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Biopolymer Molecular Weight Can Modulate the Wound Healing Efficacy of Multivalent Sonic Hedgehog-Hyaluronic Acid Conjugates

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Abstract

There is a clinical need for new therapeutics to improve healing of chronic impaired wounds. Thus, we investigated how biopolymer conjugation could be used to improve the wound healing performance of a key growth factor for tissue regeneration: Sonic hedgehog (Shh). We generated two multivalent Shh conjugates (mvShh) using hyaluronic acid (HyA) with two different molecular weights (MWs), which exhibited equivalent potency and proteolytic protection *in vitro*. Using db/db diabetic mice, we showed that mvShh made with smaller HyA MW resulted in more rapid and robust neovascularization compared to mvShh made with larger MW HyA. Further, smaller mvShh conjugates resulted in faster wound resolution compared to the unconjugated Shh. This study is the first to show how the wound healing efficacy of multivalent protein-polymer conjugates is sensitive to the polymer MW, and our findings suggest that this parameter could be used to enhance the efficacy of growth factor conjugates.

TOC Image

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Supporting Information

We have included representative characterization data for the structure of the HyA-EMCH intermediate product in our mvShh reactions. We also presented data to verify that the Shh is covalently bound to the HyA-EMCH after the mvShh conjugation reaction. Finally, we included the results of several control experiments: (1) to identify the appropriate incubation times with plasmin for predictable proteolysis of Shh, (2) to verify release of the Shh and mvShh treatments from the vehicle for *in vivo* experiments, and (3) to present wound closure data on a control cohort of non-diabetic mice.

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Notes: TH and WMJ own equity interest in Valitor, Inc.

Keywords

drug development; pharmacodynamics; hyaluronic acid; growth factor; protein conjugates; Sonic hedgehog; protease; proteolytic protection; wound healing; diabetic ulcer

INTRODUCTION

Protein-based drugs have enormous potential to promote wound healing,¹ but the clinical translation of these therapies has proven challenging. The clinical performance of recombinant proteins in the wound space is limited in part by their rapid proteolysis and clearance from wound bed *in vivo*, often resulting in an inadequate window of therapeutic activity after administration.^{2–5} For example, due to its short *in vivo* half-life (~30 minutes⁶), the development of vascular endothelial growth factor (VEGF) was halted in phase II clinical trials despite its positive safety profile,⁷ as frequent administration was required to yield only a modest treatment effect.⁸ As a second example, becaplermin (platelet derived growth factor, Smith and Nephew) received FDA approval, but it has demonstrated poor cost-efficacy,^{9–10} and has struggled with clinical adoption. Thus, better control over the duration of their treatment effect is critical to advance the development of protein therapeutics as effective wound healing products.

A variety of biomaterial and nanotechnology strategies have been implemented to improve the tissue-level distribution and stability of recombinant proteins after clinical administration.¹¹ One commonly employed strategy uses polyethylene glycol (PEG) conjugated to a protein-based drug (i.e., PEGylation) to prevent deactivation by immune cells and proteolytic enzymes via steric inhibition.¹² This method also modulates the size of the resulting macromolecular entity, thereby enabling control over its vascular permeability, tissue diffusion, and elimination routes. However, these methods to develop protein-based drugs primarily utilize a single-parameter design approach. As such, improvements made to one parameter of protein pharmacology are made at the expense of another. Using PEGylation as an example, *in vivo* half-life can be increased, but with this improvement, the potency of the protein can drop by an order of magnitude or more.¹³ While these tradeoffs in pharmacological performance have been acceptable for at least ten PEGylated protein-based drugs that have been FDA approved for systemic administration, monovalent protein-polymer conjugation has not been successfully applied for wound healing applications.

In contrast, we are developing a method of multivalent protein conjugation that offers a multiple parameter approach to enhancing protein function. We generate soluble, nanoscale clusters of tethered proteins on single-chain hyaluronic acid (**HyA**) biopolymers with stoichiometric control over the number of proteins on each biopolymer (i.e., valency).^{14–15} Previous studies showed how these multivalent conjugates (**MVCs**) increased the potency of their cargo proteins compared to equimolar concentrations of unconjugated proteins, as measured by *in vitro* assays.^{14, 16–17} The MVCs also enhanced the therapeutic function of the conjugated proteins when administered to several *in vivo* disease models.^{16–19} The observed improved *in vivo* performance of the MVCs is due, at least in part, to their increased potency, but they also likely benefited from pharmacokinetic advantages relative to the unconjugated proteins due to their larger size.

In this study, we will focus on the effect of polymer molecular weight (**MW**) to control MVC size and enable independent control over their *in vivo* bioactivity to yield greater overall therapeutic efficacy. As a model growth factor, we used Sonic hedgehog (**Shh**), which has been previously proposed as a drug to promote tissue regeneration and wound healing.^{20–22} Our overall hypothesis was that the wound healing efficacy of multivalent Shh (mvShh) conjugates would be sensitive to the MW of their HyA component. To assess this parameter in isolation, we first demonstrated that the bioactivity and proteolytic protection of mvShh could be normalized for conjugates made using HyA with different MWs. Then we selected two mvShh with different MW but equivalent *in vitro* bioactivities to evaluate using an *in vivo* wound healing model. In doing so, our study was the first to show how the biopolymer MW parameter of a multivalent protein conjugate can be used to enhance the therapeutic performance of a protein drug.

