siRNA Delivery from an Injectable Scaffold for Wound Therapy

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Significance: Aberrant overexpression of proinflammatory molecules is believed to be a key mediator in the formation of chronic skin wounds, and the inhibition of these signals may be an effective therapeutic strategy to promote healing. Small interfering RNA (siRNA) can provide gene-specific silencing and may present a safe and effective route for knockdown of inflammatory or other target proteins in chronic skin wounds.

Critical Issues: siRNA suffers from delivery barriers *in vivo* such as susceptibility to degradation, membrane impermeability, and transient activity. Therefore, a delivery strategy that stabilizes siRNA, provides intracellular (cytoplasmic) delivery, and produces temporally sustained activity is needed. The novel approach described combines pH-responsive, siRNA-loaded nanoparticles into a biodegradable polyurethane (PUR) scaffold and presents a promising platform for effective, local silencing of deleterious genes in non-healing skin wounds.

Recent Advances: The siRNA delivery barriers have been overcome using a nanoparticulate carrier that protects siRNA and responds to pH gradients in the endo-lysosomal pathway to mediate cytosolic delivery. Nanoparticle incorporation into a biodegradable PUR scaffold provides a means for controlling the delivery kinetics of siRNA-loaded carriers. Furthermore, the PUR is injectable, making it feasible for clinical use, and provides a porous tissue template for cell in-growth during tissue regeneration and remodeling. This local siRNA delivery platform can be tuned to optimize release kinetics for specific pathologies.

Future Directions: siRNA may provide a new class of biologic drugs that will outperform growth factor approaches, which have shown only moderate clinical success. The new platform presented here may provide clinicians with an improved option for wound care.

SCOPE

CHRONIC SKIN WOUNDS represent a significant clinical burden, especially in diabetic patients whose skin wounds can become ulcerated and even lead to limb amputation. The current state of the art in biologic drugs for improving wound healing is Johnson and Johnson's Regranex[®], which delivers platelet-derived

growth factor (PDGF). Regranex significantly improves wound healing, but unfortunately, it still cannot close 50% of chronic wounds.¹ Recently, small interfering RNA (siRNA) has been proposed as a potential treatment for a range of pathologies, including skin wounds, and biomaterial scaffold-based siRNA delivery has been recently

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Abbreviations and Acronyms

DMAEMA = 2-dimethyl-aminoethyl methacrylate

GAPDH = glyceraldehyde 3-phosphate dehydrogenase

LTI = lysine triisocyanate

N:P = charge ratio, the number of positively charged tertiary amines in the siRNA condensing block divided by the number of negatively charged phosphate groups in the siRNA

PAA = 2-propyl acrylic acid

PDGF = platelet-derived growth factor

PUR = polyurethane

RISC = RNA induced silencing complex

siRNA = small interfering RNA

si-SPN = smart pH responsive polymeric nanoparticle with complexed siRNA

SPN = smart pH responsive polymeric nanoparticle

TLR = toll-like receptor

sought using both natural and synthetic scaffolds.^{2–5} Here, we introduce a robust approach for siRNA delivery to skin wounds. This new platform incorporates siRNA delivering smart pH-responsive polymeric nanoparticles (SPNs) into injectable, biodegradable, porous, polyurethane (PUR)-based synthetic scaffolds.

TRANSLATIONAL RELEVANCE

Since the discovery of RNA interference in gene regulation, a large volume of research has been directed into rapidly developing siRNA for clinical use.⁶ siRNA are short double-stranded RNA, where the guide strand of the molecule is loaded onto the RNA-induced silencing complex (RISC), a cohort of proteins intrinsic to mammalian cells. The activated RISC identifies the targeted mRNA through complementary base pairing and cleaves the mRNA. The guide strand and activated RISC are conserved and may reinitiate degradation of additional mRNA molecules,⁷ making the process catalytic and thus more potent than stoichiometric inhibitors (*i.e.*, protein or small molecule). These favorable properties of siRNA have led to rapid advancement into clinical tests for a variety of conditions, including respiratory syncytial virus infection, macular degeneration, hepatitis B, renal failure, macular oedema, pachyonychia congenital, and solid tumors.^{8–10}

