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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 has been mimicked *in vitro* with thiolated hyaluronic acid (HA-SH) hydrogels, improved cardiomyocyte maturation has been observed. However, host interactions, matrix polymerization, and the 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 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.

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, 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 cellular 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 remodeling, but have demonstrated mixed results [22–24], potentially due to the consequences of stiffening [18] inducing transdifferentiation [25].

A variety of cell-adhesive scaffolds have been employed to protect cells from these adverse conditions, including fibrin [26,27], collagen [28], matrigel [29–31], alginate [32,33], and decellularized matrix [34,35]. Each scaffold has been tailored to display unique properties suitable for its application, e.g. injectability, stiffness and cellularity. 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]. Although HA is a glycosaminoglycan that can be chemically modified 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 have a negative impact on the *in vivo* performance of HA-SH hydrogels. Moreover, it is not certain whether HA-SH hydrogels will have similar crosslinking dynamics to those 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 time-dependent stiffening of a 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

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resulting from adverse host interaction with free thiols on HA. While use in the failing heart post-MI would be ideal, biocompatability 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 acid was obtained directly from a commercial source (Glycosan Biosystems). The sample was analyzed by ^{1}H 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 (M_w ~3400 Da, polydispersity index or PDI ~3, Glycosan Biosystems) in degassed (DG) phosphate-buffered saline (PBS) and 1.25% thiolated HA (Glycosan Biosystems) in DG PBS were separately mixed at 37 °C with gentle shaking for up to 30 min. To initiate polymerization, solutions were combined at a volume ratio of 1:4 PEGDA solution to 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 the University of California, San Diego Institutional Animal Care and Use Committee (protocols 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 min 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 3 mg kg^{-1} bupivacaine hydrochloride (Hospira), a local anesthetic, was delivered to the injection site in order to minimize pain. Two 200 μl injections were made on the back of Sprague–Dawley (SD) rats opposite the spinal cord using a 25 G needle attached to a 1 ml 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 subjected to mechanical (atomic force microscopy (AFM)) and histological analysis.

For intramyocardial injection, the pre-gelation time was carefully regulated, because when injected too early, the gel solution dispersed interstitially and could not be identified histologically (<~3 min); when injected too late, the syringe resistance was too great to deliver the increasingly crosslinked hydrogel (>~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 the effects of the PEGDA crosslinker, a mixture containing a tenth of the amount of 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 the slow gelation time when the acrylate crosslinker concentration is dramatically reduced or the crosslinker is absent.

Before injection, animals were anesthetized with 5% isoflurane, intubated, and maintained at 2.5% isoflurane throughout the procedure. After anesthetization, the animals were given 3 ml of lactated Ringer's solution (Hospira) for hydration during surgery. Injections were performed using a procedure described previously [26]. 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 μl 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 confirmed material injection into the myocardium, although some extrusion was not uncommon. After injection, suction of the chest cavity was performed in order to ensure that the diaphragm was tight, the abdomen was stitched up, and the animals were allowed to recover. Once alert and upright, the animals were given 0.05 mg kg^{-1} buprenorphine hydrochloride (Reckitt Benckiser Healthcare), an analgesic, prior to recovery from anesthesia. Hearts from 3–6 animals per time point were removed after 1 h 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 using PS – Power and Sample Size Calculation software, and it indicated that at least 3 rats per condition were required per experiment.

2.3. Hematology

For subcutaneous injection, 25 μl of blood was drawn from the saphenous vein of anesthetized (5% isoflurane, maintained at 2.5% isoflurane with a nose cone) rats prior to subcutaneous injection and prior to removal of the gel. Four control rats underwent the same blood withdrawal procedure without any injection. Blood samples were examined in 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 μm and mounted on glass slides. Intramyocardial injection samples and *in vitro* hydrogels were frozen in the same manner and sectioned at 10 μm. Alternating slides were stained with hematoxylin and eosin (H&E). Briefly, samples were rehydrated in deionized H_2O 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 ethanol, 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 cells present in the samples. Briefly, samples were fixed in acetone for 1.5 min, rinsed in 1× PBS, blocked with staining buffer (0.3% Triton X-100 and 2% goat serum in 1× PBS) for 20 min, and incubated with primary antibody at 1:200 in staining buffer for 1 h at 37 °C, anti-mouse Alexa Fluor® 488 (A11001, Invitrogen) for 30 min at 37 °C, and Hoescht for 10 min at room temperature. Samples were mounted with fluoromount-G (SouthernBiotech). Samples were examined using