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Injectable and protease-degradable hydrogel for siRNA sequestration and triggered delivery to the heart

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Abstract

Injectable hydrogels have significant therapeutic potential for treatment of myocardial infarction (MI) through tissue bulking and local drug delivery, particularly for small interfering RNAs (siRNAs). As siRNA targets are identified as potential treatments for MI, hydrogels may bolster efficacy through local and sustained release. Here, we designed an injectable hydrogel to respond to local upregulation in proteolytic activity after MI to erode and release siRNA against MMP2 (siMMP2), a target implicated in deleterious remodeling. Specifically, hyaluronic acid (HA) was modified with hydrazides or aldehydes and mixed to form shear-thinning and self-healing hydrogels through dynamic hydrazone bonds and with peptide crosslinkers that degrade in response to protease activity. HA was further modified with β -cyclodextrin to sequester cholesterol-modified siRNA, limiting passive diffusion. Hydrogels eroded in response to proteases and released active siRNA that knocked down MMP2 in primary cardiac fibroblasts. In a rat model of MI, hydrogels delivering siMMP2 attenuated hydrogel erosion by ~46% at 4 weeks when compared to hydrogels delivering control siRNA, ultimately improving myocardial thickness in the infarct. Delivery of the siMMP2 hydrogel led to significant functional improvements, including increased ejection fraction (27%, 66%), stroke volume (32%, 120%), and cardiac output (20%, 128%) when compared to hydrogels with control siRNAs or saline injection alone, respectively. This report demonstrates the utility of biomaterial-based RNA delivery systems for cardiac applications.

Graphical abstract

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Keywords

siRNA; myocardial infarction; hydrogel; matrix metalloprotease

1. Introduction

Myocardial infarction (MI) and ensuing heart failure continue to be a leading cause of mortality worldwide and account for approximately 17.3 million deaths globally each year and at least one in every four deaths in the United States [1]. Although 95% of patients will survive initial hospitalization from acute MI due to medical and surgical innovations [2], the majority of patients will inevitably develop chronic heart failure, which is associated with a 50% mortality rate five years after acute MI [1,3]. This mortality rate can be attributed to inadequate treatments for the pathologic sequelae that occurs after MI.

Biomaterials have emerged as therapies for treating MI, beginning with the use of polymeric meshes that are wrapped around or sutured to the infarcted tissue, restricting infarct expansion and dilation [4–6]. Towards less invasive approaches, numerous injectable hydrogels, water-swollen polymer networks, have been investigated in the heart for therapy [7–9], including some that have undergone assessment in clinical trials (NCT01226563, NCT01311791, NCT02305602). Injectable hydrogels can further be adapted for delivery through minimally invasive approaches, such as percutaneously through catheters fluoroscopically guided to the heart [9–11]. Because wall stress in the heart is inversely proportional to wall thickness according to LaPlace’s law, injectable hydrogel bulking confers a mechanical advantage to infarcted myocardium by increasing wall thickness to reduce stress [9,12].

In addition to cardiac bulking, injectable hydrogels have been designed for the therapeutic delivery of biologically active agents to the heart, which include growth factors[13,14], small molecules[15], DNA[16], and RNA [17,18]. Through a combination of diffusion, erosion, and swelling, hydrogels can release encapsulated therapeutics, with kinetics that are tuned by varying features such as polymer concentration, crosslink density, and drug-polymer interactions (e.g. electrostatics, hydrophobicity). Advanced hydrogel systems are also being developed where release can be triggered by events in the internal or external environment, including but not limited to enzymatic activity, temperature, pH, or electrical current [14,19]. Such engineered or “smart” hydrogels thus achieve additional spatiotemporal control over drug delivery.

For MI, hydrogels have been adapted to respond to protease activity within infarcted tissue [14]. Protease overexpression after MI is identified as one of the leading mechanisms for

infarct expansion, dilation, and mechanical compromise. One family of proteolytic enzymes that have been specifically implicated in these processes are the matrix metalloproteases (MMPs) [20–23]. MMPs are elevated acutely in serum after MI, as well as locally in the interstitium of cardiac tissue [24,25]. Thus, the local rise in their activity can be used as an endogenous stimulus for the on-demand release of therapeutics from hydrogels.

Therapeutics of interest to treat MI include molecules that can attenuate MMP activity [26,27]. MMP2, in particular, is significantly overexpressed after MI and is as much as 3000-fold higher in infarct tissue by 8 weeks [24]. MMP2 is also individually linked to acute mortality and cardiac remodeling after MI, as well as to cardiomyocyte apoptosis, ischemia/reperfusion damage, and inhibition of endothelialization [28–33]. While exogenous small molecules or endogenous tissue inhibitors of MMPs (TIMPs) are able to broadly inhibit MMP activity, they are not specific to single MMPs, including MMP2, and may affect activity of other unrelated proteases [34–36]. To introduce specificity to molecular targets, RNA interference therapies (RNAi) have emerged, wherein small double-stranded RNAs, known as small interfering RNAs (siRNAs) are able to precisely inhibit translation of a single, complementary messenger RNA (mRNA) target [37]. siRNAs targeting MMP2 (siMMP2) have not been explored as a therapy after MI, although their use has previously been shown to improve cardiomyocyte function and vascularization [32,33], and MMP2 knockout mice have demonstrated improved cardiac function after MI [28,29].

For the therapeutic application of siMMP2, we engineered a protease-responsive hydrogel delivery platform, which addresses many systemic delivery challenges common to siRNA therapeutics, including off-target silencing, low local bioavailability, and serum stability [37]. Here, we describe an injectable, hyaluronic acid (HA) hydrogel for the local and sustained delivery of siMMP2 that erodes in response to local protease activity, thereby releasing siMMP2.

2. Materials and methods

2.1 Materials:

Sodium hyaluronate (MW = 400 kDa, 74 kDa) was purchased from LifeCore (Chaska, MN). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless specified otherwise. All synthesis products were stored dry and under N₂ at –20°C.

2.2. siRNA sequences:

Accell siRNAs with cholesterol modifications on the 3' end of the sense strand, 2'-O-methylation, and phosphothiorates were custom-synthesized from Dharmacon (Lafayette, CO). The sequence of the sense strand of non-specific siRNA targeting GFP (siCTRL) is 5'-GCCACAACGUCUAUAUCAU-3' and the sequence of the sense strand of siMMP2 is 5'-GACGCUGCCUUUAACUGGA-3'. The siMMP2 sequence is a rat-specific sequence that was adapted as an Accell siRNA from a sequence previously used in cardiac fibroblasts [38]. All siRNAs were stored as 10 µM aliquots in PBS at –20°C.

2.3. CD-HA-ALD synthesis and characterization:

Sodium hyaluronate (400 kDa, 200 mg, 0.5 mmol) and sodium periodate (110 mg, 0.5 mmol) were dissolved in 20 mL de-ionized water (dH₂O). The reaction was stirred for 2 hours in the dark and quenched in 2 mL ethylene glycol to consume unreacted periodate. Products were dialyzed for 5 days against dH₂O (14000 MWCO) and lyophilized. To quantify aldehyde modification, aldehyde-modified HA (ALD-HA) was dissolved at 2 wt% and subsequently reacted with tert-Butyl carbazate (t-Bc, 1% in trichloroacetic acid) in dH₂O overnight as previously described [14]. The next day, the unreacted t-Bc in the HA-ALD solution was compared to t-Bc standards by reaction with 2,4,6-trinitrobenzenesulfonic acid (TNBS, 6 mM in 0.1 M sodium tetraborate, pH 8) for 1 hour. Samples were then reacted with 0.5 N hydrochloric acid and absorbance was measured at $\lambda=340$ nm on a microplate reader (Tecan). To conjugate β -cyclodextrin (CD), ALD-HA (~25 kDa, 225 mg, 0.6 mmol) was dissolved in dH₂O at 2 wt%. An aminated form of CD, mono-(6-hexanediamine-6-deoxy)- β -cyclodextrin (1.4 g, ~1.2 mmol), synthesized as previously described [39], was added in two-times molar excess to the ALD-HA and dissolved overnight at room temperature. N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide (EDC, 120 mg, ~0.6 mmol) and hydroxybenzotriazole (HOBT, 81 mg, ~0.6 mmol) were subsequently added and the reaction was stirred overnight, dialyzed for two weeks against dH₂O (14000 MWCO), filtered, frozen, and lyophilized. ¹H NMR (Bruker) at 360 MHz was used to determine final product modification by integrating the protons of the hexane linker of CD ($\delta=1.2\text{--}1.8$, 12H) relative to the protons of the methyl singlet of HA ($\delta=2.1$ ppm, 3H), confirming that 25% of disaccharide repeats in ALD-HA were modified. A t-Bc/TNBS assay was performed on the final product (3.5 wt%) to confirm that aldehydes were minimally affected during the amidation reaction.

2.4. Peptide synthesis:

A heterobifunctional MMP-cleavable peptide GCNSGGRMSMPVSNGG-hydrazide (MMP-HYD, MW=1626.3 g/mol) was synthesized using solid phase peptide synthesis. Briefly, solid-phase resin (Novabiochem) was deprotected with piperidine (20% v/v) in N,N-dimethylformamide (DMF). Fmoc-protected amino acids (Novabiochem) were activated with 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate in 0.4 M N-methylmorpholine in DMF at 4x molar excess to solid-phase resin using an automated solid phase peptide synthesizer (Protein Technologies). Tri-Boc-hydrazinoacetic acid was incorporated as the final group in the synthesis in the free acid form. The synthesized peptide was liberated from the resin by 95% trifluoroacetic acid, 2.5% tri-isopropylsilane, and 2.5% dH₂O for 3 hours at room temperature. The peptide was filtered, washed three times in cold ether (−80°C), dried against vacuum pressure overnight, reconstituted in dH₂O, frozen, and lyophilized.

2.5. MA-HA synthesis and characterization:

Maleimide-modified HA (MA-HA) was synthesized as previously described through an amidation reaction between the carboxyl of HA and an amino-modified maleimide [40]. Briefly, the tetrabutylammonium (TBA) salt of HA (74 kDa) was dissolved in DMSO with N-(2-aminoethyl)maleimide trifluoroacetate salt in benzotriazol-1-

xyloxytris(dimethylamino)-phosphonium hexafluorophosphate (BOP) for two hours at room temperature. The reaction was subsequently dialyzed (8000 MWCO) against dH₂O at 4°C for two weeks and stored frozen at -80°C. Products were lyophilized in 1.5 mL aliquots prior to use. ¹H NMR was used to determine final product modification by integrating the protons of the alkene group in maleimide (δ=6.8 ppm, 2H) relative to the protons of the methyl singlet of HA (δ=2.1 ppm, 3H), confirming that 25% of disaccharide repeats in HA were modified.

2.6. Hydrogel formation and siRNA encapsulation:

To form hydrogels (100 μL), solutions of the hydrazide and MMP-peptide containing HA (HA-MMP-HYD, 50 μL) and cyclodextrin-modified, aldehyde-modified HA, (CD-ALD-HA, 50 μL) were mixed. To form HA-MMP-HYD solution, MA-HA (1.6 mg) was lyophilized and reconstituted in PBS (50 μL) towards a final concentration of 3.1 wt%. The MMP-HYD peptide (1.6 mg) was dissolved in the MA-HA solution towards a final concentration of 3.1 wt% containing equimolar amounts of thiol and maleimide. The solution was mixed for 10 minutes at room temperature to allow the Michael addition reaction to occur, forming HA-MMP-HYD at a concentration of 6 wt%. CD-ALD-HA (2.6 mg) was separately dissolved in PBS (50 μL) towards a final concentration of 4.9 wt%. The two solutions of HA-MMP-HYD (6 wt%, 50 μL) and CD-ALD-HA (4.9 wt%, 50 μL) were manually mixed to form a hydrogel at a final concentration of 5.5 wt%, containing equimolar amounts of HYD and ALD. To form hydrogels with encapsulated siRNA, the aforementioned compounds were dissolved in an Accell siRNA solution in PBS (10 μM). To form hydrogels without CD, ALD-HA (1.5 mg) was dissolved in 50 μL PBS to a final concentration of 2.9 wt% and mixed with HA-MMP-HYD. Rheological measurements of hydrogel formation and mechanics were assessed on an AR2000 stress-controlled rheometer (TA instruments) fitted with a 20-mm diameter cone and plate geometry, 59 min 42 s cone angle, and 27 μm gap as previously described [18,39].

2.7. Fluorescence recovery after photobleaching (FRAP):

Experiments were performed on a confocal microscope (TCS SP5, Leica, Germany) with the 488 nm line of an Argon laser. Hydrogels (100 μL) were mixed and formed between two cover slips. Bleaching of a 200 μm diameter circular spot was performed under the 10x objective for 10 seconds under 50% laser power followed by post-bleach imaging every second at 2% laser power for 300 seconds. Analysis of fluorescent recovery was performed in MATLAB as previously described [41,42]. Briefly, fluorescent recovery profiles were fit by nonlinear least squares regression to the Soumpasis equation, which states the relationship between fluorescence (F) as a function of time (t) after photobleaching, the mobile fraction (k), diffusion time ($-\tau_D$), and modified zero (I_0) and first (I_1) order Bessel functions:

$$F(t) = ke^{-\frac{\tau_D}{2t}} \left[I_0\left(\frac{-\tau_D}{2t}\right) + I_1\left(\frac{-\tau_D}{2t}\right) \right]$$

Effective diffusivity was calculated from the diffusion time ($-\tau_D$) and bleach spot radius (w):

$$D_{eff} = \frac{w^2}{\tau_D}$$

2.8. Hydrogel erosion and siRNA release:

Hydrogels (100 μ L) were formed as previously described in 1.5 mL microcentrifuge tubes, vortexed, and centrifuged. Collagenase type II solutions (0, 20, and 200 U/mL) prepared in PBS supplemented with CaCl_2 (1 mM) were added to the hydrogel at 37°C. At indicated timepoints, buffer was collected and replaced. HA concentrations in releasates were quantified using a uronic acid assay as previously described [43]. For siRNA release characterization, FAM-modified siRNA (cat. no. D-001950, Dharmacon) was encapsulated in hydrogels. Releasates (50 μ L) from each timepoint and condition were diluted in PBS supplemented with CaCl_2 (150 μ L) and fluorescence (λ_{ex} =500 nm, λ_{em} =600 nm) was quantified on a microplate reader.

2.9. Cellular siRNA uptake and flow cytometry:

NIH 3T3 fibroblasts were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. All cells were seeded 24 hours prior to experiments at 1×10^5 /mL in 1 mL of media in a 24-well plate in humidified CO_2 (5%) atmosphere at 37°C. For siRNA uptake experiments, releasates (500 μ L) were added to 3T3 cells in serum-free conditions for 24 hours. For flow cytometry, cells were detached from plates manually with a cell scraper, washed three times in PBS, and transferred to polystyrene flow cytometry tubes while protected from light. Cells were analyzed on a BD FACSCalibur to quantify FAM-siRNA uptake.

