Controlled Delivery of Sonic Hedgehog Morphogen and Its Potential for Cardiac Repair

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Abstract

The morphogen Sonic hedgehog (Shh) holds great promise for repair or regeneration of tissues suffering ischemic injury, however clinical translation is limited by its short half-life in the body. Here, we describe a coacervate delivery system which incorporates Shh, protects it from degradation, and sustains its release for at least 3 weeks. Shh released from the coacervate stimulates cardiac fibroblasts to upregulate the expression of multiple trophic factors including VEGF, SDF-1α, IGF-1, and Shh itself, for at least 48 hours. Shh coacervate also demonstrates cytoprotective effects for cardiomyocytes in a hydrogen peroxide-induced oxidative stress environment. In each of these studies the bioactivity of the Shh coacervate is enhanced compared to free Shh. These results warrant further investigation of the in vivo efficacy of Shh coacervate for cardiac repair.

Introduction

Heart failure afflicts 5.7 million Americans, a prevalence that is expected to rise 25% by 2030 [1]. There is an urgent need for preventative and regenerative therapies for this deadly disease, for which current treatments only seek to slow its progression [2]. The best therapy may involve “master switch” agents which activate multiple downstream pathways [3]. Sonic hedgehog (Shh) is one such master regulator, acting as a powerful morphogen during development when it typically influences cell fate but can also affect cell growth and survival [4,5]. Shh signaling remains active in the adult mammalian heart at very low levels though it is upregulated during ischemic heart injury such as myocardial infarction (MI) and congestive heart failure [6,7]. Currently, damage to the adult heart is considered permanent as cardiomyocytes have limited proliferative capability and fibrosis occurs post-MI instead of healing [2,8]. However, during development, when cardiac tissue patterning is controlled by powerful morphogens such as Shh [9], the embryonic mammalian heart briefly displays the ability to regenerate itself [10]. Temporarily recapitulating this embryonic signaling environment through exogenous application of cardiac morphogen Shh is therefore a possible approach towards repair of the damaged adult heart.

Shh has previously been applied to the heart as a free protein. Direct injection has been shown to restore blood flow in a critical hindlimb ischemia model [11]. However, this approach is highly inefficient due to short morphogen half-lives in the body [12] and therefore requires multiple injections. High dosage is also necessary to elicit a desired effect which is expensive and may carry similar safety concerns to gene therapy. Investigations using gene therapy [7,13] and transduced cell therapy [14] have been shown to improve cardiac function following MI. However, both of these approaches carry high risk of inducing tumor formation, especially considering the potency of morphogens [15], and thus trials have not progressed beyond large animal models. On the contrary, a controlled delivery approach provides stabilization and protection of Shh and can release it slowly to maintain a constant local concentration within the therapeutic range.

Here we introduce a controlled delivery system comprised of heparin and a synthetic polycation that interact polyvalently and form stable droplets. These emulsion-like aggregations are referred to as a “coacervate”. Shh is incorporated into the coacervate by high-affinity binding to heparin and is then released in a slow and sustained fashion. We demonstrate that Shh delivered by the coacervate can have multiple beneficial effects on cardiac cells, stimulating trophic factor expression and affording cytoprotection from oxidative stress, and may therefore have the potential to protect the heart from ischemia or promote tissue regeneration following insult. This is, to the best of our knowledge, the first investigation of controlled delivery of Shh for cardiac repair.

Materials and Methods

Ethics statement

Animals were cared for in compliance with a protocol approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh, following NIH guidelines for the care and use of laboratory animals (NIH publication No. 85-23 rev. 1985).
Shh coacervate preparation
Poly(ethylene arginylaspartate diglycide) (PEAD) was synthesized as previously described [16]. PEAD and clinical-grade heparin (Scientific Protein Labs, Waunakee, WI) were each dissolved in 0.9% saline at 10 mg ml⁻¹ and 0.22 μm filter-sterilized. Heparin was initially complexed with recombinant mouse Shh (N-terminus peptide; R&D Systems, Minneapolis, MN), then PEAD was added. Self-assembly of PEAD and heparin:Shh immediately precipitated the ternary complex out of solution to form the Shh coacervate.

