibiotic with independent, broad spectrum MMP inhibition activity at sub-antimicrobial dosages[37]. An elution assay was performed in accordance to ISO 10993-5 standards for biomaterial testing to assess cytotoxicity of the modified myocardial matrices. L929 mouse fibroblasts were exposed to elution media extracted from unmodified, crosslinked, and doxycycline loaded hydrogels with 0.5% glutaraldehyde crosslinked hydrogels used as positive cytotoxic controls. After 48 hour incubation, DNA content was quantified by PicoGreen and cellular metabolism evaluated by MTT. Using both metrics, it

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was determined that the following concentrations were non-cytotoxic: 0.05% glutaraldehyde, 1 mM genipin, 120 mU/mL transglutaminase, and 1 mg/mL doxycycline (Figure 1). All results described in subsequent experiments were based on these concentrations.

Crosslinking and Doxycycline Reduce Collagenase Degradation In Vitro

To evaluate whether the concentrations of crosslinkers and doxycycline were sufficient to modulate degradation kinetics, an *in vitro* assay was performed using bacterial collagenase. Ninhydrin reactivity of the supernatant collected from hydrogels after 4 and 24 hours of collagenase incubation were used to assess the amount of peptides released (Figure 2). Degradation occurred in the no enzyme group, indicating that a portion of the hydrogel undergoes degradation in PBS, likely through dissolution of the portions of the myocardial matrix that do not undergo self-assembly to form the hydrogel. Doxycycline modified hydrogels showed significantly lower peptide concentration at both time points compared to unmodified hydrogel controls, indicative of inhibition of collagenase degradation. Similar to previous reports using ECM scaffolds and collagen hydrogels,[38,39] glutaraldehyde and genipin crosslinking were also able to reduce collagenase degradation. However, in contrast to studies using transglutaminase,[40] crosslinking of the myocardial matrix by transglutaminase had no effect.

Doxycycline Sequestration within the Myocardial Matrix Hydrogel

Since doxycycline was not covalently linked to the myocardial matrix, the release profile was determined by submerging doxycycline-loaded hydrogels in PBS and measuring the concentration of doxycycline diffusing into solution by absorbance using a spectrophotometer. The results indicated that doxycycline had a burst release profile from the myocardial matrix with most of the loaded amount diffusing out over the first few hours, as expected for a small molecule in a hydrogel (Figure 3A). However, by 8 hours this release plateaued and an undetectable amount of doxycycline was released by the hydrogel, indicating that approximately 30% of doxycycline was sequestered by the myocardial matrix hydrogel, possibly through secondary interactions. No further release occurred out to day 7 (data not shown). Since this likely represents the amount of doxycycline retained by the myocardial matrix once injected into the myocardium and the burst release of doxycycline likely inhibited collagenase in the

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previous degradation assay, the ability of this sequestered doxycycline to inhibit degradation was similarly evaluated using bacterial collagenase. After both unmodified and doxycycline loaded hydrogels underwent 24 hours of PBS to release any unbound doxycycline, the hydrogels were then subjected to collagenase digestion. Ninhydrin reactivity showed that the sequestered doxycycline in the myocardial matrix hydrogel was still sufficient in inhibiting collagenase and reducing the degradation of the hydrogel (Figure 3B). Based on our results, we estimated that 50 μg (or 70% of the total 75 μg injected dose) is burst released from the hydrogel over 24 hours. Initial released doxycycline may therefore potentially have an additional effect when hydrogels are injected *in vivo*, as it diffuses out into the surrounding tissue. Doxycycline on its own has in fact been examined for treatment post-MI. Previous studies in rats showed that oral doxycycline therapy post-MI is able to improve LV morphology and function.[41] However, others have reported that subcutaneous injections of doxycycline do not have any affect on LV remodeling or dysfunction[42] and MMP inhibition in clinical trials for patients with acute MI has shown conflicting results.[43,44] While doxycycline inhibits MMPs at a relatively low concentration of 5 μg/mL, it has been shown to reduce MMP-2 and -9 production by smooth muscle cells at higher concentrations of 40 μg/mL.[45] Thus, the minimal amount of released doxycycline is unlikely to result in significant effects on MMP activity in the surrounding tissue – as it would not sustain an elevated tissue concentration beyond the immediate hours post-injection.