EXPERIMENTAL

Multivalent Shh-HyA Conjugation

We prepared mvShh following a method described previously.¹⁴ We obtained HyA over a range of MWs from 150–860 kDa (LifeCore Research), which we have referenced by their weight averaged MW, as specified from the manufacturer based on their static light scattering analysis. 3 mg/mL of each HyA was dissolved by rotating overnight in MES buffer (0.1 M, pH 6.5). Carbodiimide substitution was initiated by adding 10 mg/mL 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), 0.3 mg/mL sulfo-NHS, and 1.2 mg/mL N-ε-maleimidocaproic acid hydrazide (EMCH) to the solution and reacting for 2 hours at 4°C, which resulted in the addition of maleimide reactive groups on the HyA (see Fig. S1). Samples were dialyzed with 50 kDa MWCO membranes against PBS buffer (930 mg/L EDTA and 10% glycerol in DPBS) twice for 4 hours and then once for 24 hours at 4°C. Activated HyA-EMCH was then stored at –20°C. Recombinant N-terminal Shh fragment with a C-terminal cysteine residue was synthesized as previously described using an *E.coli* expression system.²³ To obtain mvShh over a range of valencies, a fixed concentration of Shh-Cys was combined with HyA-EMCH at various defined feed ratios in PBS and allowed to react at 4°C for four hours. The mvShh solution was then dialyzed with 100 kDa MWCO against PBS buffer twice for 4 hours and once for 24 hours at 4°C.

Characterization of Shh-HyA Conjugates

The concentration of Shh protein for each mvShh conjugate was measured using the BCA assay (Thermo), and we verified that the concentration of HyA in the mvShh was ~100X lower than its detection limit by BCA.²⁴ Conjugation of Shh to HyA was verified by protein separation with SDS-PAGE (Mini Protean System, BioRad) using 12% acrylamide gels and staining with Bio-Safe Coomassie Stain (BioRad) or SYPRO ruby (Promega, see Supporting Information Fig. S2). mvShh conjugate MW and R_{g,z} was measured using size-exclusion chromatography with multi-angle light scattering paired with an in-line differential refractometer and ultraviolet spectrometer (SEC-MALS-RI-UV). Using a protocol previously described in detail,^{18, 25} the combined molecular mass distribution for HyA and Shh in the conjugates was determined based on both the distributions of light scattering and differential refraction (dn/dc). Then, based on the known dn/dc and UV extinction coefficients (ϵ_{ex}) for HyA and Shh, we could use the conjugate dn/dc and UV absorption values to solve for the Shh and HyA mass fractions over the total MW distribution. The HyA mass fraction indicated the final MW of the HyA after the reactions, and the Shh valency was calculated by dividing the Shh mass fraction by the known MW of Shh (23 kDa). The Shh ligand spacing was calculated by dividing the nominal HyA MW by the Shh valency.

Alkaline Phosphatase Bioassay

C3H10T1/2 embryonic murine fibroblasts (ATCC) were maintained in growth medium (GM) consisting of Eagle's minimum essential medium (ATCC) supplemented with 10% fetal bovine serum (FBS, Hyclone) in a 37°C humidified incubator with 5% CO₂. Between passages 5 and 15, cells were plated in 96-well plates at an initial seeding density of 3,200 cells/well in GM supplemented with 20 mM HEPES (Lonza). The cells were allowed to proliferate for three days after seeding, and then the media was replaced with fresh GM supplemented with 20 mM HEPES, 2% FBS, and various treatments containing Shh or mvShh. After an additional four days, the cells were rinsed once with TBS (50 mM Tris-Cl, pH 7.6; 150 mM NaCl), and after aspirating the TBS, the cells were flash frozen at -80°C. 0.1% Triton-X 100 in TBS was then added to each well and then the plate was agitated for 30 minutes to thaw and lyse the cells. ALP enzyme activity in each well was detected using the *p*-nitrophenyl phosphate (pNPP) liquid substrate system (Sigma) and normalized to the total protein concentration measured by the BCA assay. We fit the resulting dose-response curves using four-parameter logistic non-linear curves to determine the EC₅₀ for each treatment.

Enzyme Digestion Assays

Unconjugated Shh and mvShh at the indicated concentrations were incubated with various concentrations of plasmin (Sigma) at room temperature for various digestion intervals. We selected 6.25 U plasmin/mg Shh as a suitable concentration for further investigation on the effect of multivalent conjugation to prevent proteolytic deactivation of Shh (See Supporting Information Fig. S3). Proteolytic degradation was assessed using SDS-PAGE and the ALP bioactivity assays, as described previously.