siRNA is regarded to have untapped clinical potential, but one of the major challenges to harnessing RNA interference pharmaceutically is efficient cytoplasmic delivery of the siRNA biomacromolecules into target cells. Naked siRNA has a very short half-life *in vivo* due to rapid degradation by nucleases and clearance through kidney filtration. siRNA is also relatively large molecular weight, anionic, and polar, making it impermeable to cell membranes. This is problematic for initial cellular internalization and for escape from endo-lysosomal vesicles following uptake by endocytosis. Thus, siRNA carriers are required that can both package and protect the siRNA and deliver siRNA into the cytoplasm of the cell, where the RISC machinery is located. Polycations have been heavily studied as an approach to package and protect siRNA and enable endosome escape via the proton sponge effect. However, most polycations are characterized by cytotoxicity to form electrostatic polyplexes that are unstable *in vivo*.

Although siRNA activity is catalytic, it does have a finite half-life in the cell. Previous reports generally note maximum silencing at around 2 days post-transfection¹¹ with normal gene expression

restored by ~1 week in rapidly dividing cells.¹² One approach to extend silencing may be to achieve sustained, local release from scaffolds injected or transplanted onto the wound. Mostly natural materials such as alginate, collagen, and agarose have been pursued for biomaterial-based siRNA delivery to this point.^{2–5} Recently, key proof-of-concept studies were published describing effective topical siRNA gene silencing *in vivo* using agarose scaffolds loaded with siRNA packaged into the commercial transfection reagent Lipofectamine 2000. This particular approach represents a significant breakthrough, though it did suffer from the potential limitation of siRNA diffusing from the scaffold in a relatively rapid burst release. This rapid release required removal and re-application of siRNA-loaded scaffolds to achieve better and more sustained siRNA activity.^{4,5} It may be possible to achieve more optimal wound therapies with delivery systems that integrate efficient, nontoxic siRNA carriers into an injectable delivery matrix that can achieve sustained and tunable rates of siRNA release for >1 week.

CLINICAL RELEVANCE

Nonhealing diabetic skin wounds are characterized by a heightened and unresolved inflammatory phase leading to chronic ulceration. Because specific inflammatory mediators integral to this phenotype have been identified, siRNA may be a strong candidate drug for the treatment of chronically inflamed ulcers through sequence-specific gene silencing. By silencing proinflammatory genes (*e.g.*, p53, and Smad3 silencing have previously been attempted to improve wound healing)^{4,5} the wound site may be transformed into a more reparative environment that enables progression from the prolonged inflammatory phase and into remodeling and wound closure.

EXPERIMENTAL MODEL OR MATERIAL

Our work has focused on the combination of two complementary biomaterials that enable efficient, sustained siRNA intracellular delivery to skin wounds (see Fig. 1). The first class is a pH-responsive micelle, referred to here as the smart polymer nanoparticle (SPN). The SPN is capable of electrostatic loading and nuclease protection of siRNA in addition to pH-dependent membrane disruptive activity that can mediate escape from endo-lysosomal vesicles. This SPN is self-assembled from a reversible addition fragmentation chain transfer-synthesized diblock copolymer recently described.¹³ This diblock polymer (see

Figure 1. Schematic illustrating the approach described in this review. **(A)** Reversible addition fragmentation chain transfer-synthesized diblock co-polymer with the siRNA condensing block shown in gray and the pH responsive block in black. **(B)** In aqueous solutions, the diblock copolymer self-assembles into micellar nanoparticles with a positive surface charge that can be used to electrostatically condense siRNA. **(C)** Lyophilized si-SPNs are mixed into the polyol component and then added to the lysine triisocyanate and water to form a porous PUR scaffold containing embedded si-SPNs. **(D)** The si-SPNs can diffuse out of the PUR scaffold, and, upon release, si-SPNs can be internalized and efficiently delivered in a bioactive form into the cytoplasm of cells. siRNA, small interfering RNA; PUR, polyurethane; si-SPN, smart pH responsive polymeric nanoparticle with complexed siRNA.