2.10. Cardiac fibroblast isolation and MMP2 knockdown:

Rat cardiac fibroblasts were isolated as previously described from adult male *Rattus norvegicus* (Wistar) rats obtained from Charles River Laboratories (Boston, MA) and grown in DMEM/F12 with 15% FBS and 1% penicillin/streptomycin [44]. All cells were seeded 24 hours prior to experiments at 1×10^5 /mL in 1 mL of media. For MMP2 knockdown, hydrogel/siMMP2 degradation products (500 μ L) were added to cardiac fibroblasts in serum-free conditions for 24 hours, and cells were supplemented with regular media for another 48 hours. To prepare cells for flow cytometry, cells were lifted with Cellstripper™ (Corning), washed 3 times in PBS, and resuspended in 150 μ L flow cytometry buffer (PBS supplemented with 5% FBS and 0.1% sodium azide). To fix cells, 100 μ L of Fix & Perm® Medium A (Labeling and Detection Kit, Thermo Fisher) was added to cell solutions for 15 minutes at room temperature, and washed 3 times. To permeabilize cells, 100 μ L of Fix & Perm® Medium B (Thermo Fisher) was added to cell solutions for 15 minutes at room temperature. MMP2 antibody (Ab92536) was added 1:200 in 500 μ L for 1 hour at room temperature. Cells were washed 3 times and resuspended in 500 μ L of flow cytometry buffer. Goat anti-rabbit Alexa Fluor® 488 secondary antibody was added at 1:2000 in 500 μ L for 1 hour at room temperature in the dark. Cells were washed 2 times with flow

cytometry buffer, resuspended in 1 mL of flow cytometry buffer for acquisition, and analyzed on a BD FACSCalibur.

2.11. Rat infarction model:

Rats were anesthetized under 5% isoflurane in an induction chamber, intubated, mechanically ventilated, and maintained under anesthesia in 1–3% isoflurane. Buprenorphine (0.05 mg/kg) was administered subcutaneously for pain control. To induce MI, a left fourth interspace thoracotomy was performed to access the left anterior descending artery (LAD), which was ligated 1 mm below the left atrial appendage to produce an infarct that was approximately 30% of the left ventricle. Hydrogels or PBS prepared in 27G x ½” tuberculin syringes were injected ($5 \times 10 \mu\text{L}$) directly into the infarct. During closure, bupivacaine (1 mg/kg) was injected at the intercostal incision, and meloxicam (2 mg/kg) was injected subcutaneously. Animals were recovered on a heating pad, ventilated on room air until spontaneously breathing. Four weeks following MI induction, hemodynamic function was assessed using a 2 Fr pressure-volume catheter (Millar, Inc., Houston, TX) that was inserted retrograde into the left ventricle of rats under isoflurane anesthesia through the right common carotid artery. At described timepoints, hearts were explanted, embedded in OCT and frozen at -80°C . Transverse sections ($10 \mu\text{m}$) were taken from the apex to the base of the left ventricle. Section were stained using a Masson Trichrome Stain Kit (Sigma-Aldrich) according to manufacturer’s protocols. Left ventricular wall thickness was determined by averaging the distance between the endocardium and epicardium of the scar at both ends and the middle of the scar, for a total of three sections per animal taken from the mid-papillary level to above the apex. All rat procedures were performed in accordance with the Institutional Animal Care and Use Committee at the University of Pennsylvania.

2.12. Statistics:

All data are reported as mean $\pm$ standard deviation and performed in triplicate unless otherwise noted. Comparisons between two groups were performed by Students t-test with two-tailed criteria and significance determined at $p < 0.05$. For comparison between multiple groups, significance was determined by one-way ANOVA with post hoc testing. Bonferroni correction was used to account for multiple comparisons with $\alpha = 0.05$. All statistical analyses were performed in Graphpad Prism 7.

3. Results and Discussion

3.1. Macromer synthesis and hydrogel formation

Hydrogels were designed to: (i) be injectable, (ii) sequester cholesterol-modified siRNA to limit passive release, and (iii) degrade in response to proteases, leading to triggered release of siRNA. Specifically, aldehyde-modified and hydrazide-modified HA macromers were synthesized to form a hydrogel crosslinked through dynamic covalent hydrazone bonds, which has been previously used to impart shear-thinning and self-healing properties into hydrogels [45,46]. β -cyclodextrin (CD) was covalently conjugated to the HA macromers in order to sequester cholesterol-modified, self-transfecting siRNAs through supramolecular guest-host interactions between cholesterol and CD to limit passive siRNA diffusion from

hydrogels. Lastly, MMP-sensitive peptide sequences (GGRMSMPV) were incorporated within the crosslinks to enable the hydrogel to erode in response to MMP activity.

