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Degradable acetalated dextran microparticles for tunable release of an engineered hepatocyte growth factor fragment

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Abstract

Injectable biomaterials are promising as new therapies to treat myocardial infarction (MI). One useful property of biomaterials is the ability to protect and sustain release of therapeutic payloads. In order to create a platform for optimizing the release rate of cardioprotective molecules we utilized the tunable degradation of acetalated dextran (AcDex). We created microparticles with three distinct degradation profiles and showed that the consequent protein release profiles could be modulated within the infarcted heart. This enabled us to determine how delivery rate impacted the efficacy of a model therapeutic, an engineered hepatocyte growth factor fragment (HGF-f). Our results showed that the cardioprotective efficacy of HGF-f was optimal when delivered over three days post-intramyocardial injection, yielding the largest arterioles, fewest apoptotic cardiomyocytes bordering the infarct and the smallest infarcts compared to empty particle treatment four weeks after injection. This work demonstrates the potential of using AcDex particles as a delivery platform to optimize the time frame for delivering therapeutic proteins to the heart.

Graphical Abstract

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Supporting Information Available. The following files are available free of charge.

Supporting Information ACS.docx. ¹H-NMR of the FAST, MED and SLOW degrading AcDex before and after fluorescent labeling and interstitial fibrosis quantification.

Acetalated dextran (AcDex) microparticles with three distinct degradation profiles were created and injected into infarcted hearts. It was shown that the cardioprotective efficacy of an engineered hepatocyte growth factor fragment (HGF-f) was optimal when delivered over three days post-intramyocardial injection, yielding the largest arterioles, fewest apoptotic cardiomyocytes bordering the infarct and the smallest infarcts compared to empty particle treatment. This work demonstrates the potential of using AcDex particles as a delivery platform to optimize the time frame for delivering therapeutic proteins to the heart.

Keywords

acetalated dextran; HGF; myocardial infarction; tunable release; microparticle

INTRODUCTION

Injectable biomaterial therapies, in the form of hydrogels^{1–2} and nano- or microparticles³, hold promise for healing and restoring function of damaged heart tissue post-myocardial infarction (MI). One useful property of injectable biomaterials is their ability to protect and sustain release of therapeutic cargo including growth factors, enzymes and small molecule drugs^{3–4}. Processes such as angiogenesis, fibrosis and apoptosis are controlled by precisely tuned spacio-temporal presentation of certain molecules. Thus, delivering therapeutic molecules to the heart from biomaterials at the proper time could facilitate positive tissue remodeling following ischemic damage post-MI. This type of precisely tuned temporal therapy may require delivery of several molecules at different time points. Therefore, two important steps towards designing such a therapy include understanding the optimal timing for delivery of cardioprotective molecules and design of a biomaterial delivery system that can deliver such molecules over a wide range of time frames.

A few studies have looked at the effect of delivery rate of growth factors released from biomaterials in the heart post-MI^{5–8}. Most commonly, delivery rate is varied by encapsulating two factors with different affinities for the biomaterial, thus releasing one factor before the other^{5–7}. Another technique for altering release rate is by tethering growth factors to a biomaterial⁸. In this case, the tethered growth factor is released slower than the untethered version, offering a comparison of two different release rates. These approaches, however, do not allow for a wide range of delivery rates.

To facilitate an investigation into the optimal time frame for delivering protein therapeutics post-MI, we aimed to develop particles with tunable degradation that would enable delivery over a wide range of time frames. To this end, we designed acetalated dextran (AcDex) microparticles as carriers for growth factors⁹. We previously showed the particles to have

easily tunable degradation and protein release kinetics in mildly acidic *in vitro* environments⁹, as found in the infarcted heart^{10–11}, and to be biocompatible following injection in the heart. We demonstrated that protein release could be tuned from days to weeks by altering the acyclic acetal content⁹. In this study we aimed to similarly modulate particle degradation and protein release in the infarcted heart. We first validated the predicted degradation time of the particles *in vivo*. Then as a proof-of-concept for using AcDex particles to study the impact of delivery rate on healing post-MI, we chose to deliver an engineered hepatocyte growth factor (HGF) fragment (HGF-f)^{12–13}. One month after injection we assessed the impact of the delivered HGF-f on angiogenesis, fibrosis and apoptosis to determine the optimal delivery time frame.

