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Controlled and sustained delivery of siRNA/NPs from hydrogels expedites bone fracture healing

Yuchen Wang [a, b], Dominic W. Malcolm [a, b], Danielle S.W. Benoit [a, b, c, d, e, *]

[a] Department of Biomedical Engineering, University of Rochester, 308 Robert B. Goergen Hall, Rochester, NY 14627, USA
[b] Center for Musculoskeletal Research, University of Rochester Medical Center, 601 Elmwood Ave, Rochester, NY 14642, USA
[c] Department of Chemical Engineering, University of Rochester, 206 Gavett Hall, Rochester, NY 14627, USA
[d] Department of Orthopaedics, University of Rochester, 601 Elmwood Ave, Rochester, NY 14642, USA
[e] Department of Biomedical Genetics, University of Rochester, 601 Elmwood Ave, Rochester, NY 14642, USA

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ABSTRACT

Despite great potential, delivery remains as the most significant barrier to the widespread use of siRNA therapeutics. siRNA has delivery limitations due to susceptibility to RNase degradation, low cellular uptake, and poor tissue-specific localization. Here, we report the development of a hybrid nanoparticle (NP)/hydrogel system that overcomes these challenges. Hydrogels provide localized and sustained delivery via controlled release of entrapped siRNA/NP complexes while NPs protect and enable efficient cytosolic accumulation of siRNA. To demonstrate therapeutic efficacy, regenerative siRNA against WW domain-containing E3 ubiquitin protein ligase 1 (*Wwp1*) complexed with NP were entrapped within poly(ethylene glycol) (PEG)-based hydrogels and implanted at sites of murine mid-diaphyseal femur fractures. Results showed localization of hydrogels and controlled release of siRNA/NPs at fractures for 28 days, a timeframe over which fracture healing occurs. siRNA/NP sustained delivery from hydrogels resulted in significant *Wwp1* silencing at fracture callus compared to untreated controls. Fractures treated with siRNA/NP hydrogels exhibited accelerated bone formation and significantly increased biomechanical strength. This NP/hydrogel siRNA delivery system has outstanding therapeutic promise to augment fracture healing. Owing to the structural similarities of siRNA, the development of the hydrogel platform for *in vivo* siRNA delivery has myriad therapeutic possibilities in orthopaedics and beyond.

1. Introduction

Small interfering RNA (siRNA) exhibits significant therapeutic potential as it can induce potent and specific silencing of a broad range of genetic targets [1–3]. However, clinical translation of siRNA-based therapies has been hampered by delivery barriers associated with susceptibility to nuclease degradation, cell and endosomal membrane impermeability at the target site, and lysosomal degradation [3,4]. While many carriers have been developed to overcome these challenges [5–7], the most prominent barrier is tissue/cell-specific delivery of siRNA. Systemic delivery of siRNA, which is typically achieved using nanoparticle (NP)-based delivery systems, suffers from inefficiencies due to liver accumulation and reticuloendothelial system (RES) clearance, resulting in subtherapeutic concentrations at target tissues [8]. The concomitant high doses of administered siRNA required to reach therapeutic levels result in off-target effects, limiting the safety of siRNA therapeutics [9].

The incidence and burden of fractures, of which approximately 20% have impaired healing [10], is predicted to increase dramatically as the population ages, motivating the development of potent therapeutic strategies to enhance fracture healing. Accordingly, targeting genes that negatively regulate bone formation without altering bone resorption or affecting other cell types may overcome impaired bone healing [11]. Therefore, siRNA has been employed to augment osteogenic function of mesenchymal stem cells (MSCs), which play a key role in fracture repair. For example, delivery of siRNA against dickkopf-related protein 1 (DKK1) [12] or guanidine nucleotide binding protein alpha stimulating activity polypeptide 1 (GNAS1) [13] enhances osteogenic differentiation of MSCs. These data suggest that the delivery of siRNA can be used to enhance bone

* Corresponding author. Department of Biomedical Engineering, University of Rochester, 308 Robert B. Goergen Hall, Rochester, NY 14627, USA.
E-mail addresses: yuchen.wang@rochester.edu (Y. Wang), dominic.malcolm@rochester.edu (D.W. Malcolm), benoit@bme.rochester.edu (D.S.W. Benoit).