For analysis of digestion fragments using SDS-PAGE, the enzyme activity was stopped by adding Laemmli sample buffer followed by denaturing at 95°C for 10 minutes. This method

of quantification was sufficient to measure protein fragment concentrations that were at least 7.5% of the initial protein mass loaded into each well (standardized to 1 ug/well for each sample). For bioactivity analysis of the digestion fragments using the ALP assay, the enzymes were deactivated by the 2% FBS present in the GM for C3H10T1/2 embryonic fibroblasts. The results from this assay were reported as *percent bioactivity retained*, which was calculated as the EC50 for each conjugate incubated with the enzyme divided by the EC50 of a matched dose response curve for a no-enzyme control of the same conjugate. EC50s were calculated using four-parameter logistic fits, which yielded good fits ($R^2 > 0.9000$).

In Vivo Wound Healing Model

The IACUC at UCSF approved the animal procedures used in this study. Chronic, non-fasting hyperglycemia (>350 mg/dL) was verified in 10-week-old BKS.Cg-Dock7^m +/+ Lepr^{db}/J mice (Jackson Laboratory, Bar Harbor, ME) using a commercial blood glucose monitor. The mice were anesthetized with isoflurane in oxygen (1–3%, adjusted for adequate anesthesia), the dorsum was shaved and sterilized, and then skin tissue was excised from the dorsum using a dermal punch to make a full-thickness excisional wound. To minimize discomfort for the mouse, we used the smallest circular wound sizes needed for each experiment: two bilateral, 0.5-cm diameter wounds per animal for histology, or one 1.5-cm diameter wound per animal for wound closure experiments. The animals were randomized into four treatment groups on the basis on blood glucose concentration, and one of the three treatments or vehicle control was applied to each wound via a disc of 1% (w/v) dehydrated methylcellulose (MC; Sigma Aldrich) having the same diameter as the wounds. $6.25 \mu\text{g}/\text{cm}^2$ of Shh (based on wound area) was the treatment dose for both the mvShh and unconjugated Shh treatments. The MC discs were placed directly onto the open wound and covering the entire wound to ensure the treatment dose was applied evenly across the wound surface regardless of the wound size. The MC dissolved after contact with wound fluid, and we verified that the Shh and mvShh retained their bioactivity after release from the MC (see Supporting Information Fig. S4). Wound areas were subsequently imaged and measured every 3–4 days by tracing the wounds onto transparency paper and the calculating the pixel area of each trace with image analysis using ImageJ. Mice were sacrificed at the specified time points by CO₂ asphyxiation.

Following sacrifice at 24, 48, or 72 hours for histology experiments, we performed a bilateral thoracotomy to expose the heart for perfusion with PBS. Square tissue samples approximately 2 cm on each side and centered on the wound were excised, fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. The specimens were then sectioned perpendicular to the wound surface at $10\text{-}\mu\text{m}$ intervals and transferred to slides to visualize wound cross-sections through the diameter of the wound. At least two sections from each wound were stained using rat anti-mouse CD31 (BD Biosciences) with biotinylated anti-rat IgG and Vectastain Elite ABC reagent (Vectorlabs). CD31+ cell quantification was performed using previously described methods.^{26–27} We imaged the stained cells in the tissue specimens at $10\times$ magnification, which was sufficient to view the full thickness of epidermis, dermis and granulation tissues. For each specimen, the average

cell number per field was calculated from four images taken from sections across the thickness of the wound.

Statistical Analysis

All grouped data was represented as mean $\pm$ standard deviation, and the Shapiro-Wilk test was applied to all groups to verify normality. Differences between groups were assessed using one-way ANOVA with Tukey *post-hoc* analysis. Linear regression was used to assess strength of correlation between variables and to identify slope terms that were significantly non-zero. Statistical significance for all tests was assigned for $\alpha < 0.05$. Sample sizes for all analysis indicate the results from independent experiments. ANOVA, curve fits, and simple linear regression were performed using Prism (GraphPad). Multiple linear regressions were performed using R (Foundation for Statistical Computing).

RESULTS AND DISCUSSION

Macromolecular characterization of mvShh conjugates

Following an established protocol,^{14, 18} we generated mvShh for this study using HyA with MW ranging from 150 to 860 kDa and feed ratios ranging from 1 Shh:1 HyA to 60 Shh:1 HyA. We first characterized the resulting conjugates on the basis of protein concentration, and the final molar ratio of Shh to HyA correlated linearly to their molar feed ratios ($R^2=0.9176$; Fig. 1A). The average conjugation reaction efficiency was $66.7 \pm 9.6\%$ (Fig. 1B) and was not correlated to either the Shh feed ratio or HyA MW ($R^2=0.0778$, multiple linear regression with $N=41$). We verified the conjugation of Shh to HyA by comparing the protein separation of the unconjugated Shh to that of mvShh using SDS-PAGE. Unconjugated Shh migrated through the gels as anticipated (~ 23 kDa; Fig. 1C), whereas the primary protein band for mvShh conjugates was too large to enter the resolving gel. Protein bands corresponding to unconjugated Shh did not appear in the mvShh lanes. Thus, the Shh had been covalently conjugated to the HyA backbone of the mvShh samples and not more than 0.5% of the Shh in our final dialyzed product was associated with the mvShh via non-covalent interactions (see Supporting Information Fig. S2).