Shh coacervate imaging
Shh was fluorescently labeled using Alexa Fluor 488 Protein Labeling Kit (Invitrogen, Carlsbad, CA). A fluorescently labeled Shh coacervate was prepared in water with 0.2 ng fluorescently labeled Shh, 100 μg heparin, and 500 μg PEAD. The coacervate was added to a 96-well plate and imaged using an Eclipse Ti inverted microscope (Nikon, Tokyo, Japan).

Shh release profile
200 μl Shh coacervate containing 10 μg heparin, 100 ng Shh, and 50 μg PEAD was prepared in 0.9% sterile saline and was then pelleted by centrifugation at 12,100 g for 5 min. The supernatant was aspirated and stored at −80°C and 200 μl fresh saline was replaced to cover the pellet. The sampling procedure was repeated on day 1, 4, 7, 10, 14, and 21 for three separate tubes. Released Shh was analyzed by direct ELISA using rabbit polyclonal antibodies against VEGF (1:50, Peprotech, Rocky Hill, NJ), SDF-1x (1:30, Santa Cruz Biotech, Santa Cruz, CA), and IGF-1 (1:30, Santa Cruz Biotech). Results were normalized to total protein content, determined using Pierce 660 nm Protein Assay (Thermo Fisher Scientific, Waltham, MA). For Shh quantification, western blot was employed with samples being denatured and reduced. Lacking a well-accepted protein loading control for conditioned media, total protein assay results were used to determine appropriate loading volumes for each sample, such that 47.5 μg protein was loaded to each well. Following transfer, the PVDF membrane was incubated for 5 min in Ponceau-S staining solution (Sigma), rinsed in DI water and imaged. Immunoblotting was then performed using a rabbit anti-human Shh polyclonal antibody (1:200, Santa Cruz Biotech) followed by a peroxidase conjugated anti-rabbit IgG antibody (Sigma). Band intensities were measured using NIH Image J Version 1.46r software.

Cardiomyocyte oxidative stress-induced apoptosis
Isolated cardiomyocytes were cultured for 2 d after which cells were spontaneously contracting at approximately 1 beat per second. Cells were pre-treated for 48 h with 500 ng ml⁻¹ Shh or recombinant human fibroblast growth factor-2 (FGF2; Peprotech) as either free protein or delivered by the coacervate. The FGF2 coacervate was prepared similarly to the Shh coacervate. Cells were then exposed to 200 μM H₂O₂ in serum-free media to induce oxidative stress. Two control groups received no growth factor treatment and then received H₂O₂, and one did not. After 2 h, cells were harvested, lysed, and assayed using EnzChek Caspase-3 Kit #2 (Invitrogen). Immediately before harvesting the cardiomyocytes, videos were taken in the center of the wells using an Eclipse Ti inverted microscope (Nikon) using standard light microscopy settings.

Statistical analysis
Statistical analysis was performed using SPSS 16.0 software (SPSS Inc, Chicago, IL). Data was tested for normality and equal variance before analysis. Statistical differences between groups were calculated using one-way analysis of variance (ANOVA) followed by Tukey post-hoc testing. Differences were considered significant at P<0.05.

Results
Shh coacervate characterization and release
Fluorescent imaging of the Shh coacervate revealed round droplets ranging from 0.5–10 μm in size (Fig. 1). Shh release from the coacervate into saline was sustained for at least 3 weeks with 80% being released in total (Fig. 2). Less than 5% Shh was detected in the supernatant immediately after forming the coacervate and pelleting, therefore loading efficiency is greater than 95%. There was effectively no initial burst release. Of the 100 ng loaded, approximately 6 ng day⁻¹ was released until day 10, then slowing to around 2 ng day⁻¹ until day 21.

Shh coacervate upregulates growth factor secretion by cardiac fibroblasts
Isolated neonatal rat cardiac fibroblasts were stimulated with Shh, free or in the coacervate, and analyzed for expression of various growth factors over 2 days. Shh itself was detected in increasing concentrations, up to 7.8 times the initially added amount by 48 h (Fig. 3a). VEGF, SDF-1x, and IGF-1 were at 2- to 4-fold higher levels by 6 hours after stimulation with both free Shh and Shh coacervate and remained upregulated for 48 h (Fig. 3b–d).
media. This concentration of H$_2$O$_2$ is higher than has been
normally in the non-H$_2$O$_2$ treated control (Video S1), but rapidly
experiment endpoint show that cardiomyocytes were beating
accordance with our caspase-3 results.