Glutaraldehyde and Genipin Crosslinking Alters Rheological Properties of Hydrogels

Changes in the rheological properties of crosslinked and doxycycline loaded hydrogels were measured using a parallel plate rheometer at a constant strain of 2.5%. Figure 4A and 4B shows the frequency sweep from 0.25 to 10 rad/s from one experimental replicate (n = 4 per group). Since collagen fibrils show minimal shear thinning at frequencies below 0.6 rad/s,[46] the storage modulus (G') and loss modulus (G") are reported at 0.4 rad/s (Figure 4C and 4D). At this frequency, unmodified myocardial matrix had a storage modulus of 11.3 ± 2.0 Pa, similar to previously reported values,[28] and loss modulus of 2.4 ± 0.5 Pa. Both glutaraldehyde and genipin crosslinked hydrogels increased the storage modulus of the hydrogel by a similar magnitude, at 59.5 ± 9.8 Pa and 62.4 ± 17.4 Pa, respectively (Figure 4C). Both crosslinkers also significantly increased the loss modulus to 8.1 ± 1.2 Pa and 6.1 ± 0.4 Pa (Figure 4D).

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However, crosslinking with transglutaminase had no effect on either rheological property (G' = 7.9 ± 0.8 Pa, G" = 1.1 ± 0.1 Pa). A previous study using transglutaminase to crosslink an adipose tissue-derived ECM hydrogels showed that similar to our results, 100 mU/mL of the enzyme had no effect on hydrogel rheology.[47] Previous studies have shown changes in pure collagen hydrogel stiffness after transglutaminase crosslinking;[33,40] however, concentrations of collagen were several folds higher than that used for the myocardial matrix in this study. Alternatively, the difference in pepsin digestion in the processing of the myocardial matrix may be altering the peptide sequence sensitive to transglutaminase crosslinking. Doxycycline loading also did not affect the rheology of the hydrogel (G' = 8.1 ± 0.6 Pa, G" = 0.6 ± 0.2 Pa), which demonstrates that it does not chemically alter the myocardial matrix. This property may be an advantage over crosslinkers when changes in degradation rate of hydrogels need to be studied without altering the mechanical properties, which can have significant effects on cell migration and behavior.[48,49]

Crosslinking and Doxycycline Do Not Inhibit Cell Migration through the Hydrogel

The effect that crosslinking and doxycycline loading had on cell migration through the myocardial matrix hydrogel was assessed using the FluoroBlok transwell migration inserts. Fluorescently labeled L929 mouse fibroblasts, the same cells used in the cytotoxicity experiments, were plated on top of hydrogels formed in the upper chamber of transwell insert. Cells that migrated through the hydrogel and the opaque membrane were fluorescently detectable. One-way ANOVA analysis showed that while migration rates were significantly different initially, fibroblast were still able to migrate through crosslinked and doxycycline loaded hydrogels, as indicated by the non-significant differences in fluorescence levels by later time points (Figure 5A). Interestingly, post-hoc analysis using Dunnett's correction revealed that there was significantly faster migration of fibroblasts through transglutaminase crosslinked hydrogels, while other crosslinking methods and doxycycline loading had no significant effect (Figure 5B). The previous study using adipose ECM hydrogels demonstrated a change in fiber diameter of the hydrogel under scanning electron microscopy;[47] a similar change in the microstructure of the myocardial matrix may explain why fibroblasts were able to traverse the hydrogels more quickly in the transwell inserts. Further evidence that transglutaminase may affect the bioactivity of ECM hydrogel *in vivo* came from the

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same study, where crosslinked matrices induces higher vascularization than hydrogel alone.[47] Thus, transglutaminase was included in further *in vivo* characterization along with glutaraldehyde, genipin, and doxycycline despite its inability to affect degradation.

In vivo Biocompatibility Assessment

While cytotoxicity of the compounds at the chosen concentrations were determined to be negligible *in vitro* using established ISO 10993-5 standards for biomaterial testing, biocompatibility of the modified hydrogels *in vivo* was also examined. To assess the biocompatibility of crosslinked and doxycycline loaded myocardial matrix hydrogels, modified matrices were injected into healthy rat myocardium. The injection site lesion at two weeks post-injection was evaluated by a histopathologist blinded to the treatment groups, who identified the injection sites as morphologically similar among the groups, with areas of spindle cells and/or interconnected (branching) elongate cells – suggestive of degenerative/regenerative myocardial fibers, plus usually minimal inflammatory infiltrate consisting of lymphocyte-type small mononuclear cells. The doxycycline group did not exhibit a notable difference in injection site morphology suggesting that the amount of doxycycline that is released had minimal effect on the overall inflammatory response to the hydrogel. Across all the groups, only within the glutaraldehyde crosslinked group did the pathologist note moderate to marked focal non-suppurative inflammation with lymphocytic infiltrate and foreign-body giant cells (Figure 6). This result contradicted many previous *in vitro* and *in vivo* studies, which had shown that up to 0.25% glutaraldehyde to be considered biocompatible.[50-52] However, many studies evaluate the biocompatibility of the biomaterial by implantation in the subcutaneous space. In comparison, the myocardium is much more vascularized and therefore may be under increased surveillance by the immune system, resulting in heightened sensitivity towards potentially toxic compounds. This phenomenon had been previously reported in a study where thiolated-hyaluronic acid hydrogels showed minimal immune response when injected subcutaneously, but induced granuloma formation when intramyocardially injected, which the authors attributed to the high vascularity of the heart.[53] Due to this associated inflammatory response, glutaraldehyde was not evaluated further in subsequent *in vivo* experiments.

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Doxycycline Reduces Myocardial Matrix Degradation In Vivo

To more clearly identify presence of the hydrogel from histological sections, liquid matrices were fluorescently labeled with AF568. The fluorophore was conjugated to an NHS ester, allowing it to react with the primary amines on the protein component of the myocardial matrix. The reaction is rapid at neutral pH and forms a stable amide bond.[54] Labeled myocardial matrices – unmodified, crosslinked with genipin or transglutaminase, or loaded with doxycycline – were injected into healthy myocardium to evaluate effects on *in vivo* degradation. Comparisons of H&E stained sections (Figure 7A) with fluorescently scanned neighboring sections (Figure 7B) show good correlation between the morphology of the injection sites. Changes in the degradation rate of the modified myocardial matrix were approximated by the amount of hydrogel remaining at one-week post-injection, which was chosen based on previous studies showing that the unmodified hydrogel was degraded by approximately three weeks.[20] Quantification of the degree of red fluorescence through the tissues sections by ImageJ revealed that only doxycycline loading significantly increased the amount of myocardial matrix remaining (Figure 7C), suggesting that it was able to reduce *in vivo* degradation through MMP inhibition, while the crosslinkers had no significant effect. Despite showing promise *in vitro* in reducing degradation by bacterial collagenase, genipin crosslinking had no effect in altering the degradation rate. This may be due to the slower reaction rate of genipin crosslinking, which occurs over the span of hours after it is combined with the material.[55] As a result, injected genipin likely diffused away from the myocardial matrix before it was able to react. Alternatively, the crosslinker may react non-specifically to primary amines on the proteins and peptides in the surrounding myocardium.