Next, we employed an analytical method based on size exclusion chromatography and multiangle light scattering (SEC-MALS) with in-line differential refractive index (dn/dc) and ultraviolet light absorption (UV) measurements, which was developed for more sensitive analysis of multivalent protein conjugates and described in detail elsewhere.²⁵ This analysis provided simultaneous measurements for both the molecular weight of the HyA biopolymer and the concentration of the conjugated Shh. Combined, these data provide an estimate for the ratio of Shh protein directly to each HyA biopolymer chain (i.e., **valency**). Shh valency correlated linearly with the final Shh:HyA molar ratio for each conjugate ($R^2=0.9560$; Fig. 1D), and due to variability in the conjugation reaction efficiency, the measured Shh valencies were different for each mvShh conjugate. This analysis also measured the final MW of the HyA chains after conjugation, which retained greater than approximately 90% of their mass due to the facile reaction conditions (Fig. 1E). Finally, we determined the size of the mvShh macromolecules on the basis of the radius of gyration ($R_{g,z}$), and we determined that there

was a significant correlation ($p < 0.005$) between conjugate size and the MW of the HyA component (Fig. 1F).

We assessed the bioactivity of mvShh conjugates based on their ability to upregulate alkaline phosphatase (ALP) in C3H10T1/2 murine embryonic fibroblasts. We generated dose-response curves for each mvShh conjugate with various Shh valencies and HyA MWs compared to unconjugated Shh based on the total concentration of Shh in each treatment (representative plots shown in Fig. 2A and 2B). The EC50 values for mvShh having the same HyA MW were linearly correlated to the conjugate valency (Table 1, representative plots shown in Fig. 2C and 2D), but the combined EC50 data for all mvShh conjugates was only weakly correlated to either Shh valency or HyA MW ($R^2 = 0.2408$, multiple linear regression with $N = 19$; Fig. 2E). By contrast, the combined EC50 values for all data sets correlated more strongly to **Shh ligand spacing** (Defined as HyA MW divided by Shh valency, $R^2 = 0.8674$; Fig. 2F). Thus, this single parameter was sufficient to predict mvShh conjugate bioactivity while being independent of the HyA MW. In the next experiments, mvShh conjugates with different HyA MWs could be made to have equivalent potency by matching their Shh ligand spacing.

Proteolytic resistance by mvShh conjugates

We assessed the ability of mvShh to resist proteolytic degradation of Shh by incubating the conjugates with plasmin, a plasma protein involved in the wound healing process.²⁸ Given the two cleavage sites for plasmin on Shh,²⁹ there were three possible digestion products for the mvShh conjugates (see Supporting Information Fig. S3): (1) cleavage at only the N9 position would result in a *biologically deactivated* mvShh conjugate; (2) cleavage at only the C3 position would yield *bioactive* Shh fragments separated from the HyA backbone; and (3) cleavage at the N9/C3 positions would yield *deactivated* Shh fragments separated from the HyA backbone. The generation of Shh fragments after mvShh digestion with plasmin for up to an hour was assessed using SDS-PAGE (Fig. 3A). A band for the N9 fragment did not appear at any time point, as cleavage at that site would leave the inactivated Shh attached to the HyA backbone that would be trapped in the resolving gel. Bands for the C3 and N9/C3 fragments could be resolved, although they developed more slowly than was observed for the unconjugated Shh (see Supporting Information Fig. S3). We quantified each band using densitometry (Fig. 3B) and used a one-phase exponential association rate equation to fit the combined C3 and N9/C3 fragment densities for Shh ($R^2 = 0.9223$, $N = 8$) and mvShh (5 Shh: 1 HyA, MW = 150 kDa; $R^2 = 0.9816$, $N = 8$). The half-digestion times for Shh and mvShh were 47.8 and 292.1 minutes, respectively, indicating that the conjugation of Shh to HyA substantially reduced the rate of proteolytic digestion at the C-3 cleavage site.

Next, we determined the effect of Shh ligand spacing and HyA MW on the ability of mvShh to interfere with plasmin activity. We assessed the extent of Shh digestion at the C3 cleavage site by quantifying the sum of the C3 and N9/C3 band densities in SDS-PAGE gel after 1 hour of incubation with 6.25 U plasmin/mg Shh. The amount of C3 fragment cleaved from the mvShh was not strongly correlated to the HyA MW ($R^2 = 0.0431$, multiple linear regression with $N = 57$), and thus we grouped the conjugates on the basis of Shh ligand spacing (Fig. 3C). Cleavage at the C3 site was significantly reduced for the mvShh

conjugates at any of the ligand spacings compared to unconjugated Shh ($p < 0.001$, one-way ANOVA with Tukey post-hoc tests and $n = 14$). Given the proximity of the C3 cleavage site to the HyA backbone, it could be inferred that the HyA provided steric interference for plasmin digestion at this site, which was independent from Shh ligand spacing.

