Fabrication and Characterization of Immunomodulating Electrospun Fibrous Mesh

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INTRODUCTION

Significance and Background

- Scarring is associated with extra-cellular matrix (ECM) dysregulation[1] and myofibroblast activation and persistence.[2]
- Myofibroblasts are a contractile pro-fibrotic cell type critical for early remodeling and deposition of predominantly type I collagen after injury in adult healing soft tissues.[1,2,3]
- Myofibroblast apoptosis or dedifferentiation, after the proliferative and contractile phase of repair[2,4,5] is required to produce more regenerative healing outcomes.
- Nuclear factor κB (NF-κB) signaling during soft tissue injury increases myofibroblast survival.[4]
- NF-κB is also correlated with degeneration and impaired healing of rat rotator cuff tendons,[6,7] tendon scarring in humans,[4] and fibrotic diseases.[8]
- More recently, NF-κB inhibition has been shown to reduce myofibroblast activation *in vitro*[8] and promote tendon healing *in vivo*.[7]
- The small molecule 2-Amino-6-[2-(cyclopropylmethoxy)-6-hydroxyphenyl]-4-(4-piperidinyl)-3 pyridinecarbonitrile (ACHP) suppresses IKKβ, which shuts down the inflammatory arm of the NF-κB signaling pathway.[7]
- Oral ACHP administration has been shown to enhance tendon-to-bone healing in a rat rotator cuff repair model.[7]
- ACHP has also been shown to strongly attenuate TGF-β1 induced myofibroblast transition in adult human lung and dermal fibroblasts.[8]
- Preliminary studies have shown that exogenous ACHP can reduce TGF-β1-induced myofibroblast differentiation[8] and turn off inflammatory signaling in human anterior cruciate ligament (hACL) cells *in vitro* tissue culture plastic.
- This work strives to develop a targeted biomaterial approach for localized ACHP delivery in order to reduce scar and promote regeneration of ligaments and tendons.

Study NF-κB *Motivation & Hypothesis*

- NF-κB itself is a ubiquitously expressed transcriptional regulator of over 500 genes associated with cellular survival, angiogenesis, and inflammation.[9,10,11]
- As such, systemic inhibition of NF-κB has major adverse effects[9,10,11] which is why strategies that target NF-κB at the local level are necessary.
- Thus, the main objective of this work was to fabricate and optimize the incorporation and release of ACHP from collagen-based fibers to locally target NF-κB *in vitro.*
- As such it was hypothesized that small molecule IKKβ inhibitor, ACHP, could be incorporated, and released from a biopolymer fibrous mesh.

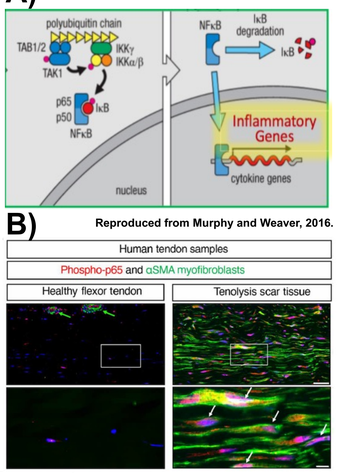

Figure 1: *A) NF-κB signaling, the canonical, rapid pathway responsible for cytokine production and apoptosis.[1,9] B) NF-κB in human tendon. C) ACHP inhibition of IKKβ.*

MATERIALS & METHODS

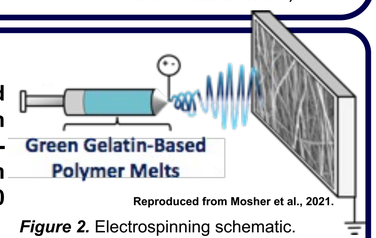

Figure 2. Electrospinning schematic.