in the Shh coacervate group than in the Free Shh group in
coacervate group (Video S3). Additionally, more cells were beating
and irregularly in the Free Shh group (Video S2) and Shh
coacervate or Shh coacervate showed significantly lower levels of
cardiomyocytes (Fig. S1). Myocytes pre-treated with FGF2
isolation yielded a highly pure population of primary neonatal rat
previously reported to induce myocyte apoptosis [17]. Cardiac cell
p = 0.083) from the H$_2$O$_2$ treated control. Videos taken at the
differences were not statistically significant (p = 0.094 and
FGF2 or Shh did reduce caspase-3 levels, however these
control was more statistically significant for Shh coacervate than

The VEGF level at the 6 hour timepoint stimulated by Shh
cocervate was significantly greater than that stimulated by free
Shh. Conditioned media from cells treated with normal culture
media had undetectable concentrations of all growth factors tested
ever timepoint (data not shown).

**Shh coacervate protects cardiomyocytes from apoptosis**

Caspase-3 is a well-known indicator of cell apoptosis due to
oxidative stress, mimicked here with 200 µM H$_2$O$_2$ in culture
media. This concentration of H$_2$O$_2$ is higher than has been
previously reported to induce myocyte apoptosis [17]. Cardiac cell
isolation yielded a highly pure population of primary neonatal rat
cardiomyocytes (Fig. S1). Myocytes pre-treated with FGF2
cocervate or Shh coacervate showed significantly lower levels of
apoptosis compared to the H$_2$O$_2$ treated control, but higher levels
than the control group not treated with H$_2$O$_2$ (Fig. 4). Additionally,
the difference in means compared to the H$_2$O$_2$ treated
control was more statistically significant for Shh coacervate than
for FGF2 cocervate (p = 0.003 vs. p = 0.012). Incubation with free
FGF2 or Shh did reduce caspase-3 levels, however these
differences were not statistically significant (p = 0.094 and
p = 0.083) from the H$_2$O$_2$ treated control. Videos taken at the
experiment endpoint show that cardiomyocytes were beating
normally in the non-H$_2$O$_2$ treated control (Video S1), but rapidly
and irregularly in the Free Shh group (Video S2) and Shh
cocervate group (Video S3). Additionally, more cells were beating
in the Shh cocervate group than in the Free Shh group in
accordance with our caspase-3 results.

**Discussion**

Ischemic damage to the heart may be mitigated by Shh through
three key mechanisms: i) Angiogenesis- the sprouting of new blood
vessels from pre-existing ones, ii) Cytoprotection of cardiomyo-
cytes under oxidative stress, and iii) Recruitment of cardiac
progenitor cells.

Shh-induced neovascularization is a result of upregulated
paracrine signaling by fibroblasts of several angiogenic factors
including vascular endothelial growth factor (VEGF) and angiopo-
etin-1 [11,18,19]. It has also been shown to increase the
contribution of bone marrow-derived endothelial progenitor cells
(EPCs) to the neovascularization process [19–21]. Additionally,
recent reports have revealed that Shh acts through the Rho-
associated protein kinase (ROCK) pathway, rather than its
traditional Gli-dependent pathway, to stimulate expression of
matrix metalloproteinase-9 (MMP-9) and osteopontin (OPN) in
endothelial cells [22,23]. These multiple complex roles of Shh
underscore its importance in ischemic revascularization. In the
present work we show that the Shh cocervate can stimulate
cardiac fibroblasts to significantly upregulate expression of VEGF.
Furthermore, Shh cocervate acutely (6 hour timepoint) stimulat-
ed significantly more VEGF production than free Shh. This may
be attributed to enhanced morphogen bioactivity when delivered
by the coacervate, as discussed later. In vivo, this upregulated
angiogenic growth factor expression may induce angiogenesis to
re-vascularize the ischemic myocardium.