The extent of mvShh digestion at the N9 cleavage site by plasmin after 1 hour was estimated based on its remaining bioactivity to upregulate ALP in C3H10T1/2 cells. EC50 values for each conjugate with and without plasmin incubation was determined from dose-response curves fitted using four-parameter logistic non-linear curves ($R^2 > 0.9400$ for all curves with $N = 9$). The percent change in bioactivity was determined by the EC50 for each conjugate divided by the EC50 for the same conjugate after plasmin incubation in a matched experiment. The change in mvShh bioactivity after plasmin digestion was not strongly correlated to the HyA MW ($R^2 = 0.0956$, multiple linear regression with $N = 54$), and thus we grouped the conjugates on the basis of Shh ligand spacing (Fig. 3D). The mvShh retained significantly greater bioactivity after plasmin digestion compared to unconjugated Shh (a, b, and c indicate groups statistically different from each other with at least $p < 0.05$, one-way ANOVA with Tukey post-hoc tests and $n = 12$), and the bioactivity of mvShh was dependent only on the Shh ligand spacing. Given that the N9 cleavage site was far from the HyA backbone, it could be inferred that the HyA biopolymer may not directly provide steric interference from plasmin digestion at this site. Rather, the adjacent Shh ligands were interfering with the action of plasmin at the N9 cleavage site, and closer Shh ligand spacing provided more effective plasmin interference. Furthermore, endogenous enzymes would have an equivalent effect on mvShh conjugates with different HyA MWs as long as they had equal Shh ligand spacing.

Effect of HyA MW on mvShh Wound Healing Function

To evaluate the wound healing function of mvShh conjugates, we used a model of impaired full-thickness excisional wound healing in diabetic (db/db) mice. The db/db mouse is a frequently used model of delayed and diabetic wound healing due to its well-characterized disease phenotype,^{22, 30–35} which includes an aggregate effect of delayed cellular wound infiltration, reduced angiogenesis, and delayed wound contraction.^{33–36} From the mvShh conjugates that were evaluated *in vitro*, we selected the two conjugates with the closest Shh ligand spacing and approximately 2-fold difference in HyA MW for further investigation *in vivo* (See Table 2). Based on the results of our *in vitro* studies, these two mvShh conjugates will exhibit equivalent potency and proteolytic protection *in vivo*, as these properties were dependent only on the Shh ligand spacing. Thus, with these conjugates, we will isolate any pharmacokinetic effects that are dependent on the MW of the HyA and may contribute to the wound healing efficacy of mvShh *in vivo*.

For our *in vivo* studies, we included two controls: a vehicle no treatment control, and an unconjugated Shh control. In previous studies, HyA alone has been shown to improve the rate of wound healing, but only when applied twice daily at doses that are approximately three orders of magnitude higher than the HyA used in the mvShh conjugates.^{37–38} Thus, we previously concluded that the HyA component of mvShh has a negligible direct effect on its wound healing performance.¹⁸ Still, to further control for any effects of the HyA, we

included HyA in the unconjugated Shh control at a concentration that was equivalent to the HyA component of the mvShh conjugates.

First, we assessed the effect of mvShh on wound neovascularization that occurs at early time points after generating 0.5-cm excisional wounds on the dorsum of db/db mice. We used CD31+ cells in the wound margins as an indicator of cells that are likely participating in the formation of new blood vessels to support wound healing. Within 48 hours, the margins of wounds treated with either Shh or mvShh were populated with at least twice as many CD31+ cells as those receiving only the vehicle control, and there were significantly more CD31+ cells in the wounds treated with either of the mvShh conjugates compared to the unconjugated Shh ($p<0.05$, one-way ANOVA with Tukey post-hoc tests and $n=5$; Fig. 4A). After 72 hours, the wounds treated with vehicle control, unconjugated Shh, and mvShh (752 kDa HyA) appeared similar to those observed 24 hours earlier. However, the population of CD31+ cells near wounds treated with mvShh (352 kDa HyA) continued to grow to nearly twice as many as were observed for wounds treated with unconjugated Shh (Fig. 4B) and were significantly larger than in wounds treated with mvShh (752 kDa HyA; $p<0.01$). These results are similar to previous studies showing that a mvShh conjugate with high Shh valency exhibited higher potency than unconjugated Shh and resulted a more robust neovascular response to wound healing.¹⁸ This experiment now shows that the size and rate of the neovascularization response after mvShh treatment is dependent on the MW of its HyA component.

To determine the effect of Shh and the mvShh conjugates on the rate of wound closure, we generated 1.5-cm wounds on the dorsum of db/db mice and measured the size of the wounds over time as they healed. There was a negligible change in wound area during the first eight days after wounding for all of the treatment groups (Fig. 5A), and the most dramatic change in wound size occurred between day 8 and day 15 (Fig. 5B). By day 12, the wounds treated with unconjugated Shh were significantly smaller than those treated with the vehicle control ($p<0.05$, one-way ANOVA with Tukey tests and $n=10$). The wounds treated with either mvShh conjugate were significantly smaller than those treated with unconjugated Shh ($p<0.05$) and approximately half the size of the controls ($p<0.05$). By day 15, the wounds treated with either of the mvShh conjugates were nearly healed and remained significantly smaller than wounds treated with the vehicle control ($p<0.01$).