- Fibrous Mesh Fabrication. A polymer melt of 40% gelatin, 50% acetic acid, and (+/-) ACHP was vortexed for 1 hour. 2.5 mL of the solution was electrospun with the following parameters. Flow Rate: 1 mL/hour, Voltage: 18-21 kV, Mandrel: 250-500 rpm, Needle Gauge: 18.5 (pink). The meshes were crosslinked in a vacuum chamber with glutaraldehyde with the vacuum on for 10 minutes and off for 20 minutes, repeated for each side.
- Release Experiment. 6 mm mesh discs were biopsy punched (n=5, Sklar Surgical Instruments) and sterilized under UV light for 20 minutes on each side. Each mesh was placed in a 24-well culture plate with 1.5 mL of F/S DMEM media at 37 °C for 24 hours. The release media was added to 1:1 Acetonitrile: Methanol and centrifuged at 16,000 x g (rcf). The supernatant was collected, dehydrated, and resuspended for liquid chromatography-mass spectrometry (LC-MS) analysis.
- Extraction. As fabricated meshes (n=4) were placed in 2:2:1 Acetonitrile:Methanol:Water and homogenized using a bead mill homogenizer. The resulting solution was centrifuged at 16,000 x g (rcf). The supernatant was collected, dehydrated, and resuspended for analysis on Acquity UPLC H-Class LC coupled to a Xevo G2 XS Q-ToF MS.
- Endpoint Analyses. Scanning electron microscopy (SEM, 5 kV, Zeiss Sigma VP) was performed on gold-palladium coated samples (4 nm, 108 Auto, Cressington Scientific).[12] SEM images (5000X, n=50-60 fibers, 10-12 images) were analyzed in ImageJ (National Institute of Health) to determine the mean fiber diameter. MatFiber, a Matlab adaptation of Fiber3, a C++ program developed by Karlon et al.[13] was utilized to determine fiber alignment. Fourier transform infrared spectroscopy in attenuated total reflectance mode (FTIR-ATR, n=3, Spectrum 100, Perkin Elmer) was used to attain an IR spectrum of scaffolds to assess small molecule – biopolymer interactions. LC-MS was utilized to determine loading efficiency and percent small molecule release of immunomodulatory fibers.

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RESULTS

A. Mircofiber Mesh ACHP Incorporation: Characterization via SEM, ATR

- SEM. ACHP incorporation decreased fiber diameter by ~35.1%, while fibers remained unaligned.
- ATR-FTIR. Spectroscopy suggests ACHP incorporation increases characteristic IR peaks, specifically at ~1000 nm.