METHODS

All experiments were completed in accordance with the Institutional Animal Care and Use Committee at the University of California, San Diego and the American Association for Accreditation of Laboratory Animal Care.

Synthesis of FAST, MED and SLOW Degrading AcDex

Acetalated dextran (AcDex) was prepared as previously described⁹. Three batches (10 g) were created with the reaction quenched after 20, 45 or 90 minutes to yield FAST, MED and SLOW polymer degradation, respectively. The cyclic to acyclic acetal ratios were determined using ¹H NMR as previously described⁹.

Particle Degradation and Protein Release Studies Post-MI

Fluorescent Labeling of FAST, MED and SLOW Degrading AcDex—FAST, MED or SLOW degrading AcDex (500 mg) was dissolved in dichloromethane (DCM; 12 mL; Sigma Aldrich, St. Louis, MO). Alexa Fluor 647 or 568 carboxylic acid succinimidyl ester (1 mg; Life Technologies, Carlsbad, CA) was dissolved in dimethylsulfoxide (DMSO; 1 mL; Sigma Aldrich, St. Louis, MO) and added to the AcDex/DMSO mixture along with triethylamine (250 μ L; Sigma Aldrich, St. Louis, MO). The dye-polymer solution was stirred for 48 hours at room temperature protected from light. Then DCM was removed with rotary evaporation and the remaining product was dissolved in tetrahydrofuran (10 mL; Sigma Aldrich, St. Louis, MO). The dissolved product was precipitated into cold basic water and vacuum filtered. Residual water was removed through lyophilization. ¹H NMR analysis of the labeled AcDex (FAST-647, MED-568 and SLOW-647) confirmed that labeling did not alter the acetal composition.

Encapsulation of AF488 Ovalbumin in FAST-647, MED-568 and SLOW-647 Degrading AcDex—Alexa Fluor 488 Ovalbumin (Ova488; Life Technologies, Carlsbad, CA) was encapsulated in AcDex microparticles as previously described⁹. Briefly, a w/o/w double emulsion was used to encapsulate Ova488 (1 mg) and heparin (1 mg; from porcine intestinal mucosa, Sigma Aldrich, St. Louis, MO) in FAST-647, MED-568 or SLOW-647 AcDex polymer (100 mg).

Intramyocardial Injections for Particle Degradation—MI was induced in female Sprague-Dawley rats as described previously (n = 4 per group per time point)¹⁴. One week following injury rats received a single 75 μ L injection (5 mg/mL in saline) of FAST-647, MED-568 or SLOW-647 degrading AcDex particles containing Ova488. Injections were made directly into the infarct in the LV-free wall using a 27-gauge needle as described previously^{14–15}. At designated time points animals were euthanized with an intraperitoneal injection of FatalPlus (Vortech Pharmaceuticals; 300 μ L) and hearts were harvested for histological detection of the particles.

Histological Assessment of Particle Degradation—Hearts were fresh frozen in Tissue Tek OCT freezing medium and sectioned (10 μ m thickness) along the short-axis every 350 μ m. One slide from each location was stained with hematoxylin and eosin (H&E) to verify the presence of an infarct and determine the location of the particles with respect to the infarcted tissue. Hearts that did not contain an infarct upon histological analysis were excluded from further analysis. One slice at each location was imaged unstained to avoid washing the particles out of the tissue. Images were taken with a Carl Zeiss Observer D.1 (Carl Zeiss, Oberkochen, Germany) using FITC and either DsRed or Cy5 filters to detect co-localization of the encapsulated protein (Ova488) within the labeled-polymer particles (Alexa568 or Alexa647). Particle presence in each heart was scored as follows: 0 = no particles, 1 = a few intact particles, 2 = low density of intact particles or medium density of partially degraded particles, 3 = medium density of intact particles, 4 = high density of intact particles.

Studies of HGF-f Delivery Rate on Angiogenesis, Apoptosis and Fibrosis Post-MI

HGF-f Preparation and Characterization—HGF-f was expressed in *Pichia pastoris* using growth and induction media conditions and protein purification methods previously described in detail^{12–13}. Protein purity was analyzed using 4–12% Bis-Tris gel (Invitrogen) and quantified using a Nanodrop 2000 (Thermo Scientific) with the extinction coefficient 51324 M⁻¹ cm⁻¹ for the HGF-f. Protein was flash-frozen in 0.1% Tween-20 in 1x phosphate buffered saline containing an additional 500 mM NaCl (PBS500) and stored at –80 °C. Thawed protein was kept at 4 °C and used within three weeks.