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regeneration, through promotion of MSC differentiation, survival, and/or tissue production [14].

Hydrogels, which are three dimensional (3D) networks formed from crosslinked hydrophilic polymers, have great potential to sustain the delivery of biomolecules, including siRNA [15]. Hydrogels have been used for drug delivery to the heart, eyes, lungs, central nervous system, and bone [15–18]. For siRNA delivery specifically, dextran and chitosan-based hydrogels have been utilized [19,20]. While these approaches sustain siRNA release for up to 10–14 days, regenerative medicine approaches, especially for bone healing, commonly require gene knockdown for a month or longer. Subsequently, biomaterial delivery depots have been developed for siRNA to promote angiogenesis [21] and inhibit tumor growth [22]. Although these approaches proved feasibility of local siRNA delivery, the challenge of delivering therapeutic siRNA to the right tissue over times commensurate with healing (approximately 3–4 weeks) still remains. Recently, nanofiber polymer scaffolds were developed that achieve sustained microRNA (miRNA) delivery for 2–4 weeks [23]. The scaffolds improved calvarial bone defect regeneration, paving the way for the development of functional materials for delivery of nucleic acid-based drugs to bone.

Herein, we report that a hybrid nanoparticle (NP)/PEG hydrogel delivery system can be used to control the release of a regenerative siRNA targeting WW domain-containing E3 ubiquitin protein ligase 1 (*Wwp1*) (Fig. 1A–E) for fracture healing in a clinically-relevant fracture model. *Wwp1* has been previously identified as a negative regulator of bone formation, as increased bone formation rate and bone mass were observed in mice globally deficient in *Wwp1* [24]. *Wwp1* siRNA electrostatically interacts with polymer diblock nanoparticles (NP) (Fig. 1A) to form siRNA/NP complexes (Fig. 1B). siRNA/NPs were then embedded in hydrolytically degradable hydrogels made from PEG-*b*-poly(lactide)-*b*-dimethacrylate (PEG-*b*-PLA-*b*-DM) (Fig. 1C), which was designed to deliver siRNA/NPs to fractures in a sustained and localized manner (Fig. 1D). We showed that hydrogel-mediated siRNA delivery resulted in prolonged knockdown compared to siRNA/NP alone (Fig. 2). We also demonstrated sustained localization and release of siRNA/NP and hydrogels *in vivo* in a murine femoral fracture model (Fig. 3). Knockdown of *Wwp1* using siRNA/NPs hydrogels showed significantly increased bone formation and accelerated healing (Figs. 4–6), highlighting the therapeutic promise of this approach.

2. Materials and methods

2.1. Diblock copolymer synthesis and characterization

The siRNA delivery copolymer consisted of two blocks. The first cationic block was composed of dimethylaminoethyl methacrylate (DMAEMA), which is designed to protect and complex with negatively charged siRNA [25–27]. pDMAEMA was synthesized by reversible addition-fragmentation chain transfer (RAFT) polymerization as previously described using the radical initiator 2,2′-Azobis(2-methylpropionitrile) (AIBN, Sigma) and chain transfer agent (CTA), ECT (4-cyano-4-[(ethylsulfanylthiocarbonyl)sulfanyl] pentanoic acid), under a nitrogen atmosphere in N,N-dimethylformamide (DMF) at 60 °C for 6 h. The second block, a tercopolymer of DMAEMA, propylacrylic acid (PAA), and butyl methacrylate (BMA), has been shown to be essential for endosomal escape of siRNA [25]. AIBN and pDMAEMA macroCTA were added to DMAEMA, PAA, and BMA (DMAEMA:PAA:BMA = 25:25:50) in DMF and the reaction was allowed to proceed at 60 °C for 24 h. The reaction was terminated via exposure atmospheric oxygen and the diblock polymers were purified via precipitation in 80:20 pentane:diethyl ether and dried under vacuum. First block and diblock molecular weights (Mn) was determined using gel permeation chromatography (GPC) (Shimadzu Technologies) using a TSKgel Guard SuperH-H guard column (Tosoh Biosciences) and a TSKgel Super HM-N for separation using a column oven at 60 °C. Polymer composition was determined using proton nuclear magnetic resonance (^{1}H-NMR, Bruker Avance400), as previously described [25].