The cytoprotective effects Shh displays for cardiomyocytes have
also been attributed to multiple mechanisms. One contributor is
insulin-like growth factor (IGF-1) which has been shown to be
upregulated in Shh-stimulated cardiac fibroblasts [7] and bone
marrow-derived cells [24]. IGF-1 interferes with the transcription
of angiotensin II, thereby preventing the formation of reactive
oxygen species during oxidative stress [25,26]. A more direct anti-
apoptotic role of Shh has also been demonstrated in vivo for
cardiomyocytes, which express the Shh receptor Patched-1 [7].
Our results seem to confirm this direct role, as recombinant Shh
protein applied alone to stressed cardiomyocytes reduced cell
apoptosis. Yet we must still consider that the cardiomyocyte
population was not entirely pure and may have contained a small
number of fibroblasts. Upregulated secretion of IGF-1 by these
fibroblasts may have therefore played a role as well. FGF2 was
included as a positive control in apoptosis experiments as it is a
well-known survival factor for numerous cell types [27–29]
including cardiomyocytes [30], and we have experience delivering
it with the coacervate [31–33]. We observed the coacervate to
enhance the bioactivity of both FGF2 and Shh compared to either

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**Figure 1. Shh coacervate imaging.** Alexa Fluor 488-labeled Shh was
incorporated into the coacervate in water and imaged by fluorescence
microscopy. (a) 10× magnification. (b) 40× magnification. Scale
bars = 100 µm.
doi:10.1371/journal.pone.0063075.g001

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**Figure 2. Shh release profile.** In vitro release of Shh from the
coacervate into saline over 21 days, quantified by sandwich ELISA.
Percent release is relative to total amount loaded. Bars indicate means
± SD.
doi:10.1371/journal.pone.0063075.g002

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**Controlled Delivery of Shh for Cardiac Repair**
factor in free form. We also found that Shh coacervate protected stressed cardiomyocytes better than FGF2 coacervate. This is significant because FGF2 expression by fibroblasts has actually been shown to be downregulated in response to Shh stimulation [11]. The anti-apoptotic effects of Shh must therefore be ascribed to non-FGF2-related pathways.

Stromal cell-derived factor-1 (SDF-1), also known as CXCL12, is a trafficking chemokine for stem and progenitor cells [34]. It has been shown to act in local recruitment of cardiac progenitor cells [35–37], as well as EPCs [38] and smooth muscle progenitor cells [39]. It is well accepted that SDF-1 plays a pivotal role in stem and progenitor cell homing to sites of injury [40]. We prove here that Shh coacervate can upregulate production of SDF-1α at least as well as equal dose of free Shh, therefore the bioactivity of released Shh is well maintained.

In vivo, high local levels of SDF-1α may encourage recruitment of blood derived EPCs to aid in neovascularization, and of cardiac progenitor cells to engender myocardial tissue repair.

Clearly, Shh acts as a “master switch” agent in many tissues including the heart, activating multiple downstream pathways in response to injury. Shh has been shown to activate the ERK1/2 [41], PKC [14], ROCK [22], and PI3K/Akt [20] signaling pathways, among others. Shh morphogen may therefore provide benefits similar to co-delivery of multiple different growth factors. However, a multi-factor approach would require extensive optimization of growth factor dosage and release rate, while Shh stimulates a natural healing environment, similar to the embryonic state when cardiac regeneration is possible. Finally, we also observed upregulated Shh expression by cardiac fibroblasts in response to stimulation with Shh, a positive autoregulation that has been previously reported [7].

Regarding the role the coacervate plays in enhancing the effects of Shh, we point to heparin as a major player. Proteoglycans associated with the extracellular matrix or cell surface play a vital role in the transduction of cell signaling pathways [42]. Heparin binds many growth factors, cytokines, and morphogens, stabilizes and protects them, and in some cases modulates their activity [43,44]. It has been shown to interact with a growth factor molecule and its corresponding cell receptor simultaneously, forming a stable ternary complex which facilitates signal activation [45]. Heparan sulfate proteoglycans have also been shown to play

Figure 3. Shh-stimulated cardiac fibroblast signaling. Near-confluent cardiac fibroblasts were incubated with Shh, free or in the coacervate, and growth factor levels in the conditioned media were assessed after 6, 12, 24, and 48 h. Data is presented as a fold-change from the stimulation media (C). Bars indicate means ± SD. (a) Quantification of Shh concentration in the cardiac fibroblast-conditioned media by western blot. Ponceau-S staining of protein bands near 27 kDa is shown as the loading control. (b) Quantification of VEGF in the cardiac fibroblast-conditioned media by indirect ELISA, *P<0.05. (c) Quantification of SDF-1α in the cardiac fibroblast-conditioned media by indirect ELISA. (d) Quantification of IGF-1 in the cardiac fibroblast-conditioned media by indirect ELISA.