Based on historical data and our internal controls (see Supporting Information Fig. S5), 1.5-cm wounds typically heal in approximately 15 days in non-diabetic mice.^{33–35} The same size wounds treated with the vehicle control in the db/db diabetic mice in this experiment required 22.9 ± 1.8 days to close completely (Fig. 5C). Wounds treated with Shh closed completely after 21.1 ± 1.4 days, but the effect of Shh was not sufficient to achieve statistical significance in this experiment. In contrast, the wounds treated with either of the mvShh conjugates healed at a substantially faster rate than the vehicle controls ($p<0.005$, one-way Anova with Tukey tests and $n=10$), requiring 17.2 ± 2.4 days for the 357 kDa HyA mvShh and 19.2 ± 1.9 days for the 752 kDa HyA mvShh. Interestingly, only wounds treated with mvShh using 357 kDa HyA exhibited a wound healing time that was significantly different from those treated with unconjugated Shh ($p<0.01$). These results suggest that both mvShh conjugates were sufficient to enhance the mechanisms of wound healing and accelerate

wound closure. However, the smaller mvShh may have been better suited to impart a therapeutic effect on the wound environment, as it appeared to enable more significant effects to enhance wound healing throughout the process until complete resolution.

DISCUSSION

Our overall hypothesis in this study was that the wound healing efficacy of mvShh conjugates would be sensitive to the MW of their HyA component. To address this hypothesis, we first performed a detailed analysis of mvShh composition and *in vitro* bioactivity. This analysis showed that Shh valency could be normalized for mvShh conjugates over a range of HyA MW using average Shh *ligand spacing* on the HyA backbone, and thus the Shh valency and HyA MW could be modulated as independent parameters to control the bioactivity of mvShh conjugates. Next, we determined that protein digestion by plasmin occurred approximately 6 times more slowly for the mvShh relative to the unconjugated Shh, and as a result, the mvShh retained 2–3 times more bioactivity compared to the unconjugated Shh. Interestingly, the level of proteolytic protection offered by the mvShh correlated directly with the Shh ligand spacing on the HyA biopolymer and was independent of the HyA MW. By contrast, the $R_{g,z}$ of the mvShh correlated to the HyA MW but not the Shh ligand spacing. MVCs with smaller HyA MW (and thus smaller $R_{g,z}$) appeared capable of a faster and more robust neovascular response after mvShh treatment of wounds in diabetic mice. From day 8 to day 15, the HyA MW did not have a detectable effect on wound healing performance for the mvShh conjugates evaluated in this study, but both mvShh conjugates resulted in accelerated wound closure compared to an equimolar concentration of unconjugated Shh. However, the smaller mvShh treatment (357 kDa HyA) resulted in the greatest reduction in wound closure time, which may be in part due to the more robust neovascular response early in the wound healing process. Thus, this report is the first to identify specific MWs for the HyA component of a multivalent protein conjugate that can be used to enhance the therapeutic function of its cargo protein *in vivo*.

Several aspects of this study support the strength of our conclusions. First, we performed a detailed characterization of our mvShh conjugates using a variety of biochemical, biophysical, and cellular bioactivity assays over a range of molecular parameters (i.e., Shh valency and HyA MW) to assess the effect of these parameters on mvShh *in vitro* bioactivity. Consequently, we could conduct multiple parameter studies to evaluate how these features of our multivalent conjugation technology would contribute to their *in vivo* wound healing performance. Finally, we performed a set of wound healing experiments *in vivo*, and using the findings of our *in vitro* assays, we selected mvShh conjugates that could best isolate the specific effect of HyA MW on their wound healing performance.

To enable a reductionist approach for studying the effect HyA MW on mvShh bioactivity, we selected *in vitro* assays based on their sensitivity for making quantifiable comparisons between treatment groups. These assays were not intended to be representative of the *in vivo* milieu at the site of administration, and the results from this study would not necessarily be useful for predicting *a priori* the therapeutic performance of multivalent protein conjugates. However, these *in vitro* assays were useful for establishing the relative effects of Shh valency and HyA MW on specific mechanisms related to mvShh function and to select conjugate

parameters that could isolate the effect of HyA MW for our *in vivo* study. The db/db mouse is a frequently used model of delayed and diabetic wound healing due to its well-characterized microvascular disease phenotype,^{22, 30–35} which includes delayed cellular wound infiltration, reduced angiogenesis, altered immune function and delayed wound contraction.^{33–36} For our *in vivo* studies, we did not make any *a priori* assumptions about which of these impaired mechanisms would be enhanced by either mvShh or unconjugated Shh. Instead, we designed the study to evaluate how our treatments could enhance the aggregate of the wound healing responses.