2.2. siRNA/NP complexation and characterization

Diblock polymers were dissolved in ethanol at 4 mg/ml concentration then diluted to 2 mg/ml in PBS. The polymer solution was dialyzed in 3500 Da molecular cutoff dialysis tubing against distilled H_2O for 24 h. The concentration of the resulting polymer solution was then determined gravimetrically after lyophilization. NP size and zeta potential were characterized via dynamic light scattering analysis (DLS) using a Malvern Zetasizer. Results of NP characterization can be found in Table 1. The NP solution was filtered (0.2 μm) then utilized to form complexes with Silencer® Select Negative Control No. 1 siRNA (Cat. # 4,390,843) and Silencer® Select *Wwp1* mouse siRNA s98891 (Cat. # 4,390,771) from Ambion®, as previously described [25,26]. Briefly, siRNA, PBS, and then diblock copolymer were added to 1.5 mL Eppendorf tubes and incubated at room temperature for 20 min for complexation. The critical charge ratio indicating the ratio between the positive charges within the pDMAEMA block (0.5 positive charge per mole since 50% of the DMAEMA residues are protonated at physiological pH) and negative charges of siRNA (42 negative charges per mole) at which there is no uncomplexed siRNA was determined by gel electrophoresis [27].

2.3. Cellular uptake of siRNA/NP

Silencer® FAM (Fluorescein derivative, 5'-carboxyfluorescein)-labeled Negative Control No. 1 siRNA (Cat. # AM4620, Ambion®) was incubated with NPs to form complexes as described. For visualization of cellular uptake, mMSCs were seeded in chamber slides (Lab-Tek®) at 15,000 cells/cm^2 one day prior to siRNA/NP treatment. FAM siRNA/NPs (40 nM) were used to treat mMSCs (derived from C57BL/6 mice, purchased from Cyagen®) at charge ratios of 4:1 for 24 h. mMSCs were washed 3 times with PBS and fixed with paraformaldehyde (PFA; 4%) for 10 min mMSCs were then washed with deionized water, and coverslipped with the addition of ProLong® Gold Antifade Mounting solution with DAPI (Thermo Fisher Scientific) to prevent quenching. mMSCs were imaged using a Nikon E600 fluorescence microscope. For quantification of cellular uptake, mMSCs were seeded at 8000 cells/cm^2 in 24-well plates one day prior to treatment. mMSCs were incubated with cell culture media (untreated), free FAM-labeled siRNA (negative control), FAM-labeled siRNA complexed with Lipofectmine2000 (positive control), as well as FAM-labeled siRNA/NPs at 40 nM and charge ratios of 4:1. After 24 h, mMSC were harvested and analyzed on an Accuri C6 flow cytometer. A total of 5000 cell events were gated for analysis and data was analyzed using FlowJo software.

2.4. Silencing efficiency of siRNA/NP

Wwp1 siRNA and control siRNA were purchased from Ambion® (ThermoFisher Scientific). For gene silencing experiments, mMSCs were seeded in 24-well plates at 8000 cells/cm^2 one day prior to siRNA/NP treatment. Nanoparticles with complexed siRNA (40 nM) were used to treat cells at charge ratios of 1:1–8:1. Cells were also treated with siRNA complexed with Lipofectamine 2000 as a positive control. After 48 h, the cells were washed with PBS (0.5 ml) twice and lysed with TRK Lysis Buffer (OMEGA Bio-Tek). RNA was