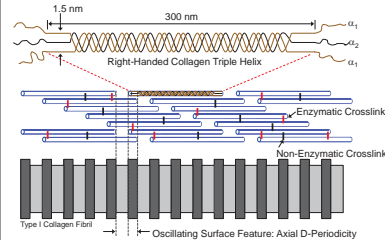


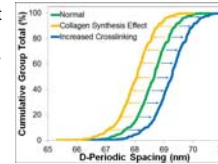
INTRODUCTION

Collagen Synthesis and Structure



- D-spacing has distribution of values in Type I collagen-based tissues and changes under a variety of conditions
- D-spacing provides information on:
 - Internal state and structure of collagen
 - Defects driven by post-translational modifications (e.g. hydroxylation)
 - Enzymatic and non-enzymatic crosslinks
- Attempts to correlate changes in D-spacing to mechanical properties inconclusive

- Disease impact on collagen synthesis shifts distributions ↓
- Crosslinking shifts distributions ↑



Bone Demineralization for Imaging

- Bone vs. Tendon: mean bone D-spacing ↓ by 2.5 nm, more than double overall ranges
- Mineral removal may allow collagen to collapse without support
 - More heterogeneous population with shifted center
- