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In Vivo Response to Dynamic Hyaluronic Acid Hydrogels

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Abstract

Tissue-specific elasticity arises in part from developmental changes in extracellular matrix over time, e.g. ~ 10-fold myocardial stiffening in the chicken embryo. When this time-dependent stiffening is mimicked *in vitro* with thiolated hyaluronic acid (HA-SH) hydrogels, improved cardiomyocyte maturation has been observed. However, host interactions, matrix polymerization, and stiffening kinetics remain uncertain *in vivo*, and each plays a critical role in therapeutic applications using HA-SH. Hematological and histological analysis of subcutaneously injected HA-SH hydrogels showed minimal systemic immune response and host cell infiltration. Most importantly, subcutaneously injected HA-SH hydrogels exhibited time dependent porosity and stiffness changes at a rate similar to hydrogels polymerized *in vitro*. When injected intramyocardially, host cells begin to actively degrade HA-SH hydrogels within 1-week post-injection, continuing this process while producing matrix to nearly replace the hydrogel within 1 month post-injection. While non-thiolated HA did not degrade after injection into the myocardium, it also did not elicit an immune response, unlike HA-SH, where visible granulomas and macrophage infiltration were present at 1 month post-injection, likely due to reactive thiol groups. Altogether, these data suggest that the HA-SH hydrogel responds appropriately in a less vascularized niche and stiffens as had been demonstrated *in vitro*, but in more vascularized tissues, *in vivo* applicability appears limited.

Keywords

cardiomyocyte; intramyocardial injection; extracellular matrix; stiffness

1. Introduction

Matrix elasticity is an important cue regulating a variety of cellular responses, including ‘durotaxis’ [1–3], adhesion [4–6], proliferation [7–9], and differentiation [1, 4, 10, 11]. Stem or progenitor cellular responses can be further improved when extracellular matrix (ECM) cues are presented in a developmentally appropriate context, i.e. matrices with appropriate temporal [12–14] or spatial changes in stiffness [1, 9, 13, 15] to encourage cell alignment,

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cell fusion [15], or striation assembly [16]. On the other hand, matrices that are stiffer than normal to mimic *in vivo* tissue fibrosis [17–19] result in aberrant cell behavior *in vitro* [20, 21]. In the case of myocardial infarction (MI), stem cell-based interventions post-MI, i.e. cellular cardiomyoplasty, are intended to attenuate negative remodelling, but have demonstrated mixed results [22–24], potentially due to consequences of stiffening [18] inducing transdifferentiation [25].

To protect cells from these adverse conditions, a variety of cell-adhesive scaffolds have been employed, including fibrin [26, 27], collagen [28], matrigel [29–31], alginate [32, 33], and de-cellularized matrix [34, 35]. Each scaffold has been tailored to display unique properties suitable for its application, e.g. injectability, stiffness, cellularity, etc. For example, stiffness increases ~10-fold during development, and mimicking this change *in vitro* using a thiolated hyaluronic acid (HA-SH)/poly(ethylene glycol) diacrylate (PEGDA) hydrogel improved cardiomyocyte maturation by more than 60% [12]. Though HA is a glycosaminoglycan that can be modified with chemistries to present specific spatial and temporal properties [9, 12, 14, 36, 37], it is not clear whether thiolated HA remains as non-immunogenic as unmodified HA *in vivo* [38]. Recently free thiols have been implicated in hematopoietic stem cell differentiation and subsequent lymphocyte activation [39], which may negatively impact the *in vivo* performance of HA-SH hydrogels. Moreover, it is not certain if the HA-SH hydrogel will have similar crosslinking dynamics as was reported *in vitro* [12]. To answer these questions and determine its utility as a cardiac tissue engineering scaffold, we sought to determine the biocompatibility and temporal stiffening of the HA-SH/PEGDA material *in vivo* using subcutaneous and intramyocardial injections. Although the material stiffens with the same kinetics and does not elicit an immune response in a subcutaneous niche, we found an unforeseen inflammatory reaction after injecting into the healthy heart, likely resulting from adverse host interaction with free thiols on HA. While use in the failing heart post-MI would have been ideal, biocompatibility issues in healthy myocardium may limit its further *in vivo* use; nonetheless, the dynamics of this hydrogel system makes it a useful *in vitro* tool to study dynamic effects on cellular behavior in a wide variety of applications.

2. Materials and Methods

2.1 Hyaluronic Acid Gelation

Thiolated hyaluronic was obtained directly from a commercial source (Glycosan Biosystems). The sample was analyzed via ^1H nuclear magnetic resonance (NMR) spectroscopy (JEOL ECA 500) to assess thiol substitution. To prepare appropriately stiff HA hydrogels to mimic heart stiffening, 4.53% (w/v) PEGDA with $M_w \sim 3400$ Da (polydispersity index or PDI ~ 3 , Glycosan Biosystems) in de-gassed (DG) PBS and 1.25% thiolated HA (Glycosan BioSystems) in DG PBS were separately mixed at 37°C with gentle shaking for up to 30 minutes. To initiate polymerization, solutions were combined at a volume ratio of 1 PEGDA solution: 4 HA solution to yield a 1% HA/0.9% PEGDA hydrogel, unless otherwise indicated.

2.2 Subcutaneous and Intramyocardial Injections

All animals received humane care in compliance with University of California, San Diego's Institutional Animal Care and Use Committee (protocol #S08172 and #S10026) and the American Association for the Accreditation of Laboratory Animal Care. HA hydrogels were prepared as above but using aseptic methods and materials warmed at 37°C for ~5 mins until the solution became viscous. For subcutaneous injections, animals were anesthetized with 5% isoflurane and maintained at 2.5% isoflurane with a nose cone throughout the procedure. Prior to injection, 3mg/kg Bupivacaine Hydrochloride (Hospira), a local anesthetic, was delivered to the injection sites in order to minimize pain. Two 200 μL injections were made

on the backside of Sprague-Dawley (SD) rats opposite the spinal cord using a 25 G needle attached to a 1mL luer lock syringe (BD Biosciences). Boluses were visible at the injection sites. Samples from four animals per time point were removed 1, 3, 5, 7 and 14 days after injection and subject to mechanical (AFM) and histological analysis.

For intramyocardial injections, pre-gelation time was carefully regulated because when injected too early, the gel solution dispersed interstitially and could not be identified via histology ($< \sim 3$ min); when injected too late, the syringe resistance was too great to deliver the increasingly crosslinked hydrogel ($> \sim 5$ min). For comparison, three other materials were also examined for intramyocardial injection. Human clinical grade HA (Restylane®) was injected as a 1% pre-gelled solution to be able to best compare its results with HA hydrogels lacking thiols. In order to examine effects of the PEGDA crosslinker, a mixture containing 1/10 PEGDA and the regular amount of HA-SH, i.e. 0.09% PEGDA/1% HA-SH hydrogel, was prepared along with a 2% HA-SH mixture without crosslinker. Both samples were prepared aseptically the day before injection and allowed to gel in the syringe at 37°C overnight due to slow gelation time when acrylate crosslinker concentration is dramatically reduced or absent.

Before injection, animals were anesthetized with 5% isoflurane, intubated, and maintained at 2.5% isoflurane throughout the procedure. After anesthetization, animals were given 3 ml of lactated Ringers solution (Hospira) for hydration during surgery. Injections were performed using a procedure described previously [40]. Briefly, an incision was made in the abdomen, the diaphragm was cut to expose the heart and the heart was held steady using forceps. A single injection of 50–75 μ L of the hydrogel was delivered into the left ventricular free wall of healthy SD rats using a 27 G needle attached to a 1 mL slip tip syringe (BD Biosciences). Blanching was observed after injection, which ensured material injection into the myocardium, though some extrusion was not uncommon. After injection, suction of the chest cavity was performed in order to ensure the diaphragm was tight, the abdomen was stitched up, and animals were allowed to recover. Once alert and sternal, animals were given 0.05 mg/kg of buprenorphine hydrochloride (Reckitt Benckiser Healthcare), an analgesic, prior to recovery from anesthesia. Hearts from 3–6 animals per time point were removed after 1 hr or less and 3, 7, 14 and 30 days after injection and subjected to histological analysis. A statistical power analysis set to a threshold of 0.8 was performed by “PS - Power and Sample Size Calculation” software indicated at least 3 rats per condition were required per experiment.

2.3 Hematology

For subcutaneous injections, 25 μ L of blood was drawn from the saphenous vein of anesthetized (5% isoflurane and maintained at 2.5% isoflurane with a nose cone) rats prior to subcutaneous injection and prior to removal of the gel according to the time course. Four control rats underwent the same blood withdrawal procedure without any injection. Blood samples were examined on a Hemavet Hematology Analyzer (UCSD Hematology Core). Whole blood cell, neutrophil and monocyte counts were examined by comparing pre- vs. post-injection values for each time point. Statistical analyses compared each time point of non-injected and HA-injected samples.

2.4 Histology

For subcutaneous injections, one of the bilateral injections was removed from each rat at the indicated time points and frozen in Tissue Tek Optimal Cutting Temperature (OCT) solution. Subcutaneous injection samples were sectioned on a Cryocut 1800 (Leica) at 10 μ m and mounted on glass slides. Intramyocardial injection samples and *in vitro* hydrogels were frozen in the same manner as mentioned and sectioned at 10 μ m. Alternating slides

were stained with hematoxylin and eosin (H&E). Briefly, samples were rehydrated in DH₂O for 3 min, stained in hematoxylin (Fisher Scientific) for 2.5 min, rinsed in water for 3 min, dipped in bluing reagent (Protocol) 20 times, dehydrated in EtOH, stained in Eosin (Fisher Scientific) for 2.5 min, dehydrated once more, and cleared in HistoClear (National Diagnostics). Samples were mounted with Cytoseal-60 (Richard-Allan Scientific), examined using a Carl Zeiss Observer D.1, and analyzed with AxioVision software. H&E stained samples were corrected for white balance.

2.5 Immunohistochemistry (IHC)

Remaining slides from subcutaneous and intramyocardial injections were stained with antibodies for a lymphocyte marker, CD45 (ab10558, Abcam), or a macrophage marker, CD68 (ab31630, Abcam), and Hoescht (33342, Invitrogen) as indicated in order to visualize any inflammatory cell response present in the samples. Briefly, samples were fixed in acetone for 1.5 min, rinsed in 1X PBS, blocked with staining buffer (0.3% Triton-100X and 2% goat serum in 1X PBS) for 20 min, and incubated with primary antibody at 1:200 in staining buffer for 1hr, anti-mouse Alexa Fluor[®] 488 (A11001, Invitrogen) for 30 min, and Hoescht for 10 min at room temperature. Samples were mounted with fluoromount-G (SouthernBiotech). Samples were examined using a BD Carv II confocal microscope (BD Biosciences; San Jose, CA) mounted on a Nikon Eclipse TE2000-U microscope with Metamorph 7.6 software. Since the hydrogels were not stained, they could be visualized using bright field in order to confirm injected hydrogel and host tissue position for fluorescence imaging.

2.6 Atomic Force Microscopy

The other subcutaneous bilateral injection was removed intact, rinsed in 1X PBS and mounted on a glass slide for AFM analysis. AFM indentation was performed as previously described [41] using 20 pN/nm pyramidal tips (Olympus TR400PB) or 2 μ m radius 120pN/nm spherical tips (Novascan), a tip approach velocity of 2 μ m/s, and a deflection trigger of 100 nm. To minimize tip-sample interaction, measurements were performed in 1% bovine serum albumin in PBS. Force vs. displacement curves were analyzed using a non-contact, linearized Hertz method [42], assuming a Poisson's ratio $\sim$ 0.5. No significant difference was observed between values obtained from the two different cantilever geometries for all hydrogels.

2.7 Statistical Analysis

All experiments were performed using three biological replicates unless otherwise noted and reported as a mean $\pm$ standard error of the mean. Significance was assessed by student's t-test at a significance threshold of $p < 0.05$ or lower as indicated. Data where $p > 0.05$ is not statistically different and is reported to emphasize similarities between treated and sham conditions in hematological analysis.

3. Results

3.1 Subcutaneous Injections

Hematological analysis of total white blood cell as well as monocyte and neutrophil counts, normalized to their pre-injection value, were not found to be elevated in either the HASH/PEGDA injected or non-injected conditions (Fig. 1). Given the lack of statistical difference between injected and non-injected rats for our sample size, sham-injected rats were not measured. Hydrogel polymerization and local immune response were also assessed in order to examine hydrogel biocompatibility after injection into the host. The HA-SH and PEGDA crosslinker formed a continuous network subcutaneously (Fig. 2, top). Lymphocytes were

present at low levels within and surrounding the hydrogel as indicated by CD45 staining (Fig. 2, middle, white arrows).

HA-SH hydrogels have been previously shown to undergo time-dependent stiffening *in vitro* [12], and thus the dynamics previously measured *in vitro* were verified in this subcutaneous injection model. Hydrogel porosity was compared via histological sections between identical samples either injected *in vivo* or maintained *in vitro* with both conditions resulting in comparable changes in porosity over time (Fig. 3). Similar crosslinking dynamics between *in vivo* and *in vitro* samples should result in similar changes in stiffness over time; after removing subcutaneously injected samples, direct comparison of the two samples by AFM indicated that HA-SH/PEGDA stiffening dynamics were preserved after injection (Fig. 4).

3.2 Intramyocardial Injections

HA-SH hydrogel stiffening mimics cardiac developmental stiffening [12], so HA-SH hydrogels were also examined after intramyocardial injection into the healthy heart. HA-SH hydrogels were found to be intact and well distributed throughout the injection site through 1-week post-intramyocardial injection with host cells beginning to infiltrate and degrade the material (Fig. 5, 1 week; nuclei stained blue). The hydrogel appeared to be more porous at early time points compared to subcutaneous or *in vitro* controls (Fig. 5, 30 min vs. Fig. 3, 1 day). At one month, degraded hydrogel with host cell infiltration was observed along with new matrix deposition (Fig. 5, 1 month); however, the response was indicative of chronic inflammation.

To determine the inflammatory source, hydrogels comprised of non-thiolated HA (Restylane[®]), HA-SH, or HA-SH with high or low PEGDA crosslinker were injected into the myocardium. Restylane[®] did not elicit a significant, chronic immune response surrounding the injection site, as assessed by H&E staining and the lack of lymphocytes (CD45) and macrophages (CD68) within the injection site (Fig. 6A, leftmost column). In contrast, staining of standard HA-SH hydrogels, i.e. 0.9% PEGDA/1% HA-SH, indicated a dramatic increase in host cell infiltration from H&E staining and the presence of CD45 and CD68 positive cells within the injection site (Fig. 6A, left center column). These data implicated thiol and acrylate groups within the hydrogel as the inflammatory source. Therefore, a hydrogel with lower crosslinking concentration (0.09% PEGDA) was injected to determine if free acrylates induced inflammation, and a similar immune response with CD45 and CD68 positive cells was observed within weeks of injection (Fig. 6A right center column). In the absence of PEGDA but with a higher HA-SH concentration (to allow gelation via thiol-thiol bonding), CD45 and CD68 positive cells were still visible, indicating that the thiol may be an inflammatory source (Fig. 6A, rightmost column). A lack of CD45 and CD68 staining in non-injected regions of the heart ensured that no inflammation was present outside of the injection area (Fig. 6B).

4. Discussion

HA is a durable glycosaminoglycan for tissue engineering due to its ECM-derivation and ease of modification, e.g. methacrylation [9], thiolation [37], etc. More specifically, thiol modification of HA and subsequent crosslinking with acrylates in a Michael-type addition reaction has been well described by Prestwich and co-workers [36, 37, 43]. Recently using specific HA-SH and PEGDA concentrations and molecular weights, time-dependent crosslinking dynamics have been described where slow thiol-acrylate and thiol-thiol reactions account for the temporal change in stiffness [12]. We documented here that in a less vascularized, subcutaneous niche, these same formulations are capable of time-dependent changes in porosity and stiffness without significant immunogenicity. In much more vascularized tissues, an adverse host interaction, likely due to the presence of free

thiols, and perhaps subsequent oxidation [44, 45], created an inflammatory reaction that will limit its myocardial compatibility. Despite these results in vascularized tissues, less vascularized niche where dynamic changes occur, e.g. cartilage [46], may be suitable for thiolated HA hydrogels.

4.1 Subcutaneous versus Intramyocardial HA-SH Injections

Assessment of local and systemic immune responses after subcutaneous and intramyocardial HA-SH injection provides important information on host interactions, matrix polymerization, and stiffening kinetics *in vivo*. Subcutaneously, limited systemic immune response and small fibrotic capsule surrounding the material demonstrate that the hydrogel does not elicit a significant local and systemic response. These results are consistent with prior subcutaneously-injected hydrogels composed of other thiolated glycosaminoglycans [47], though specific formulations, concentrations, and crosslinkers differ. Most importantly, AFM and porosity measurements confirm that the HA-SH hydrogel's temporal stiffening occurs when injected subcutaneously *in vivo*. However, direct evaluation of porosity may be limited by host cell infiltration and potential sectioning differences between tissues. Despite this, AFM data was consistent between *in vitro*-polymerized and subcutaneously-polymerized measurements, though both are subject to little strain when compared to the myocardium in which the gel is more compressed and subject to rhythmic contraction. In the higher strain niche of the myocardium, non-linear strain stiffening may occur, which could alter gelation kinetics, although significant cell infiltration and hydrogel degradation precludes measurement.

In more vascularized tissues, i.e. myocardium, the formation of large, CD68-positive granulomas and inflammatory cells within and surrounding the hydrogel were observed, indicating a significant immune response not present in subcutaneous injections. Though non-thiolated HA did not elicit the same adverse response intramyocardially, such hydrogels lack the benefit of cell-mediated degradation and HA-SH's temporal stiffening. Closer inspection of the biocompatibility issue appears to indicate that the adverse interaction was specific to thiol-containing hydrogels. Thiol oxidation is cytotoxic *in vitro* [44, 45], and while capping exposed groups post-polymerization *in vivo* via iodoacetamide would limit oxidation, it would also prevent additional Michael-type reactions and thus block beneficial temporal stiffening of the hydrogel. While thiol-based Michael-type reactions *in vitro* have been extensively documented [48–50] and cells typically interact with a matrix layer covalently attached to the hydrogel's surface [12], there are limited studies using these materials *in vivo* [47] with results that show mixed biocompatibility, particularly in highly vascularized locations such as the eye [51] or in the myocardium of immune-compromised mice post-infarct [52]. This study, along with some of these recent studies, suggests that thiols may not produce biocompatible responses in highly vascularized tissues, and therefore this crosslinking mechanism may be limited to *in vitro* applications or specific tissues.

4.2 Alternative Approaches

Despite limitations in vascularized tissues, HA-SH hydrogels appear useful in less vascular settings and as an *in vitro* culture substrate. We have previously shown that varying hydrogel components, e.g. HA concentration, crosslinker concentration, and crosslinker molecular weight, can affect overall stiffness as well as stiffening kinetics [12]; therefore, HASH hydrogels could be used in conjunction with soluble factors to guide *in vitro* cell behavior for applications that require softer or stiffer hydrogels with slower or faster kinetics. For example, dynamic culture conditions could be utilized for stem cell cardiac differentiation, wound healing or fibrosis [12, 18, 53], keeping in mind that *in vitro*, cells attach to a layer of collagen covalently bound to the HA-SH hydrogel.

An alternative approach to our hydrogel system may be to modify HA with thioethyl ether derivatives, which appear to protect against oxidation as it reduces cell apoptosis upon exposure to hydrogen peroxide *in vitro* [36]. To ensure that it would have dynamic stiffening like the HA-SH hydrogel, such material would need to have approximately the same substitution efficiency of HA-SH, e.g. ~40% [12], be used at the same concentrations, etc.

5. Conclusions

It has previously been shown that time-dependent stiffening enhances cardiomyocyte maturation [12] and thus, the combination of cells and developmentally appropriate matrix cues in regenerative therapies may significantly restore tissue function relative to cell-only therapies, e.g. cellular cardiomyoplasty. Injections into less vascularized tissues here indicate that these same HA-SH hydrogels can at least achieve stiffening and crosslinking as *in vitro* hydrogels, without significant host response, and therefore may be useful as a subcutaneous *in vivo* model. In highly vascularized regions, i.e. myocardium, injections show that HA-SH can assemble and degrade over time; however, the continual recruitment of inflammatory cells likely due to interactions with free thiol in the scaffold implies that HA-SH based hydrogels have limited biocompatibility, adding to recent evidence that popular thiol-based Michael-type reactions may not be suitable for many *in vivo* applications. However, these materials may still be applicable as an *in vitro* tool to study the effects of dynamic mechanical cues on cellular behaviors where cells on top of the hydrogel bind to a covalently attached matrix layer and ‘feel’ the stiffening beneath them rather than interact with the free thiols within the hydrogel.

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Abbreviations

HA	Hyaluronic Acid
Pa	Pascal
PEGDA	poly(ethylene glycol) diacrylate
DG PBS	degassed phosphate buffered saline
AFM	atomic force microscopy

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Figure 1.

Hematological cell counts of subcutaneously HA-injected (black) and non-injected rats (gray) for whole blood (top), monocytes (middle) and neutrophils (bottom) are shown. Values are post-injection at each indicated time point, were always taken prior to sample removal, and were normalized to pre-injection values. None of the subcutaneous HA-injected data was statistically different from non-injected rats, i.e. whole blood had $p > 0.06$, monocytes had $p > 0.14$, and neutrophils had $p > 0.16$ as determined by student's t-test. $n = 4$ for each group.

Figure 2. (top) Hematoxylin and eosin (H&E) stained sections of subcutaneously injected samples at 1 (left) and 14 (right) days post-injection. (middle) Immunohistochemical staining for CD45 (green) and Hoescht (blue) shows the presence of a limited number of inflammatory cells within and around the hydrogel (arrowheads). (bottom) Accompanying brightfield images for the middle panel of images. Scale bars are 500 μm for the top and 100 μm for the middle and bottom images. The dashed white line denotes the host tissue (T) and injection (I) regions.

Figure 3. Hematoxylin and eosin stained cross-sections of *in vitro* (left) and subcutaneously injected *in vivo* (right) samples are shown as a function of time. Scale bars are 500 μm.

Figure 4.

Elastic modulus of subcutaneously injected HA (gray) compared to *in vitro* (black) on a log-log plot. Data points are fitted with a power law: $y=x^A$, where $A=0.36$ *in vivo* and $A=0.32$ *in vitro*. Student's t-tests analysis indicates that these data are not statistically different ($p>0.97$). *In vitro* and *in vivo* samples had $n = 3$ and 6 , respectively.

Figure 5. (A) HA-SH hydrogel in myocardium 30 min (left), 1 week (middle) and 1 month (right) post-injection (indicated by arrowheads in top panel). (B) Images of the area indicated by the black dashed box have been enlarged. 1 month post-injection shows material degradation and cell infiltration (3). Scale bars in panels A and B are 500 and 250 μm , respectively.

Figure 6.

(A) Histological sections of hearts injected with Restylane® (left), 2% HA-SH hydrogels (left center), 0.09% PEGDA/1% HA-SH hydrogel (right center), and 0.9% PEGDA/1% HA-SH (original formulation, right) are shown 3 weeks post-intramyocardial injection. For H&E stained sections, dashed box in the top row (scale bar is 2000 μm) indicates the enlarged area depicted in the second row (scale bar is 100 μm). Immunohistochemical staining 3 weeks post-intramyocardial injection for CD68 (green, third row) and CD45 (green, fourth row) show macrophages and lymphocytes, respectively, as well as other invasive cells (Hoescht-labeled nuclei in blue) present within and around the hydrogels as indicated. The dashed line separates the injection (I) from surrounding host tissue (T). (B) Immunohistochemical staining for CD45 (left) and CD68 (right) in non-injected regions of the myocardium. Image intensity is scaled according to the injected images in panel A. Scale bars are 50 μm for all immunofluorescent images.