Development of a 3D *in vitro* model of osteoarthritic cartilage for screening siRNA-loaded polymeric nanoparticles

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INTRODUCTION: Osteoarthritis (OA) is a painful and disabling joint disease caused by progressive tissue degeneration in articulating joints[1]. It affects over 500 million people worldwide and imposes an estimated economic burden of nearly $140 billion[2, 3]. Despite this widespread prevalence and substantial cost, there are no disease-modifying OA therapies approved by the FDA[4]. To address this clinical need, short interfering RNA (siRNA), which post-transcriptionally silences specific genes of interest, has been explored for its ability to precisely target biological pathways and undruggable proteins[5]. Although this is a promising therapeutic approach, delivery has posed a barrier to clinical translation. To overcome this challenge, our lab previously developed poly(dimethylaminoethyl methacrylate)-*block*-poly(dimethylaminoethyl methacrylate-*co*-butyl methacrylate-*co*-propylacrylic acid), a diblock tercopolymer that self-assembles into siRNA-loading polymeric nanoparticles[6]. However, there is no effective way to screen siRNA candidates for therapeutic effects on the human cartilage phenotype. Therefore, this study tested siRNA-loaded polymeric nanoparticles in a 3D *in vitro* model of osteoarthritic cartilage to establish a system for rapid testing of therapeutic siRNA. Specifically, siRNA-loaded polymeric nanoparticles were evaluated for two key design criteria: 1) more than 70% gene silencing during chondrocyte differentiation and 2) no significant effect on glycosaminoglycan content.

METHODS: Polymer Synthesis & Characterization: Reversible addition fragmentation-chain transfer polymerization was used to synthesize the diblock tercopolymer poly(dimethylaminoethyl methacrylate)-*block*-poly(dimethylaminoethyl methacrylate-*co*-butyl methacrylate-*co*-propylacrylic acid) (**Fig. 1A**). Nuclear magnetic resonance spectroscopy and gel permeation chromatography characterized polymer molecular weight and composition. Nanoparticle Self-Assembly & Characterization: Polymer was dissolved in ethanol, then self-assembled into nanoparticles in saline. Diameter, zeta potential, critical micelle concentration, and siRNA loading capacity were characterized by dynamic light scattering, electrophoretic light scattering, solvatochromic reporter detection, and gel electrophoresis. Chondrocyte Culture & Treatments: Articular cartilage from the femoral condyle of a healthy human female donor was obtained with approval from the University of Oregon's institutional review board. Following articular cartilage digestion, chondrocytes were isolated and passaged three times. Chondrocytes were then treated in monolayer with 60 nM GAPDH or non-targeting siRNA. After 24 hours, chondrocytes were centrifuged into 3D pellets and cultured in differentiation media for 7-14 days. To model osteoarthritic cartilage and diminish the healthy cartilage phenotype, pellets were stimulated with 1 ng/mL IL-1β and 1 ng/mL TNF-α for three days. Endpoint Assays: Dimethylmethylene blue (DMMB) and PicoGreen were used to quantify glycosaminoglycans and DNA in digested pellets. Pellets were also fixed, embedded, and sectioned for Toluidine Blue O staining, which binds to proteoglycans. Expression of the chondrogenic genes COL2A1 and ACAN and the target gene GAPDH was measured using RT-qPCR with RPLP0 as the housekeeping gene. Statistics: Significant differences were determined by two-way ANOVA with Tukey's post hoc test ($\alpha = 0.05$).

RESULTS: Polymer characterization confirmed a first block molecular weight of 38.2 kg/mol, a second block molecular weight of 13.4 kg/mol, and a second block composition of 33% dimethylaminoethyl methacrylate, 48% butyl methacrylate, and 19% propylacrylic acid. Nanoparticle average diameter was 52 nm, zeta potential was 26 mV, and critical micelle concentration was 0.03 mg/mL. DMMB and Toluidine Blue O staining measured glycosaminoglycans, an essential component of articular cartilage responsible for water retention and matrix organization, produced by chondrocytes differentiated in 3D at days 7 and 14 (**Fig. 1B-D**). As expected, there were significantly more glycosaminoglycans produced on day 14 vs day 7. Furthermore, glycosaminoglycans were significantly depleted on both days 7 and 14 in response to three days of treatment with inflammatory cytokines IL-1β and TNF-α. This is corroborated by initial gene expression analysis, which showed downregulation of COL2A1 and ACAN, genes associated with healthy cartilage matrix, in response to inflammatory cytokine treatment. Following these initial assays, which defined phenotypic changes, gene silencing and glycosaminoglycan production mediated by siRNA-loaded nanoparticles were measured in this model. Chondrocytes were treated with GAPDH or non-targeting siRNA for 24 hours, then pelleted to induce chondrocyte differentiation. After two days, gene expression analysis measured 74% gene silencing relative to cells treated with PBS (**Fig. 1E**). After 7 days, DMMB and PicoGreen showed no change in glycosaminoglycan production or DNA content (**Fig. 1F**).