Our findings are consistent with previous studies that have evaluated the use of protein conjugation to prevent proteolytic deactivation of therapeutic proteins. Resistance to proteolysis is one of the key mechanisms attributed to sustained *in vivo* bioactivity of PEGylated proteins.³⁹ PEGylation of the therapeutic enzyme L-asparaginase was extended approximately 6-fold during incubation with proteases occurring endogenously within human serum.⁴⁰ PEG-L-asparaginase, superdioxide dismutase,⁴⁰ and PEG-catalases⁴¹ also retained ~60–80% of their bioactivity after incubation with a variety of proteolytic enzymes, including pronase, trypsin, and pepsin, compared to ~0–20% bioactivity retained by the unPEGylated controls. Conjugation to hyaluronic acid has also been shown to retain ~60–80% bioactivity for human growth hormone (hGH)⁴² and interferon-alpha⁴³ after incubation with human serum. Similar results were observed after matrix metalloprotease-7 digestion of a multivalent conjugate based on a VEGF receptor antagonist.⁴⁴ In this study, our mvShh conjugates could retain 57.4–83.1% of their bioactivity after incubation with plasmin, depending on the Shh ligand spacing, as compared to 30.0% bioactivity retention for unconjugated Shh. Thus, mvShh conjugates were capable of providing proteolytic protection that was equivalent to similar methods of protein-polymer conjugation.

Finally, this study is the first to specifically evaluate the effect of HyA MW on the *in vivo* performance of a HyA-protein conjugate. In previous studies, mvShh with 860 kDa HyA was shown to improve the rate and quality of dermal wound healing compared to unconjugated Shh,¹⁸ but this study was focused on the effect of Shh valency using a fixed HyA MW. Our findings for CD31+ cell proliferation during early wound healing and for overall wound resolution time are similar to this previous study. However, our findings here are unique in that we have isolated the pharmacokinetic effects of HyA MW *in vivo* by using two mvShh conjugates that exhibit equivalent *in vitro* bioactivity. In a second similar study, multivalent conjugates of epidermal growth factor (EGF) and 200 kDa HyA also accelerated wound closure compared to unconjugated EGF when incorporated in a HyA hydrogel and applied to full-thickness wounds in rats.¹⁹ The HyA appeared to facilitate proteolytic protection, transdermal transport, and wound tissue penetration of HyA-EGF compared to the unconjugated EGF, but the HyA-EGF did not appear to exhibit enhanced potency to that of the unconjugated EGF. This discrepancy may be due to the lower MW of EGF relative to Shh, and thus EGF does not appear to have the same trend between ligand spacing and potency. Thus, by comparison, the mvShh provided a wider set of therapeutic enhancements for the conjugated Shh.

Based on this study, we have narrowed the range of conjugate parameters that will maximize the wound healing efficacy of mvShh. The highest *in vitro* bioactivity of mvShh has been

achieved when the Shh ligand density has been maximized, and this requirement could be more easily met for lower MW HyA. Previous *in vitro* studies using HyA protein conjugates have also shown that the $R_{g,z}$ is dependent on the HyA MW, and their transport properties through *in vitro* hydrogels is related to $R_{g,z}$.⁴⁴ Thus, our findings suggest that it may be possible to optimize the mvShh parameters to present multiple Shh at a sufficiently high ligand spacing and also enhance its transport into the wound matrix. As a part of future studies, we will further investigate the mechanisms by which the HyA contributes to the pharmacokinetic properties of mvShh, as these studies will yield critical data to optimize their wound healing performance and advance their translation as a clinical therapy.

CONCLUSIONS

Multivalent conjugation offers a strategy for controlling the potency, tissue level distribution, and enzymatic protection of protein-based therapeutics. Others have shown that mvShh potency is dependent on Shh valency, and the potency of mvShh can exceed that of unconjugated Shh at equimolar concentrations.^{14, 18, 45} Here we have demonstrated that Shh ligand spacing is a more descriptive parameter for relating mvShh potency and enzymatic resistance to Shh valency over a range of HyA MWs. Independently, mvShh size ($R_{g,z}$) is primarily related to the MW of the HyA component and will likely contribute to the pharmacokinetic properties of the conjugates. Enhanced potency, enzymatic resistance, and macromolecular size offer important mechanisms to enable sustained bioactivity of multivalent growth factor conjugates *in vivo*, and these likely contributed to the enhanced therapeutic effect and wound healing performance of mvShh conjugates in disease specific animal models here and in previous studies.^{18–19} This study is the first to show how the wound healing efficacy of a HyA-protein conjugate is sensitive to the HyA MW, and our findings here have narrowed the range of MW that we expect will maximize the efficacy of growth factor conjugates. The continued development of this technology platform for multivalent conjugation will likely be useful to improve the therapeutic performance for a variety of similar protein-based drugs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Characterization of mvShh conjugates. (A) The final molar ratio of Shh to HyA after the conjugation reaction correlated linearly to the molar feed ratio of Shh to HyA ($R^2=0.9176$) with a significantly non-zero slope of 0.658 ± 0.040 ($p<0.0001$, $N=26$). (B) The average reaction efficiency of the conjugation reaction was $66.7 \pm 9.6\%$ (shown as a dotted line plotted against the Shh feed ratio), and the conjugation reaction efficiency was not correlated to either the Shh feed ratio or HyA MW ($R^2=0.0778$, multiple linear regression with $N=41$). (C) Based on a representative sample of mvShh conjugates, unconjugated Shh appeared in SDS-PAGE gels as expected in a band at approximately 23 kDa whereas the mvShh conjugates remained near the loading well or within the stacking gel. (D) Shh valency correlated with the final Shh:HyA molar ratio ($R^2=0.9560$, multiple linear regression with $N=19$) with a significantly non-zero slope of 0.676 ± 0.057 ($p<0.001$), and there was no significant effect of HyA MW on valency. (E) The final HyA MW correlated linearly to its initial MW ($R^2=0.9560$, $N=16$) with a significantly nonzero slope of 0.920 ± 0.057 ($p<0.001$). (F) The radius of gyration ($R_{g,z}$) correlated linearly to the MW of the HyA

component ($R^2=0.643$, $N=16$) with a significantly non-zero slope of 0.042 ± 0.014 ($p<0.005$).