■(-) ACHP

▣(+) ACHP

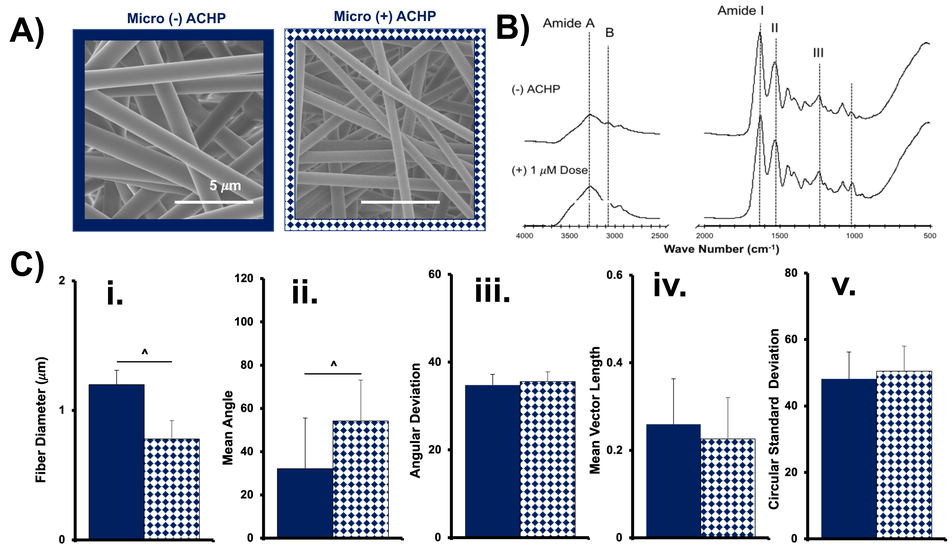

Figure 3: ***A)*** *SEM 5000x images of (-)ACHP & (+)ACHP microfibers.* ***B)*** *ATR-FTIR spectrum of (-)ACHP & (+) ACHP.* ***C) i.*** *Fiber Diameter (n=50-60 fibers, 10-12 images).* ***ii.*** *Mean Vector Angle (n=10-12 images).* ***iii.*** *Circular Standard Deviation (n=10-12 images).* ***iv.*** *Angular Deviation (n=10-12 images).* ***v.*** *Mean Vector Length (n=10-12 images). All error bars represent standard deviation.*

Table 1. Fiber Diameter and Alignment

Group MICRO	Gelatin (%wt/vol)	50% Acetic Acid Vol (mL)	Flow Rate (mL/hr)	Needle Gauge	Fiber Diameter (nm; n=50-60 fibers, 10-12 images)	Mean Vector Angle (n=10-12 images)	Angular Deviation (n=10-12 images)	Mean Vector Length	Circular Standard Deviation
Control	40%	2.5	1	18.5	1200 ± 109 ^	32.17 ± 23.42 ^	34.79 ± 2.42	0.26 ± 0.10	48.14 ± 8.13
(+)ACHP	40%	2.5	1	18.5	779 ± 143 ^	54.17 ± 18.95 ^	35.58 ± 2.22	0.22 ± 0.09	50.47 ± 7.59

^ indicates significance between groups

B. Fibrous Mesh ACHP Incorporation: Extraction & Release via LC-MS

- LC-MS Extraction. The actual and theoretical mass of ACHP loaded was quantified to determine the ACHP incorporated scaffold had a loading efficiency of 72.7%.
- LC-MS Release. LC-MS was performed to determine the mass of ACHP released. The released ACHP mass was compared to the loaded ACHP mass to determine 70.9% of ACHP was released into media after 24 hours.

Table 2. LC-MS Extraction and Release

Microfiber Gelatin Mesh Extraction	Loaded ACHP (ng; n=4)	Theoretical Loading (ng)	% Loading Efficiency (norm. by scaffold weight)
	1412.18 ± 397.76	1941.56 ± 334.10	72.7%
Microfiber Gelatin Mesh 24 Hour Release F/S DMEM	24 Hour Release (ng)	24 Hour Release (μM)	% Release 24 hours
	1000.55 ± 200.32	2.496	70.9%

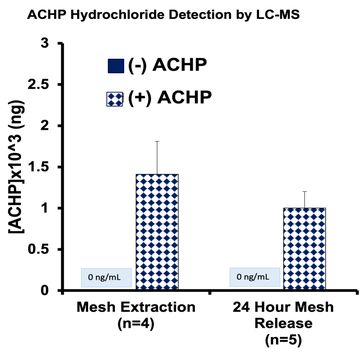

Figure 4: *ACHP mass (ng) from Extraction & 24-Hour Release.*

DISCUSSION & CONCLUSIONS

- Overall, we developed and characterized mechanically stable and physiologically-relevant collagenous substrates.
- We demonstrated that ACHP can be incorporated into electrospun biopolymer scaffolds at high loading efficiency and a majority of the small molecule can be released within 24 hours.
- Specifically, we characterized the (-)ACHP & (+)ACHP scaffolds and determined that while fiber diameter decreased due to ACHP incorporation, the fiber alignment was random for both groups.
- Many papers have investigated approaches to alter drug release kinetics such as varying diameter[14] and the polymer blend ratio.[15]
- Future research is required to achieve controlled release for a more sustained delivery of ACHP.
- The successful incorporation of ACHP into biopolymer scaffolds offers a potential method to target the NF-κB inflammatory pathway.