This result highlighted the importance of evaluating degradation *in vivo*; while degradation *in vitro* can be simulated with use of various proteases, allowing relative comparisons of multiple materials and concentrations, the results often do not correlate with *in vivo* degradation rates.[31] A careful survey of literature on reagents used for collagen-based materials show that an ideal crosslinker for modifying injectable ECM-derived hydrogels *in vivo* does not currently exist. Like genipin crosslinking, glycation crosslinking occurs too slow to have an effect.[56] Faster reacting reagents such as di-isocyanates, carbodiimides, and azides occur at conditions that are not physiological – higher temperatures, acidic or basic pH, or use of solvents and surfactants – making them unsuitable to be injected for *in situ*

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crosslinking.[57-62] In addition, these reagents were typically developed for crosslinking scaffolds, intended for implantation, rather than hydrogels that are injected into host tissue. This makes cytotoxicity an even higher priority since unreacted crosslinkers can diffuse away from the injected bolus, which may have contributed to the adverse inflammatory reaction seen with glutaraldehyde-injected groups. Previous studies demonstrated that carbodiimide-crosslinked ECM induced polarization of macrophages to a M1 phenotype, which is associated with a vigorous inflammatory response, while non-crosslinked ECM elicited a predominantly M2 response, indicative of a pro-regenerative environment.[63,64] The difference in response to crosslinking between previous reports and this study is likely due to tissue site and the form of ECM, as previous work involved intact decellularized scaffolds that are not further processed and therefore have significantly longer degradation times. Lastly, while there have been previous reports of synthetic hydrogels loaded with doxycycline, the goal of these studies was to deliver doxycycline as a therapeutic in diseases where MMP inhibition is desirable.[65-67] In contrast, this is the first study to our knowledge to employ doxycycline as an MMP inhibitor to modulate the degradation rate of a naturally derived material. Given the potential other activities of doxycycline,[68,69] it may be beneficial to incorporate more specific MMP inhibitors to similarly slow degradation of ECM hydrogels.

4. Conclusions

We have demonstrated through *in vitro* characterization that the properties of the myocardial matrix hydrogel could be easily modified by various crosslinking methods commonly used for collagen-based materials. However, *in vivo* experiments on biocompatibility and degradation kinetics showed that there are commonly used crosslinkers are not able to modulate injectable ECM-derived hydrogels *in situ*. Thus there is a need for the development of new crosslinkers with the following criteria: 1) Rapid reaction kinetics, 2) ability to react at physiological conditions including 37 °C, neutral pH, and without surfactants or solvents, and 3) minimal cytotoxicity. Alternatively, loading the hydrogel with a biocompatible MMP inhibitor allows for degradation to be modulated, both *in vitro* and *in vivo*, without affecting other properties of the hydrogel. This provides a novel method to tailor the degradation rate of injectable ECM-based hydrogels and study the effect on tissue regeneration without affecting material stiffness.

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Disclosure Statement

KLC is a co-founder, board member, and holds equity interest in Ventrix, Inc.

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Figure 1. Cytotoxicity of crosslinked and doxycycline loaded hydrogels. Effect of elution products collected from myocardial matrix hydrogels crosslinked with 0.05% glutaraldehyde (GA), 1 mM genipin (Gen), 120 mU/mL transglutaminase (TG), and 1 mg/mL doxycycline (Dox) on L929 fibroblasts cell viability (measured by MTT assay) and cell number (measured by PicoGreen). Unmodified hydrogel and 0.5% GA crosslinking served as negative and positive controls, respectively ** $p < 0.01$ compared to unmodified hydrogels.

