Intra-discal Delivery of RhoA-activator For Treatment of Intervertebral Disc Degeneration

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INTRODUCTION: Intervertebral disc (IVD) degeneration is a major factor for low back pain, characterized by an imbalance in cellular and biochemical homeostasis that affects the biomechanical functions of IVD[1]. Cytoskeletal proteins, specifically F-actin, has been shown to play a key role in degeneration-induced dysregulation in cell mechanobiology [2,3]. Moreover, activation of RhoA *in vitro* was shown to confer mechanoprotection against degradative effects of TNF-α in nucleus pulposus (NP) cells[4]. However, systemic delivery of RhoA-activators, such as CN03, is challenging due to the avascular nature of NP and short half-life of protein. While intradiscal injection may enhance CN03 bioavailability, its rapid diffusion results in short-lived benefits and may require repeated dosing, increasing the risk of toxicity and injury to the disc due to needle delivery[5]. Poly(lactic-co-glycolic acid) (PLGA) is an FDA-approved biodegradable copolymer; Micron-sized PLGA particles have been used as drug carriers for IVD degeneration therapies[5]. However, PLGA microparticles (PLGA MPs) suffer from significant diffusion out of the NP and are localized in the annulus fibrosus of IVD[6]. To circumvent short intra-NP residence time, we designed cationic PLGA-MPs to confer electrostatic interactions with negatively charged proteoglycans. Based on the negative fixed charge density (FCD) of NP, our previous research has developed cationic avidin-dextran conjugate that resulted in a month-long retention in bovine NP explants[7]. In this work, we use amphiphilic DSPE-PEG(2000)-Biotin (DPB) as an insertion to surface engineer PLGA MPs and synthesize cationic particles by conjugating Avidin utilizing non-covalent interaction with Biotin. The overall goal is to enable at least a month-long NP retention of PLGA MPs and achieve a sustained CN03 release to enable treatment with only a single dose against IVD degeneration.

METHODS: Two CN03-loaded polymeric formulations are synthesized using water-in-oil-water (**W1/O/W2**) double emulsion with PLGA(38-54 kDa) while optimizing viscosity and volume of W1: **PLGA** (100µL W1-CN03 in DI water) and **LW1 PLGA** (20µL W1-CN03 in 0.5% PVA). Encapsulation efficiency (EE%) was estimated by the ratio of loaded amount of CN03 in particles to its total amount in emulsion systems. *In vitro* release kinetics of CN03 from PLGA MPs (n=3) were investigated by resuspending the particles at 37°C in PBS over 21 days. The bioactivity of CN03 released from MPs by day 9 was confirmed by measuring RhoA- activation potential in NP cells (n=3) after a 24-h treatment. To synthesize cationic PLGA MPs (**PLGA-Avidin**), DPB was added to W2 phase during the emulsification, and particles with surface-bound Biotin were reacted with Avidin. Hydrodynamic size, zeta potential, polydispersity index (PDI), and loading of avidin on PLGA surface were measured. Surface conjugation was further confirmed by fluorescence microscopy with labeled PLGA (Alexa Fluor; AF) and Avidin (Texas Red). To investigate NP retention of cationic PLGA MPs, dye-loaded PLGA and PLGA-Avidin were injected into the center of bovine healthy and degenerated (40% GAG-depletion using 1 mg/mL trypsin-EDTA digestion) caudal NP explants (6 mm x 1 mm; n=4), and distribution area of PLGA was measured over 21 days using IVIS. For comparisons between groups, two-tailed student's t-test was used. Data are presented as mean ± SD, and $p < 0.05$ is considered statistically significant.