Preparation and Characterization of AcDex Particles Containing HGF-f—HGF-f (1.3 mg) was encapsulated with heparin (1.3 mg) in FAST, MED and SLOW degrading AcDex (100 mg) using previously described methods⁹. Using the same procedure, non-loaded MED particles were prepared as a control (Empty). Particle size and morphology were subsequently determined using the Beckman Coulter Multisizer 4 and an Agilent 8500 FE-SEM, respectively. The loading efficiency of HGF-f was determined using a HisDetector Western Blot Kit (24-00-02, KPL, Gaithersburg, MD). HGF-f was encapsulated in and fully released from the particles, then run on an SDS-PAGE gel and detected using Western blot according to manufacturer specifications.

Western Blot to Determine Particle-Released HGF-f Activity—HGF-f-loaded particles were suspended (10 mg/mL) in phosphate buffered saline (PBS; pH 6 for FAST and pH 5 for MED and SLOW) containing heparin (1.8 μ g/mL) and Tween 80 (0.1 wt%)

and incubated at 37°C to release the HGF-f. Following particle degradation, the supernatant was collected and saved as the “HGF-f Released from FAST/MED/SLOW” treatment groups. Released HGF-f and unencapsulated HGF-f were diluted to 2 nM concentrations in serum free media (4.5 g/L glucose-Dulbecco’s modified Eagle’s medium (DMEM), 0.1% bovine serum albumin (BSA), 0.5% pen-strep).

Meanwhile primary rat aortic smooth muscle cells were cultured to 50% confluence in 6-well plates and serum starved for 24 hours. Then cells were treated with either released HGF-f, unencapsulated HGF-f, or no HGF-f (serum free media only) for 10 minutes. Following treatment, media was removed and cells were washed with 1x PBS. NP-40 lysis buffer (75 μ L; 20 mM Tris pH 8.0, 137 mM NaCl, 10% glycerol, and 1% NP40) was added for five minutes to rupture cell membranes. Cellular contents were collected and centrifuged (13400 x g, 20 min) to remove insoluble debris. A BCA protein assay was run to determine protein content in each cell lysate and equal protein concentrations were run on an 8% SDS-PAGE gel. Western blot was performed to probe for Met, phosphorylated-Met (p-Met) and GAPDH. After transfer to a nitrocellulose membrane, immunoblotting was completed using a mouse anti-GAPDH monoclonal antibody (ab8245; 1:5000; Abcam, Cambridge, MA), rabbit anti-Met polyclonal antibody (sc-161; 1:500; Santa Cruz Biotechnology, Dallas, TX) and rabbit anti-P-Met monoclonal antibody (#3077; 1:500; Cell Signaling, Danvers, MA) followed by the appropriate horseradish peroxidase-conjugated mouse or rabbit IgG (ab97023 and ab97051, respectively; 1:2000; Abcam, Cambridge, MA).

Intramyocardial Injections for HGF-f Delivery—MI was induced in female Sprague-Dawley rats as described previously (n = 12 per group) ¹⁴. One week following injury rats received a single 75 μ L injection of FAST, MED or SLOW degrading AcDex particles containing HGF-f (10 mg/mL in saline), free HGF-f, or empty particles. Injections were made directly into the infarct in the LV-free wall using a 27-gauge needle as described previously ^{14–15}. Four weeks after injection animals were euthanized with an intraperitoneal injection of FatalPlus (Vortech Pharmaceuticals; 300 μ L) and hearts were harvested for histological analysis. Some mortality occurred following MI and injection surgeries (MED n = 2; SLOW n = 1; empty n = 3; free n = 2).

Histological Analysis Following HGF-f Delivery—At the time of sacrifice, the hearts were excised, arrested in a solution containing 25 mM NaHCO₃, 2 mM CaCl₂, 5 mM Dextrose, 2.7 mM MgSO₄, 22.8 mM KCl, 121.7 mM NaCl, and 20 mM 2,3 butanedione mon-oxime, fresh frozen in Tissue Tek OCT freezing medium and sectioned (10 μ m thickness) along the short-axis every 350 μ m.