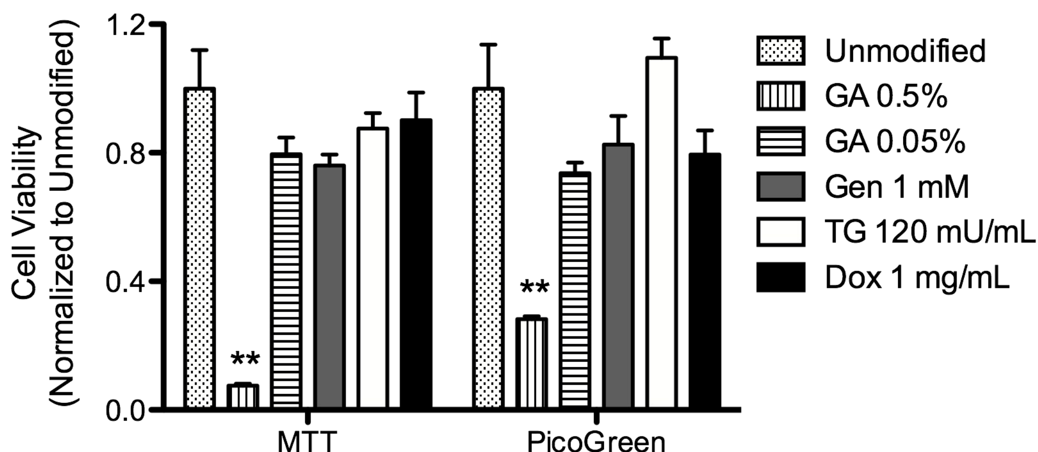

Figure 2. *In vitro* bacterial collagenase degradation. Degradation of unmodified, crosslinked, and doxycycline loaded hydrogels by bacterial collagenase were measured by amount of amines released in the supernatant through ninhydrin reactivity. Degradation in PBS alone, without addition of collagenase, is shown as negative control. GA: glutaraldehyde, Gen: genipin, TG: transglutaminase, Dox: doxycycline. * $p < 0.05$, ** $p < 0.01$ compared to unmodified hydrogel controls.

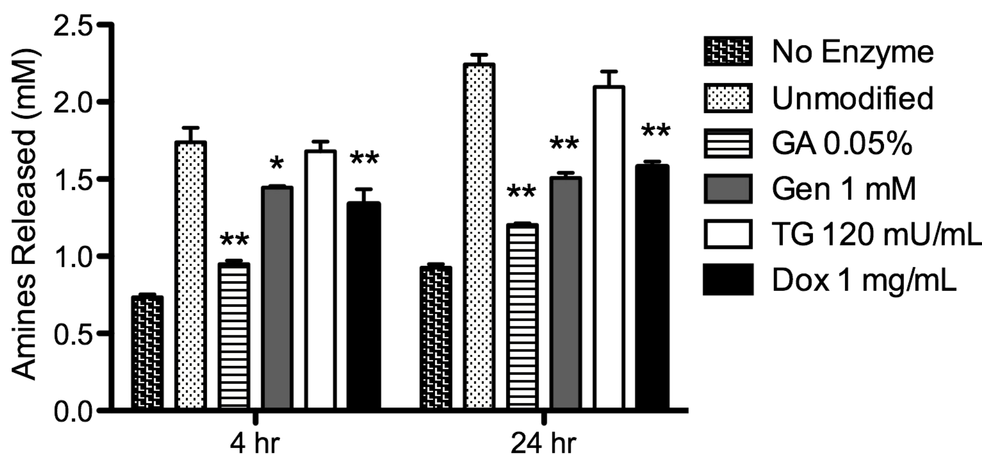

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Figure 3. Doxycycline sequestration with the myocardial matrix hydrogel. A) Release profile of doxycycline from myocardial matrix hydrogels follows the typical burst release profile, but approximately 30% of loaded doxycycline is sequestered by hydrogel. B) Degradation of the same hydrogels after burst release has occurred showed that the sequestered doxycycline is still sufficient to inhibit bacterial collagenase degradation. * $p < 0.05$.