Towards this design, a CD-and aldehyde-modified HA macromer (CD-ALD-HA) was first synthesized in two steps (Fig. 1). Sodium hyaluronate was first oxidized by periodate to form an aldehyde-modified HA (ALD-HA). CD was coupled to ALD-HA by attaching a mono-aminated CD derivative (mono-(6-hexanediamine-6-deoxy)- β -cyclodextrin) to the carboxyls of ALD-HA using EDC and HOBT. The final CD-modified, aldehyde-modified HA (CD-ALD-HA) contained CD groups on ~25% of disaccharides and aldehyde groups on ~25% of disaccharides (Fig. S1). Notably, secondary imine bonds formed between aminated CD and aldehydes were broken during dialysis against water.

An MMP-degradable, hydrazide-modified HA macromer (HA-MMP-HYD) was then synthesized by reaction between maleimide-modified HA (MA-HA) and a heterobifunctional peptide (MMP-HYD) containing, from C-terminus to N-terminus, a free thiol group (cysteine), an MMP-sensitive sequence (GGRMSMPV), and a hydrazide group (Fig. 1B). Rapid Michael addition reaction between thiols and maleimides allowed immediate coupling when the two groups were matched stoichiometrically, such that there was one MMP-HYD peptide attached to every maleimide, which was on ~25% of all HA disaccharide repeats (Fig. S2).

To form hydrogels, CD-ALD-HA and HA-MMP-HYD were dissolved and mixed so that aldehyde and hydrazide groups were present in equimolar amounts to a final concentration of 5.5 wt% (Fig. 2A). Using shear oscillatory rheometry, mixtures of CD-ALD-HA and HA-MMP-HYD were found to rapidly form hydrogels ($G' > 3$ kPa), while the individual macromers did not ($G' < 10$ Pa for CD-ALD-HA; $G' < 1$ Pa for HA-MMP-HYD) (Fig. S3A, Fig. S3B). Dynamic hydrazone bonds could break in response to shear forces during injection (shear-thinning) and slowly reform after cessation of shear (self-healing). These properties were demonstrated by rheometry, where hydrogels exhibited decreasing viscosities in response to increasing shear rate, characteristic of shear-thinning (Fig. 2B). Alternating cycles of low and high strain were further used to demonstrate the shear-thinning and self-healing nature of the hydrogels, where the storage modulus (G') and loss modulus (G'') changed in response to deforming strains (Fig. 2C, Fig. S3C).

3.2. Cyclodextrin sequestration of siRNAs

The hydrophobic host cavity of CD has been well described in hydrogel systems, where it can be used to interact with other hydrophobic molecules, or guests, in supramolecular complexes [42,47]. Of these guests, cholesterol has been one of the most commonly described, where its ability to complex CD has been used for a variety of biomaterial applications [48–50]. Thus, we hypothesized that CD inclusion would sequester cholesterol-modified siRNAs and attenuate their passive release from hydrogels, paralleling previous reports of the use of CD to retain and sustain small molecule release [42,47]. Notably, cholesterol-modified siRNAs do not require transfection vehicles or viral vectors for cellular entry. To investigate whether CD could be used to sequester cholesterol-modified siRNAs in hydrogels (Fig. 3A), fluorescence recovery after photobleaching (FRAP) experiments were performed on hydrogels loaded with fluorescein amidite (FAM)-modified, cholesterol-

modified siRNAs. Recovery of fluorescence was measured after bleaching a 200 μm diameter spot in a thin hydrogel film and was observed to attenuate when CD was incorporated into the hydrogel (Fig. S4). Nonlinear least squares regression was used to fit the recovery profiles to the Soumpasis equation, from which effective diffusivities were calculated. Using this method, hydrogels with CD were found to significantly decrease diffusivity of siRNAs ($14 \mu\text{m}^2/\text{s}$) compared to hydrogels without CD ($19 \mu\text{m}^2/\text{s}$), highlighting the guest-host interaction between cholesterol and CD that slows diffusion within the hydrogels (Fig. 3B). To investigate the effect of CD on siRNA release from hydrogels, FAMmodified, cholesterol-modified siRNAs were encapsulated into hydrogels that were incubated in PBS; releasates were collected every 2 days and total siRNA released was quantified by FAM fluorescence measurements. Hydrogels with CD had significantly slower siRNA release, where <40% of total siRNA released in 2 weeks, compared to hydrogels without CD, where >50% of total siRNA released in 2 weeks (Fig. 3C). Thus, CD inclusion slowed siRNA release, where <40% of total siRNA released in 2 weeks, compared to hydrogels without CD, where >50% of total siRNA released in 2 weeks (Fig. 3C). Thus, CD inclusion slowed siRNA diffusion and reduced passive release from hydrogels; in these formulations, CD is present at a concentration of $\sim 40 \text{ mM}$, while cholesterol is present at a concentration of $10 \mu\text{M}$, such that CD is many orders of magnitude higher than cholesterol. There is likely a secondary effect from the hydrophobicity of cholesterol where it can interact with itself in the hydrogel, explaining the sustained release of siRNA from the hydrogels without CD.