To assess angiogenesis and cardiomyocyte apoptosis, immunohistochemistry was performed on slides from the three locations containing the largest infarct for each heart. For vessel analysis cryosections were fixed with acetone, incubated with anti-smooth muscle actin antibody (Dako, Carpinteria, CA; 1:75 dilution) and von Willenbrand Factor antibody (AbCam, Cambridge, MA; 1:400 dilution) and then stained with Alexa Fluor 568 anti-mouse and Alexa Fluor 488 anti-rabbit antibodies (Life Technologies, Carlsbad, CA; 1:500 dilution). For apoptosis analysis cryosections were fixed with acetone, incubated with cleaved-caspase 3 antibody (Cell Signaling, Danvers, MA; 1:25 dilution) and α -actinin

antibody (Sigma Aldrich, St. Louis, MO; 1:800 dilution) and then stained with Alexa Fluor 568 anti-rabbit and Alexa Fluor 488 anti-mouse antibodies (Life Technologies, Carlsbad, CA; 1:500 dilution). Nuclei were visualized with fluorescent Hoescht 33342. Slides were imaged using a Leica DM6000B (Leica Microsystems Inc, Buffalo Grove, IL) immunofluorescent microscope at 10x magnification. For vessel analysis five 20x images were taken for each slide from within the infarct. Capillaries were quantified using a custom macro in ImageJ. The area stained positive for von Willebrand Factor was divided by total area within the infarct region to determine capillary density. Arterioles were defined as having co-positive von Willebrand Factor and smooth muscle actin staining and a visible lumen > 10 μm diameter^{15–16}. Arterioles within the infarct area were counted and the diameter was quantified by averaging the shortest and longest visible dimension of the lumen. Caspase positive cardiomyocytes bordering the infarct were manually counted around the entire infarct by an investigator blinded to the treatment groups.

Five slides evenly distributed throughout the infarct were stained with Masson's trichrome and used for fibrosis quantification. Slides were scanned at 20x magnification using an Aperio ScanScope CS2 slide scanner (Leica). The 'Positive Pixel Count V9' algorithm within ImageScope (Aperio) software was used to detect non-nuclear blue staining. Interstitial fibrosis was measured in non-infarcted septal wall regions as a percentage of muscle area. Infarct length was calculated as a percentage of the midline circumference that contained infarcted tissue. For both interstitial fibrosis and infarct length measurements, results from the five slides were averaged for each heart. Hearts with lesions that were <10%, which indicated an insufficient infarction procedure, were excluded (FAST: n = 1, MED: n = 2, SLOW: n = 2, empty: n = 2, and free: n = 2). Forty-three animals were included in the study (FAST: n = 11, MED: n = 8, SLOW: n = 9, empty: n = 7 and free: n = 8).

Statistical Analysis

A one-way analysis of variance (ANOVA) test with a Dunnet post-hoc test analysis was used to detect differences between treatment groups and the empty particle control for blood vessel, apoptosis and fibrosis measurements. All measurements were reported mean $\pm$ SEM, unless otherwise specified. Significance was accepted at $p < 0.05$.

RESULTS

In Vivo Particle Degradation and Model Protein Release

Three AcDex batches were prepared using 20, 45 and 90 minute reaction times, which led to 43, 54, and 57% cyclic acetals, respectively. ¹H-NMR for each batch can be found in Figure S1. Based on the relationship between cyclic content (x) and protein release rate (y) in mildly acidic environments that we determined from our previous study ($y = 7\text{E-}04\text{e}^{0.179x}$),⁹ these cyclic acetal percentages correlated to release rates of approximately 2, 10 and 20 days, respectively. To validate the predicted degradation rates of the particles in the heart post-MI, these FAST, MED and SLOW degrading AcDex labeled with fluorescent dyes were injected into the heart post-MI. Cyclic to acyclic ratios of the AcDex were maintained following labeling (Figure S1 D, E and F, respectively). H&E stained slides were used to confirm that the fluorescent particles were located within the infarcted tissue (Figure 1; right