DISCUSSION: In this study, chondrocytes were differentiated in a 3D *in vitro* model of osteoarthritic cartilage and treated with siRNA-loaded polymeric nanoparticles to measure gene silencing and model compatibility. Inflammatory cytokines modulated markers of a healthy cartilage phenotype, and significant gene silencing was mediated by siRNA-loaded polymeric nanoparticle treatment without inducing glycosaminoglycan depletion. This work is informing the screening of therapeutic siRNA-loaded polymeric nanoparticles that is currently underway in this model system.

SIGNIFICANCE/CLINICAL RELEVANCE: OA is a prevalent joint disease without an FDA-approved disease-modifying drug. Here, siRNA-loaded polymeric nanoparticles are tested in a 3D *in vitro* model of human osteoarthritic cartilage to establish model compatibility and siRNA screening feasibility. An effective model and siRNA delivery strategy will enable rapid testing of therapeutic siRNA candidates with the potential to modify OA progression.

REFERENCES: [1]Hunter et al. *Lancet*. 2019. [2]Long et al. *Arthritis Rheumatology*. 2022. [3]Murphy et al. *Arthritis Care Research*. 2020. [4]Cho et al. *Experimental Molecular Medicine*. 2021. [5]Traber et al. *Molecular Pharmacology*. 2024. [6]Convertine & Benoit et al. *J Controlled Release*. 2009.

IMAGES & TABLES:

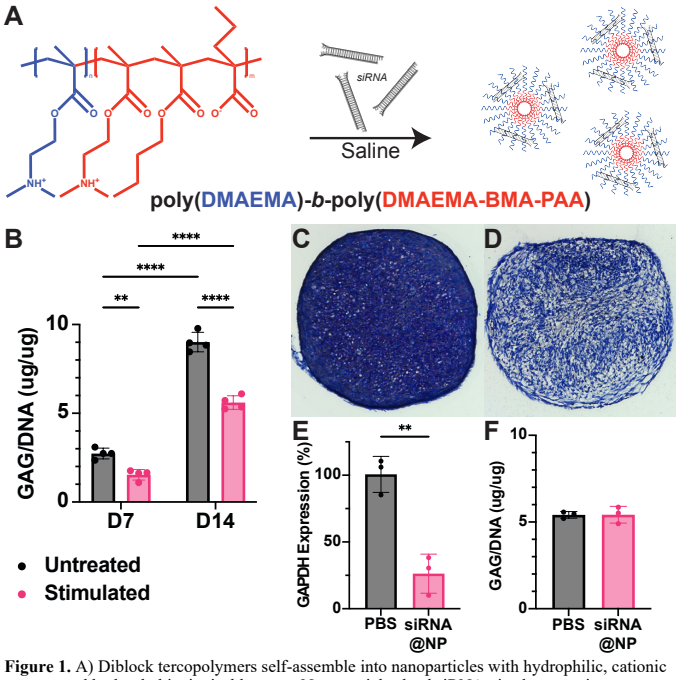

Figure 1. A) Diblock tercopolymers self-assemble into nanoparticles with hydrophilic, cationic coronas and hydrophobic, ionizable cores. Nanoparticles load siRNA via electrostatic interactions in the corona. B) Glycosaminoglycans are produced by chondrocytes in pellet culture. Inflammatory cytokine stimulation depletes glycosaminoglycans on days 7 and 14. C) Proteoglycans in untreated pellet are stained by Toluidine Blue O on day 14. D) Proteoglycans in stimulated pellet are stained by Toluidine Blue O on day 14. E-F) GAPDH siRNA-loaded nanoparticles mediate gene silencing after 48 hours of pellet culture without affecting glycosaminoglycan production. Significant differences were determined by two-way ANOVA w/ Tukey's post hoc test (* indicates $p < 0.05$, $n = 4$).