RESULTS: Particles synthesized by varying viscosity and volume of W1 (PLGA and LW1 PLGA) showed an EE of 75.1 ± 1.3% and 80.7 ± 1.5%, respectively, suggesting that the majority of the protein was loaded within the particles. PLGA suffered from high burst release (65%), and encapsulated CN03 was released completely in 6 days (**Fig.1A**). Whereas LW1 PLGA reduced burst release (13%) within 24 h (red dashed arrow), following which a constant release of about 77.2 ± 10.6 ng/day (**Fig.1A**). The bioactivity of released CN03 cumulated on day 9 showed no significant difference in its potential to activate the Rho signaling pathway in NP cells compared to an equivalent dose of free CN03 (ns, non-significant, two-tailed t-test) (**Fig.1B**). About 160 x 10^5 Avidin motifs were loaded per PLGA MP surface, which reduced the native negative charge of PLGA MP from -26.6 ± 1.6 mV to 0.6 ± 0.4 mV with no obvious change in the size of particles (**Fig.1C**). Incorporation of Avidin on the PLGA surface was also affirmed by the overlap of red fluorescence from Avidin around the surface of green fluorescence from the PLGA core (**Fig.1D**). PLGA Avidin showed a slower diffusion of dye in healthy (**Fig.1E**) and degenerated NP explants (**Fig.1F**), indicating a more localized distribution at the NP compared to PLGA. Thus, cationic PLGA showed stronger intra-NP binding and retention.

DISCUSSION: By tuning the formulation properties of PLGA MPs, we achieved a high protein encapsulation and demonstrated a controlled release of CN03 for over 21 days. Our study also showed that CN03 released from PLGA MPs was functional on the Rho-activation of NP cells to restore F-actin assembly. Moreover, we successfully surface-engineered PLGA MPs with Avidin to impart a cationic charge and demonstrated enhanced retention of PLGA-Avidin in healthy and degenerated NP explants compared to PLGA. The efficacy of CN03-loaded cationic PLGA MPs will be evaluated for restoring biophysical and mechanobiological functions in an inflammation-induced organ culture model as well as in an injury-induced IVD degeneration rat model.

SIGNIFICANCE: Our engineered cationic PLGA MPs have the potential to enhance intra-NP retention and enable controlled release of CN03 for long-term therapeutic benefits with a single dose. Hence, our system obviates the frequency of intra-discal injections and has potential to repair degenerated discs.

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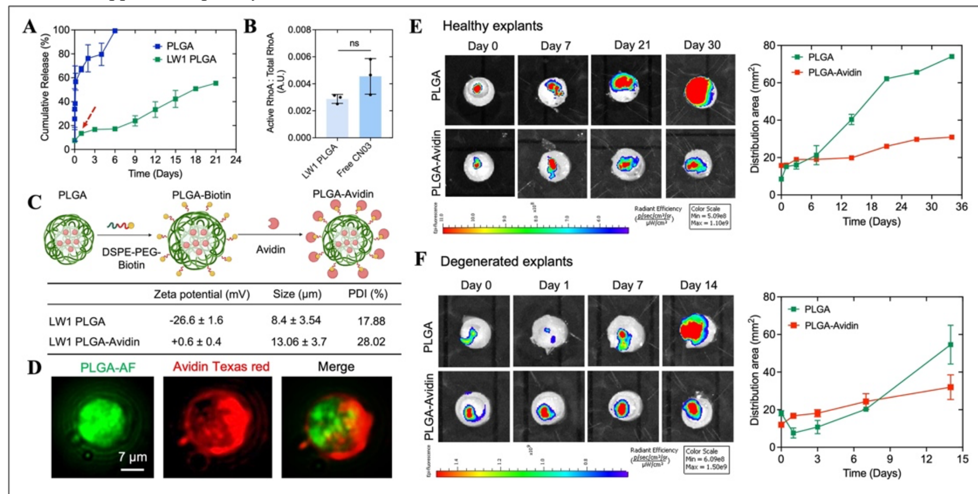

	Zeta potential (mV)	Size (µm)	PDI (%)
LW1 PLGA	-26.6 ± 1.6	8.4 ± 3.54	17.88
LW1 PLGA-Avidin	+0.6 ± 0.4	13.06 ± 3.7	28.02

Fig1. A. Cumulative release of CN03 from PLGA and LW1 PLGA formulations. **B.** Normalized Active RhoA protein after 24-h treatment of NP cells with LW1 PLGA and free CN03. **C.** Schematics of surface modification of PLGA MPs using DSPE-PEG-Biotin insertion and Avidin conjugation. Table depicting the surface charge of unmodified and surface-modified PLGA MPs. **D.** Fluorescent images confirming the surface conjugation of Avidin. Representative IVIS images showing the distribution of PLGA and PLGA-Avidin in **E.** healthy and **F.** degenerated NP explants and quantification of distribution area (in mm^2).