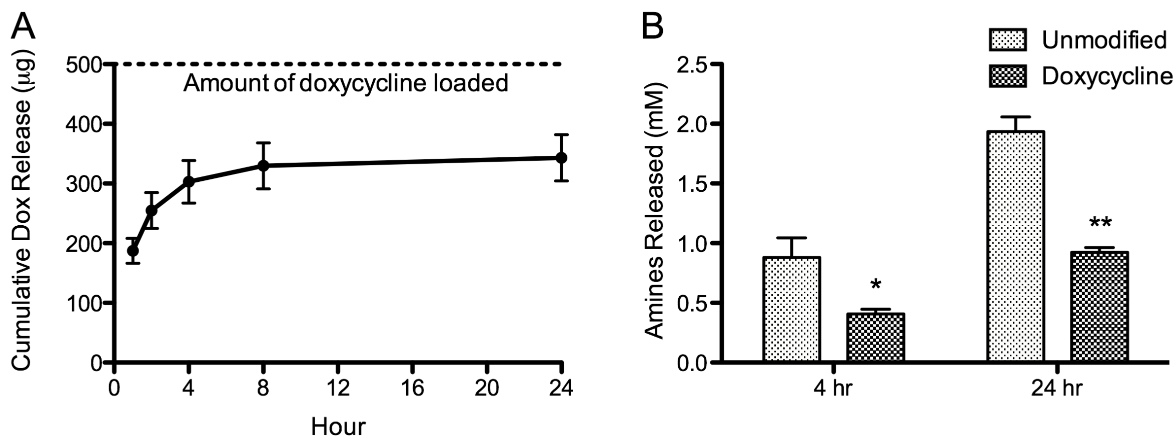

Figure 4. Rheological properties of crosslinked and doxycycline loaded hydrogels. Storage modulus (A) and loss modulus (B) of hydrogels measured by a parallel plate rheometer from a frequency sweep of 0.5 to 10 rad/s. At 0.4 rad/s, both 0.05% glutaraldehyde (GA) and 1 mM genipin (Gen) crosslinking significantly increased storage modulus (C) and loss modulus (D), while transglutaminase (TG) and doxycycline (Dox) had no effect. ** $p < 0.01$ compared to unmodified hydrogel controls.

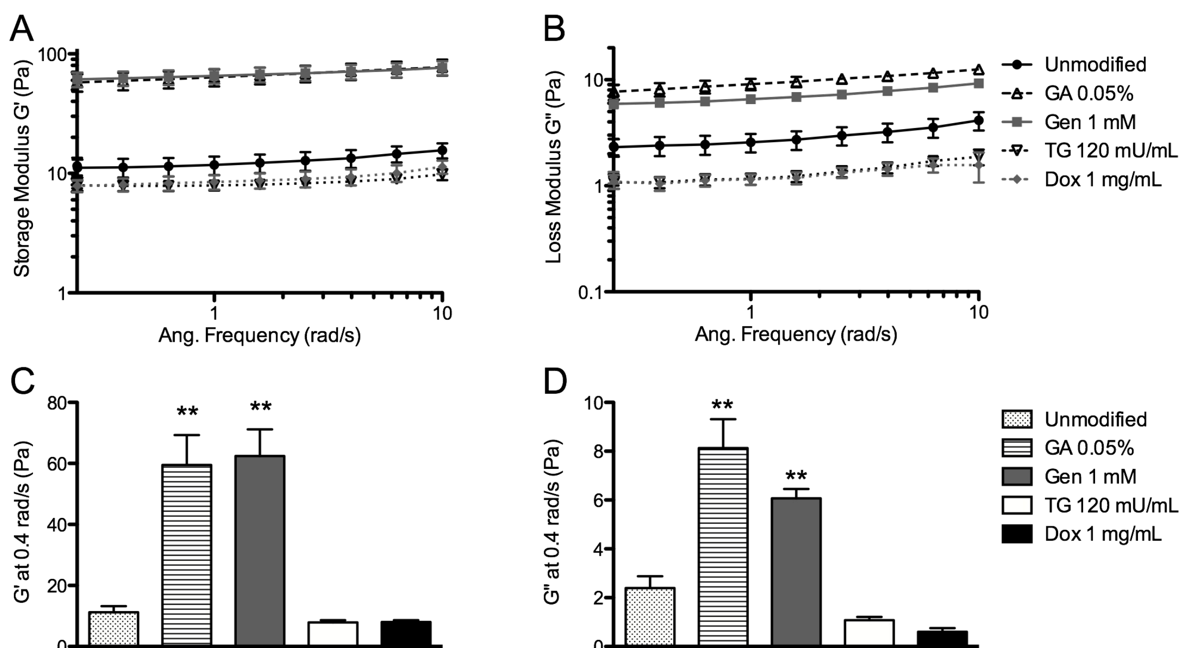

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Figure 5. Cell migration through crosslinked and doxycycline loaded hydrogels. A) Fluorescently labeled L929 migration through unmodified, 0.05% glutaldehyde (GA), 1 mM genipin (Gen), 120 mU/mL transglutaminase (TG), and 1 mg/mL doxycycline (Dox) hydrogels within transwell migration inserts over 24 hours (* $p < 0.05$ for one-way ANOVA). B) Migration through transglutaminase crosslinked hydrogels was significantly faster at earlier time points, hour 2 and hour 6, compared to unmodified matrices (* $p < 0.05$).