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Figure 2.

In vitro bioactivity of mvShh conjugates. C3H10T1/2 cells were treated with mvShh conjugates with various Shh valencies and HyA MWs and compared to unconjugated Shh based on the total concentration of Shh in each treatment. Shh-induced transcriptional activity was assessed on the basis of up-regulation of alkaline phosphatase (ALP). Representative dose-response curves are shown for mvShh with (A) 150 kDa HyA and (B) 752 kDa HyA and with various Shh valencies. Plots were fitted using four-parameter logistic non-linear curves ($R^2 > 0.9200$ for all curves with $N=27$). EC50 values were determined from the fits and used to compare the bioactivities of each conjugate to unconjugated Shh (normalized to 1; mvShh with relative EC50 < 1 were more potent than unconjugated Shh). Representative plots are shown comparing EC50 values for mvShh with (C) 150 kDa HyA and (D) 752 kDa HyA and with various Shh valencies. EC50 values were correlated with Shh valency ($R^2 > 0.6200$ for all fits with $N=32$ to 45 , see Table 1), and all of the fitted lines included significant, non-zero slopes ($p < 0.005$ for all lines, see Table 1). (E) Relative EC50 data combined for all mvShh conjugates correlated weakly to Shh valency and HyA MW

($R^2=0.2408$, multiple linear regression with $N=51$). (**F**) Relative EC50 correlated more strongly to Shh ligand spacing ($R^2=0.8674$, $N=51$).

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Figure 3.

Digestion and deactivation of mvShh conjugates by plasmin. (A) mvShh was incubated for up to 60 minute with 6.5 nM plasmin, and the digestion fragments were evaluated using SDS-PAGE. (B) One-phase exponential association rate equation fits for Shh ($R^2=0.9223$, $N=8$) and mvShh ($R^2=0.9816$, $N=8$). Half-digestion times for Shh and mvShh were 47.8 and 292.1 minutes, respectively. (C) The extent of Shh digestion at the C-3 cleavage site for mvShh conjugates with varying ligand spacing was significantly lower than for unconjugated Shh (* $p<0.001$, one-way ANOVA with Tukey post-hoc tests and $n=14$). (D) The extent of Shh digestion at the N-9 cleavage site was estimated based on ALP upregulation in C3H10T1/2 cells was assessed after digestion with plasmin at various concentrations for 1 hour. EC50 values were determined from dose-response curves fitted using four-parameter logistic non-linear curves ($R^2>0.9400$ for all curves with $N=9$), and the percent change in bioactivity was determined by the EC50 for each conjugate divided by the EC50 for the same conjugate after incubation with plasmin in a matched experiment performed simultaneously. mvShh retained significantly greater bioactivity after plasmin digestion compared to unconjugated Shh (groupings a, b, and c are significantly different from each other with at least $p<0.05$ using one-way ANOVA with Tukey post-hoc tests and $n=12$).

Figure 4.

The effect of HyA MW on CD31+ cell recruitment by mvShh during early wound healing. (A) The number of CD31+ cells per 10× microscopic field in the wound margin immediately adjacent to the wound bed at 24, 48, and 72 hours after generating a full-thickness excisional wound in db/db diabetic mice. (groupings a, b, c, d were significantly different from each other at each time point with at least $p < 0.02$ using one-way ANOVA with Tukey post-hoc tests and $n = 5$). (B) Representative images of CD31+ cells in 10× microscopic fields at the wound margin 72 hours after generating a full-thickness excisional wound in db/db diabetic mice (bar=100 μm).

Figure 5.

The effect of HyA MW on wound resolution by mvShh in db/db diabetic mice. (A) Representative wound images of full thickness excisional wounds at various time points after initial wounding at day 0 (bar=0.5 mm). (B) Wound areas normalized to their initial size at 8, 12, and 15 days after wounding (one-way ANOVA with Tukey post-hoc tests and $n=10$). (C) Total days required for complete wound closure of diabetic wounds receiving each treatment (one-way ANOVA with Tukey post-hoc tests and $n=10$).

TABLE 1

The significance of Shh valency to mvShh bioactivity based on linear regression fits to EC50.

HyA MW (kDa)	R ² of linear regression	significance of non-zero slope
150	0.8974	p<0.0005
215	0.9387	p<0.01
357	0.9327	p<0.0001
752	0.6208	p<0.005
860	0.7021	p<0.0001

TABLE 2

The molecular parameters of mvShh conjugates used for *in vivo* studies.

HyA MW (kDa)	Conjugation Efficiency (%)	Shh Valency	Ligand Spacing (HyA MW per Shh)
357	68.8	19.6	18.2
752	68.9	41.4	18.1