structure in Fig. 1A) is composed of siRNA condensing block consisting of 2-dimethylaminoethyl methacrylate (DMAEMA). This block is the relatively hydrophilic block and forms the corona of the micelle. It has pendant tertiary amines that are $\sim 50\%$ protonated at physiologic pH, which enables electrostatic loading of siRNA into the shell of the SPN (Fig. 1B). The second block is a more hydrophobic and approximately charge neutral terpolymer block. This terpolymer contains approximately equimolar quantities of 2-propyl acrylic acid (PAA) and DMAEMA to maintain charge neutrality at physiologic pH. Butyl methacrylate is the third monomer and is incorporated to increase the hydrophobic character and drive micelle self-assembly in aqueous solution. Importantly, both the PAA and DMAEMA monomers are pH-responsive (*i.e.*, environmental pH dictates their protonation state and affects their physical properties), and it is this characteristic that mediates escape from endo-lysosomes upon acidification of these vesicles following cellular internalization of the carrier (Fig. 1D).

The second biomaterial is an injectable PUR scaffold composed of a polyol component that is 60% poly(ϵ -caprolactone), 30% poly(glycolide), and 10% poly(D,L-lactide), and a hardening component, lysine triisocyanate (LTI). The polyol and the LTI react to form urethane bonds, and water is added to the reaction to produce CO_2 that creates scaffold porosity (Fig. 1C). PUR formulations have significant clinical potential because they can be directly injected into a defect where they cure into mechanically robust, biodegradable scaffolds that conform precisely to the shape and size of the wound. Also, the PUR adheres to underlying tissue, does not elicit significant inflammation,¹⁴ and biodegrades at tunable rates into biocompatible products.¹⁵ Furthermore, the scaffold morphology is highly porous, which allows for ingrowth of granulation tissue and promotes tissue remodeling. The PURs have been previously used for controlled delivery of growth factors, but have not yet been employed for release of carriers for intracellular-acting biologic drugs.

DISCUSSION OF FINDINGS AND RELEVANT LITERATURE

The composition and relative block lengths of the diblock copolymer described can be tuned to spontaneously form micelle SPNs in aqueous conditions.^{13,16} The cationic corona effectively condenses siRNA and protects it from serum nucleases. The micelle core is pH-responsive and triggers pH-dependent membrane disruption in a range relevant to endo-lysosomal trafficking. To validate the pH-dependent activity, a red blood cell hemolysis assay was performed. In this assay, siRNA-loaded SPNs (si-SPN) were exposed to red blood cells in buffered solutions that mimic extracellular (*i.e.*, blood) (7.4), early endosome (6.6), and late endosome (5.8) environments. The red blood cell, in this case, models the endosomal membrane, and the degree of RBC hemolysis is a surrogate marker for pH-driven endosomal membrane disruption. As shown in Fig. 2A, there is a sharp, switch-like change in hemolysis as the pH is lowered from

physiologic to slightly acidic, which is indicative of a cytocompatible but highly efficient endosomolytic carrier. The SPNs were also found to significantly increase cytoplasmic delivery of siRNA, which was confirmed by the presence of FAM-labeled siRNA in the cytoplasm of human cervical carcinoma cells using flow cytometry (Fig. 2B). Experiments in human cervical carcinoma cells also demonstrated significant gene silencing of the model gene *GAPDH* after 24 h of incubation with si-SPNs. Degree of knockdown was found to be dependent on the polymer to siRNA charge ratio (N:P) (Fig. 2C). There was also a concentration-dependent effect, with 50 nM siRNA silencing gene expression by nearly 90% (Fig. 2D). This polymer-mediated gene knockdown efficacy was similar to the commercial reagent HiPerFect, which showed poorer cyto-compatibility (data not shown). These promising *in vitro* validations indicated that the SPNs are a promising component for our skin wound siRNA delivery system.