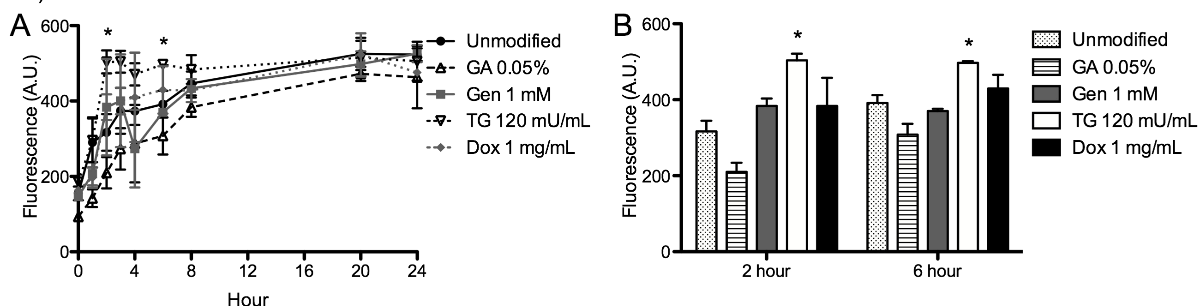

Figure 6. Biocompatibility of crosslinked and doxycycline loaded myocardial matrix *in vivo*. Representative H&E images of the myocardial matrix with 0.05% glutaraldehyde (A), 1 mM genipin (B), 120 mU/mL transglutaminase (C), or 1 mg/mL doxycycline (D) injected into healthy myocardium. Injection sites were made up of elongate spindle to branching cells suggestive of degenerative/regenerative myocardial fibers, plus usually minimal inflammatory infiltrate consisting of lymphocyte-type small mononuclear cells. Only the glutaraldehyde crosslinked hydrogel injection site showed marked lymphocytic infiltrate and foreign body giant cells (arrows). scale bars = 100 μm.