doi:10.1371/journal.pone.0063075.g003

Figure 4. Cytoprotection of cardiomyocytes from oxidative stress-induced apoptosis. Myocytes were pre-treated for 48 h with FGF2 or Shh, free or delivered in the coacervate. Cells were then exposed to oxidative stress conditions with H2O2 for 2 h. Two control groups were not pre-treated with any growth factors and then one received H2O2 for 2 h (+) and one did not (−). Cell apoptosis levels were measured by caspase-3 activity. *P<0.05, **P<0.01 compared to the +H2O2 control group.

doi:10.1371/journal.pone.0063075.g004
a vital role in developmental patterning and specifically in regulating Hedgehog distribution and pathway activation [46,47]. Heparin binds Shh with high affinity (K_D ~ 99 nM) [48] and may inherently enhance its bioactivity by mimicking the in vitro signaling environment. Indeed, the biological activity of Shh has been shown to be directly related to its affinity for heparin [49]. We suggest that utilizing heparin in our delivery system may thereby potentiate the activity of Shh. This is supported by our previous reports using the coacervate to deliver FGF2 [32,33], neuronal growth factor [31], and heparin-binding EGF-like growth factor [49]. However, the efficacy of the coacervate at maintaining or enhancing the bioactivity of Shh in an in vivo environment requires further evaluation.

The activity of bound factors is further preserved within the coacervate, which is in a separate phase from water. This isolation provides further protection from proteases. The heparin:Shh complex is soluble in water and if injected in vivo would therefore be quickly diluted and removed from the injection site. To maintain the complex locally, an insoluble coacervate is formed using a synthetic polycation, PEAD. This arginine-based polycation was designed to imitate the highly cationic heparin-binding domain [45]. Heparin-bound factors are incorporated into the coacervate and protected from proteolysis and denaturation. Release from the coacervate is based on slow hydrolysis of the polycation [16], as well as dissolution of the complex in an ionic environment. We expect the release of Shh from the coacervate to be accelerated in vivo in the presence of enzymes. We have previously shown this polycation to be highly biocompatible and to complex with heparin to control the release of many different growth factors [16]. The results presented here suggest that a simple liquid coacervate has high potential as a system of controlled delivery for heparin-binding morphogens, in addition to growth factors.

Conclusions
The results of this study indicate that PEAD-heparin coacervate can load Shh with high efficiency and sustain its release for at least 21 days. Shh released from the coacervate displays bioactivity equal to or higher than that of free Shh. We demonstrate the ability of Shh coacervate to protect cardiomyocytes from oxidative stress and to upregulate secretion of multiple growth factors by cardiac fibroblasts. These results warrant further investigation of Shh coacervate for treating cardiac ischemia.

Supporting Information
Figure S1 Neonatal rat cardiac cell populations were separated by pre-plating technique. Slow-adhering cardiomyocytes did not adhere to the pre-plated while fast-adhering cardiac fibroblasts did adhere. Both populations were immunofluorescent stained for myosin heavy chain (MHC) muscle cell marker (red) and counterstained with DAPI for cell nuclei (blue). Scale bars = 100 μm. (TIF)

Video S1 Cardiomyocytes in the – H_2O_2 control group not receiving any Shh pre-treatment and not treated with H_2O_2 in the apoptosis assay. (WMV)

Video S2 Cardiomyocytes in the Free Shh group pre-treated for 48 h with 500 ng ml^{-1} free Shh protein and then treated with H_2O_2 for 2 h in the apoptosis assay. (WMV)

Video S3 Cardiomyocytes in the Shh coacervate group pre-treated for 48 h with 500 ng ml^{-1} Shh in the coacervate and then treated with H_2O_2 for 2 h in the apoptosis assay. (WMV)

Acknowledgments
The authors would like to thank Scientific Protein Laboratories LLC for their kind donation of the clinical-grade heparin.

Author Contributions
Conceived and designed the experiments: NRJ YW. Performed the experiments: NRJ. Analyzed the data: NRJ YW. Contributed reagents/materials/analysis tools: NRJ YW. Wrote the paper: NRJ YW. Financial support: YW.

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