Figure 2. (A) si-SPNs demonstrate finely tuned, pH-dependent membrane disruption in a red blood cell hemolysis assay. Red blood cells incubated without si-SPNs showed no hemolysis at tested pH values.¹³ (B) The si-SPNs provide enhanced intracellular uptake relative to naked siRNA as shown by flow cytometry on human cervical carcinoma cells delivered FAM-labeled siRNA.¹³ (C, D) Real-time reverse transcriptase–polymerase chain reaction indicates that si-SPNs are capable of delivering siRNA against the model gene *GAPDH* and efficiently reducing target expression by nearly 90% at a charge ratio of 4:1 and siRNA concentration of 50 nM.¹³ These si-SPNs have been subsequently incorporated into PUR scaffolds with (E) interconnected, porous morphology as shown via SEM.¹⁴ Wound healing after application of empty PUR scaffolds (F–H) has been shown to be accelerated when PDGF is incorporated for sustained release into the wound (I–K).¹⁴ *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; PDGF, platelet-derived growth factor; SEM, scanning electron microscopy.

The injectable PUR scaffold has been previously described for sustained delivery of PDGF to skin wounds.¹⁴ Scanning electron microscope imaging of the scaffold revealed interconnected porous morphology, which allows diffusion throughout the scaffold and cellular in-growth (Fig. 2E). PDGF was incorporated into the matrix of the PUR scaffold that was applied to excisional wounds in mice. The incorporation of PDGF encouraged the migration of both fibroblasts and mononuclear cells, accelerated the degradation rate of the PUR scaffold, and significantly decreased the time required for formation of granulation tissue. The acceleration in wound healing can be seen in the temporal differences between tissue formation and remodeling in empty scaffolds (Fig. 2F–H) and scaffolds containing PDGF (Fig. 2I–K). In addition to the results shown here, the release of the PDGF reached 80% of the total payload over 21 days, and the release kinetics can be tuned across a broad range by altering the composition of the scaffold and pre-encapsulating the protein into biodegradable microspheres.

Our current work focuses on incorporation of si-SPNs into the injectable PUR scaffold for sustained, tunable gene silencing in skin wounds. Figure 1 illustrates the general approach to this study. siRNA is condensed onto the SPNs, which are subsequently lyophilized and mixed with the other PUR components at the time of scaffold fabrication. Like in the PDGF application, the si-SPNs have been found to be efficiently released from the PUR, and TEM has demonstrated that the PUR releasate contains what appear to be intact si-SPNs. Furthermore, real-time reverse transcriptase–polymerase chain reaction suggests that gene silencing bioactivity of the si-SPNs is maintained postrelease from the PUR scaffold, as has been recently reported.¹⁷ Further development of this siRNA delivery platform is expected to serve as a useful research tool for studying the effects of temporally controlled silencing of target genes and also represents a potentially translatable system for clinical use.

INNOVATION

Currently, there are no clinically-utilized intracellular-acting biomacromolecular drugs (*i.e.*, growth factors act on extracellular receptors). The delivery requirements for intracellular-acting biomolecules like siRNA are more rigorous because they cannot cross cellular membranes, and when endocytosed, the predominant fate is enzymatic degradation in lysosomes or recycling and extra-

cellular clearance. Here, we describe a smart polymer carrier that recognizes environmental changes to become membrane disruptive in the lower pH environment of endosomes. This innovative approach to gene inhibition may enable a new level of pharmaceutical breadth and specificity that would overcome many of the shortcomings of small molecule drugs and also allow manipulation of intracellular targets that were previously considered undruggable. The majority of recent applications of siRNA have focused on *in vitro* validation, systemic *in vivo* delivery, or *in vivo* applications where siRNA formulations have been injected locally in saline with no regard for persistence of sustained bioactivity (*i.e.*, intraocular or intratumoral injection, lung inhalation). The use of siRNA for regenerative applications could be tremendously enhanced by means for its sustained, local delivery from scaffolds that serve as porous tissue templates. The innovative combination of si-SPNs and PUR scaffolds provides a porous scaffold template for cell in-growth, ease of delivery for clinical applications (injectability), multiple levels of tunability for release kinetics, and, ultimately, the ability to optimize siRNA activity for specific target genes and/or different pathologies.