column). In order to compare the degradation rates of FAST, MED and SLOW particles, a semi-quantitative scoring system was used (Figure 1). Representative images from each time point are also shown in Figure 1. Hearts injected with FAST particles were harvested after 0, 2 or 3 days given the expected degradation rate of ~2 days. At time zero a high density of intact particles were visible and all contained the encapsulated model protein, Ovalbumin (Figure 1; n = 2). At day 2 the density of visible particles was slightly reduced and displayed a partially degraded morphology, but still contained the protein (Figure 1; n = 3). No particles were visible at day 3 (n = 3). Hearts injected with MED particles were harvested at 5, 7 or 14 days post-injection given the expected degradation rate of ~10 days. At day 5 the particle density was less than the FAST at day 0, but particles were intact and all contained encapsulated protein (Figure 1; n = 2). The number of visible particles was further reduced on day 7, but remaining particles still contained protein (Figure 1; n = 2). No MED particles were visible 14 days after injection (n = 2). Hearts receiving injection of SLOW degrading particles were harvested 14 or 21 days after injection given the expected degradation rate of ~20 days. Protein-containing particles were visible at day 14, with similar density to the MED particles at day 7 (Figure 1; n = 3). Only two SLOW particles were visible in a whole heart at day 21 (n = 2).

Characterization of HGF-f-containing AcDex Particles

FAST, MED and SLOW degrading particles contained 12 ± 1.2 , 11 ± 1.2 and 11 ± 1.0 μg HGF-f per mg particle, respectively. To assess activity of HGF-f encapsulated in and released from AcDex microparticles, its ability to activate the c-met receptor on rat aortic smooth muscle cells was determined using Western blot. HGF-f was released from FAST, MED and SLOW degrading particles under mildly acidic conditions *in vitro* and added to cells. As expected all cells contained the c-Met receptor (Figure 2). Only lanes containing unencapsulated or released HGF-f showed appreciable bands for the phosphorylated c-Met receptor indicating that the released HGF-f maintained its activity (Figure 2).

Particles were visualized with SEM and appeared spherical and non-porous (Figure 3). Particle diameter of FAST, MED and SLOW particles according to Multisizer analysis was 27.6 ± 1.52 , 28.3 ± 1.52 , and 32.2 ± 1.41 μm , respectively. Non-loaded MED (Empty) control particles were 28.88 ± 1.66 μm .

Effect of HGF-f Delivery Rate on Angiogenesis, Apoptosis and Fibrosis Post-MI

Four weeks after injection of free HGF-f (n = 8), FAST (n = 11), MED (n = 8), SLOW (n = 9) or empty (n = 7) particles containing HGF-f into the infarct there was no statistical difference in capillary density or arteriole density between groups (Figure 4A, p = 0.95 and B, p = 0.05, respectively). However, the HGF-f delivered in the FAST degrading particles yielded the largest arterioles with a significantly greater average arteriole diameter compared to the empty control (p < 0.04; Figure 4C). Sample images from the FAST and empty treated hearts are shown in Figure 4D and E, respectively.

We also assessed the number of apoptotic cardiomyocytes in the borderzone. Significantly fewer apoptotic cardiomyocytes, as identified by co-staining with anti-cleaved caspase and anti- α -actinin were found in hearts treated with HGF-f in FAST and MED degrading AcDex

particles compared to empty particle treated hearts ($p < 0.005$; Figure 5A). Example images are shown of infarcts from FAST and empty particle treated hearts in Figure 5B & C, respectively.

Lastly, to evaluate the effects of modulating HGF-f delivery timing on negative LV remodeling, we assessed infarct size and interstitial fibrosis in the remote myocardium. Infarct size, as measured by infarct length, was significantly reduced in hearts treated with HGF-f in FAST degrading AcDex particles when compared to empty particle treatment ($p < 0.02$; Figure 6A). HGF-f delivered in MED and SLOW degrading particles also reduced infarct size compared to empty particle treatment, though not significantly. Representative infarcts are shown from FAST and empty particle treated hearts in Figure 6B & C, respectively. A trend toward reduced interstitial fibrosis in FAST particle treated hearts compared to empty particle treated hearts was observed, though differences were not significant ($p = 0.1$; Figure S2).