3.3. Protease-dependent hydrogel erosion and siRNA release

Next, hydrogels were investigated for their ability to erode and release siRNA in response to MMPs (Fig. 4A). Hydrogels with siRNA were incubated in buffer solutions containing three different concentrations of collagenase (0, 20, 200 U/mL) for quantification of both erosion and siRNA release. As expected, hydrogel erosion was dependent on collagenase concentration (Fig. 4A) with minimal erosion observed (<40% by 2 weeks) without collagenase and complete erosion observed (100% by 4 days) in response to 200 U/mL collagenase. In the absence of collagenase, minimal erosion was still observed due to the dynamic nature of the hydrazone bond, in addition to HA hydrolysis. siRNA release profiles were similar to the hydrogel erosion profiles, suggesting erosion as a method of release (Fig. 4B). Minimal siRNA release was observed (40% by 2 weeks) without collagenase, whereas complete release (100% by 4 days) was observed in response to 200 U/mL collagenase (Fig. 4C). To ensure that released siRNAs could lead to a collagenase-dependent uptake response in cells, releasates collected from each 2-day interval were added to 3T3 fibroblasts in culture. After 24 hours, cellular uptake of siRNAs was assessed by flow cytometry from FAM-modification of the siRNA for fluorescence (Fig. 4D). Low fluorescence was observed in cells from hydrogel releasates in the absence of collagenase ($\sim 100\%$ fluorescence increase), whereas high siRNA uptake was observed in cells containing hydrogel releasates from 200 U/mL collagenase ($\sim 3000\%$ increase). This suggests that siRNA release and cellular uptake from hydrogels with dosing in response to protease levels. To confirm that unreleased siRNA was still present in hydrogels in the absence of collagenase, hydrogels were eroded from D14-D16, and releasates were added to cells. Cells had significantly

higher uptake of siRNA from hydrogels without collagenase, suggesting additional siRNA was retained in these hydrogels.

3.4. MMP2 knockdown in cardiac fibroblasts

To investigate whether siMMP2 could be used after MI, we investigated the effect of siMMP2 on cardiac fibroblasts. Cardiac fibroblasts are the most abundant cell type in the heart, accounting for up to 70% of the cell population by number [51]. Importantly, they respond after MI by differentiating into myofibroblasts, and are thought to account for the majority of MMP2 expression after MI, with levels much greater than cardiomyocytes and infiltrating inflammatory cells [52,53]. Thus, primary rat adult cardiac fibroblasts were assessed for MMP2 expression and siRNA uptake (Fig. 5A). Immunocytochemistry for MMP2 expression demonstrated its abundance throughout the cytoplasm of cardiac fibroblasts. FAM-modified, cholesterol-modified siRNAs were further assessed for their ability to autotransfect cardiac fibroblasts and high levels of fluorescently-labeled siRNAs were observed in these cardiac fibroblasts. To ensure that MMP2 expression could be knocked down in cardiac fibroblasts, siMMP2 was designed and synthesized in accordance with a previously published rat sequence [38]. When added to primary rat cardiac fibroblasts, siMMP2 led to dramatic MMP2 reduction by flow cytometry (Fig. 5B). To confirm that bioactivity was retained when the siRNA was released from hydrogels, hydrogels with siMMP2 were fully eroded in collagenase and added to cardiac fibroblasts, which significantly decreased MMP2 protein expression by over 50% from flow cytometry (Fig. 5C, Fig. 5D).

3.5. In vivo hydrogel delivery of siMMP2 after myocardial infarction

To assess the potential of siMMP2 delivery from hydrogels on treatment of MI, hydrogels with siMMP2 (gel/siMMP2) or a control siRNA sequence against GFP (gel/siCTRL) were assessed in a rat MI model. Following infarct induction by ligation of the LAD, hydrogels were injected ($5 \times 10 \mu\text{L}$) directly into the infarct to release siMMP2, as MMP2 is known to increase directly within the infarct region (Fig. 5A) [24]. After 28 days, rat hearts were processed for Masson's trichrome staining to evaluate infarct size (Fig. 5B). Hearts injected with a saline control exhibited significant wall-thinning, whereas hearts injected with both gel/siCTRL or gel/siMMP2 had significantly increased wall thickness in the infarct region when compared to the saline control (Fig. 6C). Notably, hearts injected with gel/siMMP2 also had significantly thicker infarct walls than those injected with gel/siCTRL, suggesting an effect from siMMP2. Of note, hydrogels had not fully eroded and were still present in hearts treated with gel/siCTRL and gel/siMMP2 by 28 days. Hydrogel area was quantified from Masson's trichrome sections and showed significantly greater hydrogel area in gel/siMMP2 treated hearts when compared to gel/siCTRL treated hearts (Fig. 6D, Fig. 6E). Thus, the hydrogel presence was greater in the gel/siMMP2 group when compared to the gel/siCTRL group. In accordance with the literature [53], MMP2 activity increases quickly following infarction, initiating rapid siMMP2 release from the hydrogel, potentially sufficient to silence MMP2 activity in accordance with our in vitro data and limit further hydrogel degradation.