CAUTION, CRITICAL REMARKS, AND RECOMMENDATIONS

Technologies for intracellular delivery of siRNA have advanced rapidly and are potentially approaching widespread adoption for clinical use. However, it should be cautioned that intracellular delivery of siRNA can lead to recognition by toll-like receptors (TLRs). TLRs recognize molecular patterns that are associated with pathogens, for example double-stranded RNA, which can be representative of the viral genome. This effect could result in pathological symptoms clinically, and it has also led to the false interpretation of pre-clinical results in studies related to viral repression, oncology, angiogenesis, and inflammation.¹⁸ As a result of this phenomenon, it is recommended that siRNA studies carefully look for potential TLR-mediated effects. It is also advisable to replicate studies with multiple siRNA sequences against the gene of interest to ensure that any phenotypic changes are solely attributable to silencing of the target gene. Importantly, there is also ongoing work to create siRNAs that avoid immune activation entirely, and we are optimistic that these nonspecific effects will become more completely understood and entirely avoidable. For example, it is thought that immune recogni-

tion is sequence dependent¹⁹ and that carefully selected siRNA sequences may avoid activation of the immune system. Also, chemical modifications of siRNA with 2-OMe nucleotides can help to eliminate TLR activation while producing negligible effects on gene silencing efficacy.¹⁸ Finally, the need to design properly controlled experiments is further highlighted by the previous finding that transfection reagents that enter the cytoplasm by lysosomal lysis may activate the NALP3 inflammasome and induce inflammation.²⁰

FUTURE DEVELOPMENT OF INTEREST

Molecular biologists have made tremendous advances toward understanding the mechanistic control of wound healing. This body of work helps to identify a variety of genes whose silencing could be potentially sought in wound healing therapies. One future requirement will be to identify the most therapeutically useful target genes for different pathological situations (*i.e.*, chronic wound vs. burn or trauma victim, etc.). Further, variation of release kinetics from the PUR scaffold will be required to establish the optimal therapeutic window for siRNA delivery. Finally, another potential future advance will be development of methods to achieve cell type-specific targeting of siRNA in the skin wound. For example, in hyperinflammatory wounds, one may be able to optimize therapeutic benefit and/or minimize nonspecific effects by targeting of siRNA specifically to leukocytic cell types.

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TAKE-HOME MESSAGES

Basic science advances

siRNA has strong potential to be translated into therapeutic use for many applications, in particular, the knockdown of overexpressed proinflammatory genes in chronic nonhealing skin wounds. Technologies for delivery of siRNA *in vivo* are the major limitation because it is susceptible to degradation, membrane impermeable, and produces only transient bioactivity. To overcome these barriers, siRNA has been loaded onto pH-responsive SPNs that have been subsequently incorporated into an injectable PUR scaffold. This delivery platform is capable of sustaining the release and intracellular delivery of siRNA for extended, clinically relevant timeframes.

Clinical science advance

After validation and optimization of siRNA delivery and bioactivity *in vitro*, pre-clinical and clinical work will follow. This delivery platform will be utilized to test the therapeutic benefit of silencing target genes and hopefully provide an improved standard of care relative to current wound treatments.

Relevance to clinical care

Current approaches for clinical management of traumatic and chronic wounds are far from optimal, and development of products that enable use of siRNA drugs for gene-specific silencing at the wound site may provide significant benefits. Currently, molecular biologists and pharmaceutical biochemists are working toward the design of more stable siRNA sequences with enhanced activity and half-lives and reduced side effects. Concurrently, we and others are working to develop improved delivery techniques that will be utilized to rapidly translate these optimized siRNA sequences into clinically utilized therapeutics.

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