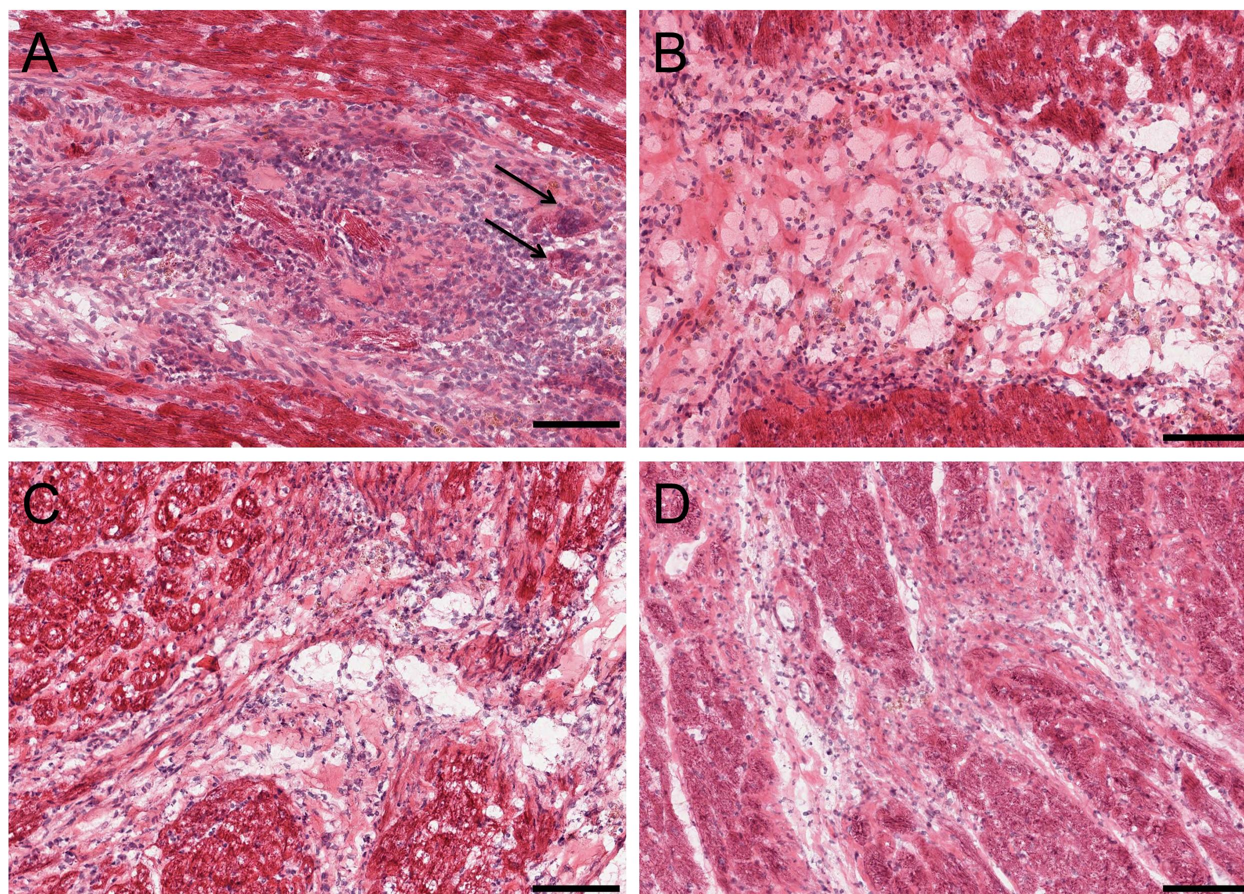

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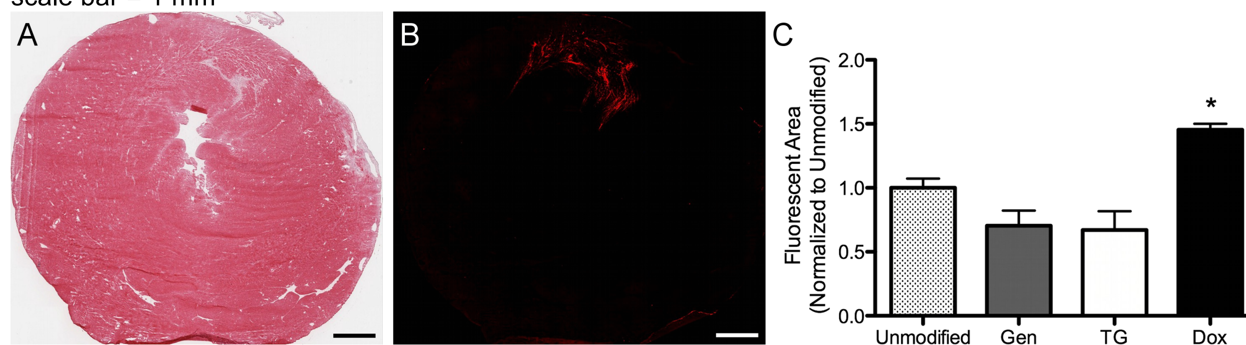
Figure 7 Doxycycline reduces myocardial matrix degradation in vivo. A) H&E image of a heart section showing the injection site. B) Alexa Fluor 568 (red) labeled myocardial matrix is visible fluorescently. C) Quantification of the amount of fluorescence remaining at one-week after injection. Gen: genipin, TG: transglutaminase, Dox: doxycycline; * $p < 0.05$ compared to unmodified hydrogel controls; scale bar = 1 mm



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