The effect of siMMP2 can be attributed at least partly to improved preservation of hydrogel volumes by limiting local hydrogel degradation through the silencing of MMP2 protein expression. MMP2 has high enzyme cleavage kinetics ($K_{cat}/K_M = 24,000 \text{ M}^{-1}\text{s}^{-1}$) for the peptide sequence (GGRMSMPV) in this hydrogel, and the highest kinetics of the MMPs that would be overexpressed in the infarct [24,54,55]. MMP2 activity is also required to activate other MMP2 molecules, suggesting that silencing a small amount of MMP2 may inhibit additional MMP2 activity [56]. MMP9 has a higher K_{cat}/K_M for the peptide sequence used in the hydrogels, but MMP9 is not overexpressed after MI and only rises in the remote and border zones [24]; MMP9 also requires MMP2 for activation [57]. Thus, attenuating MMP2 activity would have a large role in limiting subsequent hydrogel erosion both directly by MMP2 and indirectly through other MMPs. Other erosion mechanisms include hydrogel digestion by other MMPs, in addition to erosion due to both hyaluronidases and hydrazone bond breakage. We note that the preservation of hydrogel volumes alone would positively affect outcomes after MI, as bulking a thin cardiac wall may reduce wall stress and remodeling [9,12].

3.6. In vivo hemodynamic assessment of cardiac function

To assess whether hydrogel delivery of siMMP2 had an effect on cardiac function, pressure-volume catheterization was performed to assess hemodynamics at 28 days. Ejection fraction (EF) was significantly enhanced in the gel/siMMP2 group compared to gel/siCTRL and PBS controls. Notably, EF in gel/siMMP2 treated animals was comparable to healthy controls (Fig. 7A). dP/dt_{max} , a volume-independent measure of contractility, demonstrated statistical improvements in both hydrogel groups, gel/siCTRL and gel/siMMP2, compared to PBS, and both groups were comparable to healthy values, although they were not statistically different from each other (Fig. 7B). Cardiac output (CO) and stroke volume (SV) in gel/siMMP2 groups were both significantly increased compared to PBS controls and were comparable to healthy values, while CO and SV in gel/siCTRL animals were not statistically different from PBS controls (Fig. 7C, Fig. 7D). Absolute measures of end diastolic (EDV) and end systolic volumes (ESV) were not statistically different between all three groups (Fig. S5). The lack of reduced ventricular volumes with hydrogel treatment may be due to the retention of large plugs of hydrogel in the ventricular wall after injection and over the course of 28 days, where they can distend muscle and actually elevate volumes.

The functional improvement from hydrogel delivery of siMMP2 can be attributed to a number of factors. As mentioned, one of the results of hydrogel delivery of siMMP2 is that hydrogel volumes are maintained in the infarct wall due to decreased hydrogel erosion. The ability to maintain a thicker wall in the infarct will decrease wall stress and therefore lead to functional improvements in accordance with LaPlace's law. The delivery of siMMP2 likely also attenuates infarct expansion and remodeling through MMP2 silencing; MMP2 is known to directly cause remodeling and wall-thinning from experiments in knockout mice [28,29]. Additionally, MMP2 has been shown to act on the level of the cardiomyocyte, promoting apoptosis and decreasing contractility in response to ischemia/reperfusion injury [30–32]. Thus, while a primary effect of siMMP2 is the preservation of cardiac volume and bulking, secondary effects include the attenuation of remodeling and improvement in cardiomyocyte function.

Notably, a marked improvement was observed in response to both hydrogel conditions for all measures of cardiac function observed, corroborating a previous report that also demonstrated similar hydrogels alone were able to improve function in a pig model of MI [14]. Compared to other shear-thinning, physically crosslinked hydrogels, these hydrogels, which are assembled through dynamic covalent crosslinks, are significantly stiffer and may lead to improved wall bulking [9]. Thus, for many outcomes, both hydrogel treated groups were comparable to healthy controls without infarct. Additionally, the incorporated peptide sequences could serve as substrates for proteases in the heart, limiting their activity on endogenous matrix components.

4. Conclusion

Here, we present a hydrogel that is able to achieve on-demand release of siRNA in response to proteolytic activity after myocardial infarction. Silencing of MMP2 expression significantly preserves hydrogel volumes and wall bulking, and may influence remodeling and cardiomyocyte function, all of which lead to improved hemodynamic function. These data corroborate the importance of injectable hydrogels as therapeutics after MI, and further highlight the pathogenic role of MMPs. This is one of few examples that demonstrate the utility of hydrogels as siRNA delivery platforms in the heart, where hydrogels potentiate siRNA silencing through local and sustained release. Further investigation can be performed to optimize the peptide sequence towards additional specificity for MMPs, controlling hydrogel erosion and siRNA release. Additional studies are required to investigate this hydrogel formulation in large animal models of MI and in combination with other siRNA or miRNA therapeutics, which may have synergistic effects with siMMP2.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Synthesis of CD-ALD-HA and HA-MMP-HYD macromers.

A) CD-ALD-HA is synthesized from the reaction of HA with sodium periodate to form ALD-HA, and the reaction of ALD-HA with a mono-aminated CD derivative (mono-(6-hexanediamine-6-deoxy)- β -cyclodextrin) in EDC and HOBT. B) HA-MMP-HYD is formed by first synthesizing MA-HA, as previously described [40], and coupling the cysteine of MMP-HYD to MA-HA through a Michael addition reaction.

Figure 2. Hydrogel formation between CD-ALD-HA and HA-MMP-HYD.

A) CD-ALD-HA and HA-MMP-HYD form hydrogels through reaction between hydrazide (HYD) and aldehyde (ALD), which form a hydrazone bond. B) Shear oscillatory rheometry measurements of declining viscosity as a function of shear rate, characteristic of shear-thinning. C) Rheometry measurements of mechanical properties in response to low (0.5%, white) and high strain (250%, blue), where G' mechanics declines in response to high strain and rapidly recovers upon cessation, characteristic of self-healing.

Figure 3. CD sequestration of cholesterol-modified siRNA through guest-host chemistry.

A) Schematic demonstrating siRNA-cholesterol association with hydrogel via cholesterol/CD interactions. B) FRAP diffusivity measurements of hydrogels with encapsulated FAM-modified cholesterol-modified siRNA, without (-) and with (+) CD, indicating attenuated diffusion in presence of CD, $**p < 0.01$. C) Cumulative FAM-modified, cholesterol-modified siRNA release from hydrogels with or without CD, indicating the ability of CD to attenuate passive siRNA release, $*p < 0.05$ compared to (+) CD.

Figure 4. Erosion and siRNA release in response to MMP activity.

A) Schematic of hydrogel erosion in response to MMPs. B) Erosion of hydrogels with FAM-modified, cholesterol-modified siRNA in response to collagenase (0, 20, 200 U/mL) measured by uronic acid. Any remaining hydrogel in all groups was digested in 200 U/mL collagenase from D14-D16 (gray) to determine the total amount released. * $p < 0.05$ compared to 0 U/mL, # $p < 0.05$ compared to 20 U/mL. C) Quantification of siRNA in releasates in response to collagenase (0, 20, 200 U/mL) measured by FAM fluorescence. Any remaining siRNA in hydrogels was obtained by digesting hydrogels in 200 U/mL collagenase from D14-D16 (gray). * $p < 0.05$ compared to 0 U/mL, # $p < 0.05$ compared to 20 U/mL. D) Schematic demonstrating releasate collection for up to 16 days, where collagenase was used to completely degrade hydrogels from D14-D16, and addition to 3T3 cells. Flow cytometry was used to quantify siRNA uptake from releasates, where fluorescent increase was calculated by normalizing the increase in geometric mean fluorescence intensity to the geometric mean fluorescence intensity values of untreated cells. Values in B,C do not necessarily reach 100% due to technical limitations and sensitivity of the measurements when compared to the standard curve. * $p < 0.05$ compared to 0 U/mL, # $p < 0.05$ compared to 20 U/mL.

Figure 5. Characterization of primary rat cardiac fibroblasts and siMMP2 response.

A) Immunocytochemistry demonstrating MMP2 expression in primary rat cardiac fibroblasts (left) and FAM-modified, cholesterol-modified siRNA uptake (right). B) Assessment of MMP2 knockdown from siMMP2 in rat cardiac fibroblasts by flow cytometry after 72 hours with representative immunocytochemistry images (inset). C) Assessment of MMP2 knockdown from hydrogels releasing siMMP2 in rat cardiac fibroblasts by flow cytometry. Hydrogels with siMMP2 were fully eroded in collagenase (200 U/mL) and added to rat cardiac fibroblasts, which were assessed by flow cytometry 72 hours later. D) Quantification of siMMP2 knockdown in rat cardiac fibroblasts based on geometric mean fluorescence intensity (mean $\pm$ SD *p<0.05).

Figure 6. In vivo investigation of hydrogels delivering siMMP2 after MI.

A) Hydrogel is injected into rat infarcts following LAD induction and assessed at 28 days.

B) Representative Masson's trichrome sections (3 representative sections from ligation to apex from left to right in 2 representative animals per group, 1 animal per row).

C) Quantification of infarct thickness from Masson's trichrome sections across three representative axial/transverse sections per animal (mean $\pm$ SD, * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001, PBS: n =6, gel/siCTRL: n =7, gel/siMMP2: n =6) and presented in context of healthy controls (orange, mean $\pm$ SD, n =7). D) Quantification of hydrogel area from Masson's trichrome sections across three representative axial/transverse sections per animal (mean $\pm$ SD, * p <0.05, PBS: n =6, gel/siCTRL: n =7, gel/siMMP2: n =6). E) Representative high-magnification images of hydrogels in infarct regions 28 days after MI.

Figure 7. Functional outcomes from hydrogel delivery of siMMP2 after MI.

Pressure volume catheterization measurements of A) ejection fraction (EF) ((EDV-ESV)/EDV $\times$ 100), B) dP/dt_{max} , C) cardiac output (CO), and D) stroke volume (SV). Values presented are mean $\pm$ SD, * p <0.05, ** p <0.01, *** p <0.001, PBS: n =7, gel/siCTRL: n =10, gel/siMMP2: n =11 and are presented in the context of healthy controls (orange, mean $\pm$ SD, n =7).