DISCUSSION

Acetalated dextran (AcDex) is a unique biomaterial because its degradation rate and consequent payload release time frame can be easily tuned in mildly acidic conditions. AcDex particles have been utilized in a number of studies to deliver therapeutics to cells^{17–19}, but have not been employed for *in vivo* applications. Previously we thoroughly characterized this tunable release under *in vitro* conditions mimicking an infarct. Herein we again designed AcDex microparticles with three distinct degradation and consequent protein release profiles in order to determine if particle degradation and protein release could be tuned within the infarct and to study how changing delivery rate impacted the efficacy of a growth factor known to have cardioprotective properties.

We confirmed previous reports that the degradation rate of acetalated dextran (AcDex) can be easily tuned by changing the acetalation reaction time, thus enabling tunable release of protein payloads^{9, 17}. Changing the acetalation reaction time changes the ratio of cyclic to acyclic acetals along the polymer backbone, thereby affecting degradation properties and the stability of the microparticles. Cyclic acetals both form and degrade more slowly than acyclic acetals, thus increasing the reaction time makes the AcDex more stable. In this study we wanted to create particles with three distinct degradation rates in infarcted hearts. Previously we showed that running the acetalation reaction for increasing duration yielded AcDex batches that could release a protein payload from microparticles between a few days and one month *in vitro* under pH 6 conditions⁹, simulating the infarct^{10–11}. We found that the relationship between cyclic content and protein release rate in the previous study could be described by an exponential relationship ($y = 7E-04e^{0.179x}$). Thus given the cyclic acetal content of the new FAST, MED and SLOW batches we were able to predict the release rate/particle degradation to be ~2, 10 and 20 days, respectively. These predicted rates correlated with the *in vivo* results showing degradation in 3 days, ~1.5 weeks and ~2.5 weeks, respectively. Under the simulated infarct *in vitro* conditions in the previous study it was determined that protein release occurred over the same time frame as particle degradation⁹. Here, to investigate whether or not a protein payload would be retained in the particles as they degraded in the infarct, we also encapsulated fluorescently tagged ovalbumin.

Ovalbumin was selected as a model protein because its molecular weight (45 kDa) is similar to that of the HGF-f (38 kDa). While these two proteins have different isoelectric points, our previous work showed that payload charge did not affect release kinetics of similarly sized payloads from the AcDex particles⁹. Here, the encapsulated model protein remained inside the particles at all the time points the particles were present within the tissue, confirming that protein release did not precede particle degradation via rapid diffusion. Additionally, we showed previously with bFGF that AcDex microparticles are able to encapsulate and release active growth factors⁹. HGF activates numerous signaling pathways including proliferation, survival and migration through binding to the c-MET receptor²⁰. Here, HGF-f released from FAST, MED and SLOW degrading particles maintained c-met agonist activity (Figure 2). Heparin was co-encapsulated with the HGF-f to help stabilize the growth factor structure.

Confirmation that degradation of AcDex and protein release could be tuned in the infarcted heart enabled an analysis of the impact of protein release duration on healing post-MI. Specifically, we were able to investigate how delivery rate impacted the efficacy of a known cardioprotective growth factor, HGF. Despite the benefits of HGF therapy, including increased angiogenesis^{6, 21–27}, reduced apoptosis^{6, 22–26, 28}, and reduced fibrosis^{6, 21–22, 24, 26–28}, the use of recombinant HGF is limited by poor stability and high cost and thus a more cost effective and practical alternative is to deliver HGF-f, which has shown enhanced stability and expression yield, comparable c-met activation compared to recombinant HGF¹², and efficacy post-MI when delivered in a biomaterial¹³. In all measured outcomes it was shown that delivering HGF-f over a three-day time frame was optimal. Delivery of HGF-f in the FAST degrading particles resulted in the largest arterioles, the lowest number of apoptotic cardiomyocytes neighboring the infarct and the smallest infarcts compared to empty particle delivery. HGF-f delivered over 1.5 weeks in the MED degrading particles also reduced apoptotic cell number significantly compared to the empty particle treatment and showed a modest but non-significant reduction in infarct size. Delivery of HGF-f over 2.5 weeks in the SLOW degrading particles resulted in only a modest (not significant) reduction in infarct size with no appreciable impact on arteriole size or number of apoptotic cardiomyocytes. These results suggest that especially for increased vessel size and cardiomyocyte salvage HGF-f must be delivered quickly. This was further supported by the free HGF-f performing better than the SLOW released HGF-f in both the arteriole diameter and apoptotic cell analyses. The rapid clearance of the free HGF-f, however, likely prevented it from having a significant effect. It has been shown previously that free HGF is ~90% cleared in 5 hours and almost completely cleared in one day after injection into infarcted hearts²⁹. The presence of low apoptotic cell number in the borderzone in combination with small infarct size suggests that infarcts were relatively stable in the FAST group, indicating a long-term benefit of HGF-f delivery even when it is delivered over a short period of time. In comparison the infarcts were larger in the empty particle group, as indicated by high apoptotic cell number in the borderzone. In a previous study delivering the HGF-f¹³, saline treatment produced results similar to that of free HGF-f with regard to blood vessel development, but showed greater interstitial fibrosis and cardiomyocyte area. Thus overall saline treatment was equivalent to or worse than free HGF-f treatment. Here empty particles were used as a negative control instead of saline. The

results appear similar in that for all measurements empty particles treatment was either similar to or worse than free HGF-f treatment.

We loaded an equivalent amount of HGF-f in all three batches of AcDex particles allowing isolation of time frame of release as the only variable between groups. The total loaded HGF-f was 11–12 μg per mg particle, resulting in 85–90% encapsulation efficiency. For a 75 μL injection of 10 mg/mL of particles this resulted in 8–9 μg of HGF-f per injection. The same dose was administered for the free HGF-f. Since the HGF-f has a potency of about 1/10 that of recombinant HGF¹² this equated to about a 1 μg dose of HGF per injection. This dose was selected due to its prior use in studies delivering HGF to the heart post-MI in biomaterials^{13,21}. While it is unknown what the minimum dose of HGF needs to be to observe physiologic changes, another study delivered an order of magnitude less (170 ng per injection) and still observed changes in angiogenesis, apoptosis, and fibrosis⁶. Here the free HGF-f yielded the highest dose/day over the shortest number of days (as mentioned above, clearance likely occurred in less than one day). The FAST, MED and SLOW degrading particles consequently delivered progressively lower dose/day for a progressively higher number of days. The failure of the free HGF-f to reduce apoptotic cardiomyocytes or reduce fibrotic scar size suggests that an early high dosage is insufficient if not sustained for multiple days as with the FAST degrading particles. We observed that HGF-f delivery in MED degrading particles reduced apoptotic cardiomyocyte number significantly even though the dose per day was lower than with FAST degrading particles. Furthermore HGF-f released from the MED and SLOW degrading particles slightly reduced infarct size, suggesting that a lower dose/day sustained over a longer time still impacted the fibrosis pathway. Thus while it is important to consider the impact of differences in administered dose/day with the different formulations, the presence of a high initial dose cannot fully explain the efficacy of the FAST degrading particles.

The mechanisms utilized by the FAST degrading particles to induce the largest arterioles, the fewest apoptotic cardiomyocytes and the smallest infarcts are as of yet unknown. Yet while we did not perform studies to elucidate a mechanism of action of the FAST released HGF-f, we hypothesize that the enlargement of the arterioles within the infarct may be due to an interaction of HGF-f and angiopoietin-1 (Ang-1). When overexpressed, Ang-1 has been shown to increase the diameter of blood vessels without increasing vessel density³⁰. Furthermore, HGF has been shown to be a mediator of Ang-1-induced smooth muscle cell recruitment and vascular maturation *in vivo*³¹. Regarding apoptosis, elevated cleaved-caspase-3 expression has been shown to be elevated in rats models through 7–10 days post-MI compared to 4 weeks post-MI³². We hypothesize that the sustained presence of the HGF-f during the end of this time frame via either FAST or MED degrading particles helped to reduce this early phase of apoptosis. In contrast the majority of the HGF-f from the SLOW particles was delivered after this peak apoptotic window. And finally, collagen deposition begins as early as 3 days post-MI in rats and continues through one month^{33–34}. While the effect was not significant, the SLOW degrading particles were more effective in reducing scar size compared to reducing cardiomyocyte apoptosis or increasing arteriole diameter, perhaps due to this longer window of fibrosis development. Each of these hypotheses requires further study to understand why the three-day release was most effective.

