In vitro atomic force microscopy-based analysis of fibroblast-produced type I collagen

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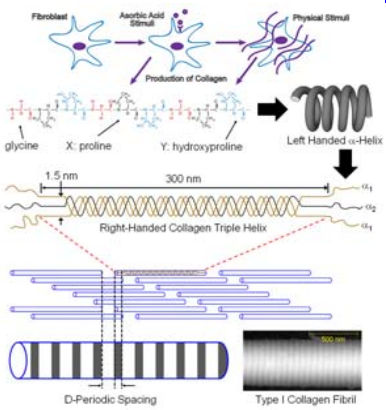
INTRODUCTION

Type I Collagen



▪Orgel et al., PNAS, 2006; 103 (24): 9001-9005
▪D. Bird, M. Allen Basic and Applied Bone Biology, 2013

Collagen nanomaterial

- Tropocollagen: consists of 2-α_1 and 1- α_2 helices forms in the cell, is secreted, pro collagen ends are cleaved and then collagen fibrils form external the cell
- Collagen produced in vivo has defined D-spacing
  - Previous studies using AFM
- Hydration plays an important role in the nanoscale morphology and mechanical properties of collagen
- Need: Effective assessment of collagen produced in vitro

Atomic Force Microscopy Imaging

- High resolution imaging requiring minimal sample preparation

Mechanical Indentation Using AFM

- Calibrated probe is pushed into surface to a known load or displacement
- Extract nanoscale mechanical properties

HYPOTHESIS

Atomic force microscopy can be used to quantitatively assess morphology and mechanical integrity of collagen produced by NIH-3T3 fibroblasts.

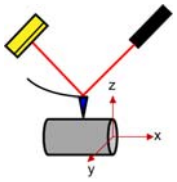
MATERIALS AND METHODS

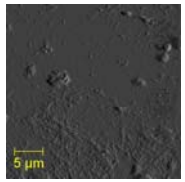
Preparation for Imaging

- Samples rinsed using 1X PBS followed by a 1-2 min treatment with trypsin
- Collagen matrix was broken up via vigorous pipetting in .5mL of H20
- Matrix solution was seeded on mica and allowed to dry

AFM Imaging and Indentation

- Imaged dry samples for greater image quality
- Rehydrated samples for indentation
  - Tip radius ~2 nm, k = 0.7 N/m, α = 17.5°
  - Indented to 5 nN: 10+ indents per 10 μm^2 location





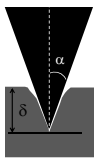
Cells and Collagen formation

- NIH-3T3 fibroblast, Passage # used for testing between 2 – 8
- Cells treated with complete media (10% FBS, 1% Pen strep) supplemented with 50µg/mL of ascorbic acid for 7–10 days

Mechanical Analysis

- Probe calibrated determining deflection sensitivity and spring constant
- Indentation depth and energy dissipation taken directly from force-separation curve
- Indentation modulus (E_s): curve fitting the middle 80% of unloading curve
  - Wet: Sneddon Model – indent depth is much larger than tip radius
  - Poisson's ratio (ν_s): assumed to be 0.35

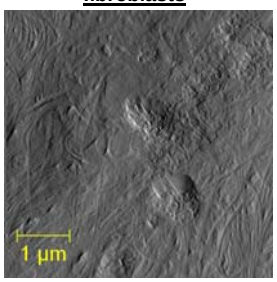
Sneddon Model



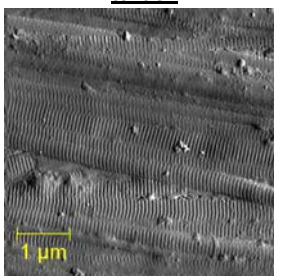
$$F = \frac{2}{\pi} \cdot \frac{E_s}{1-\nu_s^2} \cdot \tan\alpha \cdot \delta^2$$

RESULTS

AFM Image of collagen produced by NIH-3T3 fibroblasts

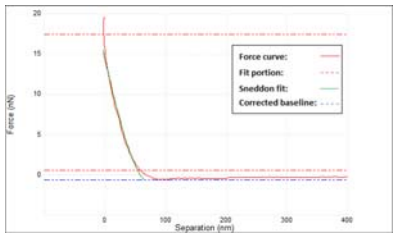


AFM Image of collagen dissected from a rat tail tendon

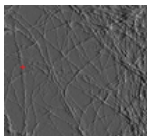


- Produced collagen (left) compared to prepared rat tendon not related to study (right) both show fibrous structures
- Collagen produced by NIH-3T3 is noticeably smaller than WT rat tendon
- No consistent D-spacing visible in the produced collagen

Mechanical Properties



- Representative force curve of one fiber indented from image below
- R^2= 0.9932
- Young's Modulus: 4.87 MPa



- Young's Modulus for re-hydrated collagen fibrils is between 1-5 Mpa
- Reduced Modulus: 5.36 Mpa

DISCUSSION

- Major differences can be observed between WT collagen and NIT-3T3 fibroblast produced collagen, such as considerably smaller diameters and less prominent D-spacing.
- Mechanical tests showed that the Young's Modulus of the collagen matrix produced by the NIH-3T3 fibroblasts was within standard physiological ranges found in the literature.
- In vitro produced collagen differs in diameter compared to vivo produced collagen potentially from a lack of physiological factors.
- Limitations
  - Wet Imaging: cells and collagen tend move under the force of the AFM probe
  - Viscous nature of collagen matrix – matrix tends to "stick" to the imaging probe
  - Cell extraction buffer lyses cells, leaving debris on top of the collagen matrix making it impossible to clearly image.

Collagen produced by NIH-3T3 fibroblasts over 7-10 days can be imaged and mechanically tested, suggesting a quantitative method to assess the quality of the in vitro-produced extracellular matrix

FUTURE STUDIES

- Research is being conducted to test how collagen produced by fibroblasts can change when subjected to external forces
- Creation of a flow chamber has been completed and several factors will be tested to observe how differing shear stresses will effect the collagen.
  - The chemical composition will be tested with quantitative PCR comparing control NIH-3T3 fibroblasts to ones subjected to shear
  - Mechanical testing of collagen will be performed to assess changes in collagen quality under loading.