CONCLUSIONS

Here we designed microparticles with three distinct degradation time frames in order to modulate *in vivo* protein release and subsequently study how duration of release of a cardioprotective growth factor to the heart post-MI impacted healing. We showed that AcDex particles could be designed to degrade in 3 days, ~1.5 weeks or ~2.5 weeks in the infarct and found that the optimal time duration of release for delivering HGF-f was three days, which lead to a significant increase in arteriole diameter, decrease in cardiomyocyte apoptosis and decrease in scar size compared to empty particle treatment. This work will help inform the design of future HGF-based therapies and provides a platform for studying additional cardioprotective growth factors to optimize their therapeutic efficacy. The versatility of AcDex particles in encapsulating payloads of different sizes and charges⁹ and the positive results observed in this study with HGF-f suggest that AcDex particles could be used to study the optimal delivery time frame of other therapeutic proteins, and could enable the delivery of multiple factors over different time frames.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Particle degradation and protein release in infarcted hearts. Graph displays semi-quantitative analysis of particle presence in the infarct at each time point. On this scale 0 = no particles, 1 = a few intact particles, 2 = low density of intact particles or medium density of partially degraded particles, 3 = medium density of intact particles, and 4 = high density of intact particles. Images display particles within the infarct with fluorescence on the left and H&E staining on the right. The infarct can be denoted in the H&E images by the disordered myocardium resulting from myocardial necrosis as well as high nuclei density indicative of the inflammatory response. Images of FAST-647 (pink) particles 0 and 2 days post-injection show a high density of intact and a medium density of partially degraded particles, respectively. MED-568 (orange) degrading particles 5 and 7 days after injection show reduced numbers of particles over time (medium and low density, respectively) and particle retention through one week. A low density of SLOW-647 (pink) particles was visible in the infarct 14 days after injection. In all images in which particles were visible within the infarct, the encapsulated protein, Ova488 (green), was still present as visualized by the colocalization of green and pink or orange. H&E images from each respective location show particles residing within the infarcted myocardium. Scale bar: 200 μ m.

Figure 2.

Activity of HGF-f released from particles. Rat aortic smooth muscle cells were treated with HGF-f released from FAST, MED and SLOW particles as well as unencapsulated HGF-f and no HGF-f as positive and negative controls, respectively. Cellular lysates were analyzed with Western blot to probe for total c-met and phosphorylated c-met receptor content. All cells expressed the c-met receptor on their surface. Only cells treated with HGF-f, whether encapsulated or not, expressed the phosphorylated c-met receptor, indicating that the released HGF-f maintained activity after encapsulation in and release from the particles. GAPDH was used as a control for total cellular protein.

Figure 3. AcDex particles for HGF-f delivery post-MI visualized with SEM. All particles, (A) FAST, (B) MED, (C) SLOW and (D) Empty appeared spherical and non-porous. Scale bar: 10 μm .

Figure 4.

Vessel quantification. No differences were observed in (A) capillary area or (B) arteriole density within the infarct between groups. (C) Arterioles within the infarct of hearts treated with HGF-f delivered in FAST degrading AcDex particles had a larger average diameter compare to the empty particle control (* $p < 0.04$). Sample images from (D) FAST and (E) empty treated hearts display the difference in average arteriole diameter. Alpha-SMA (red) and vWF (green) stain smooth muscle and endothelial cells, respectively. Scale bar: 100 μm.

Figure 5.

Apoptosis quantification. (A) The number of caspase positive cardiomyocytes bordering the infarct was significantly lower when HGF-f was delivered in FAST and MED degrading particles compared to empty particles (* $p < 0.005$). Sample images of (B) FAST and (C) Empty particle treated hearts are shown. Cleaved-caspase 3 (red) and alpha-actinin (green) visualize apoptotic cardiomyocytes (denoted with white arrows). Nuclei are counterstained with Hoescht 33342 (blue). Scale bar: 100 μ m.

Figure 6. Infarct size quantification. (A) Infarct length was significantly shorter in hearts treated with HGF-f in FAST degrading particles compare to those treated with empty particles ($p < 0.02$). Representative infarcts from (B) FAST and (C) empty particle treated hearts are shown. Fibrotic tissue is stained blue and healthy myocardium is stained red. Scale bar: 1 mm.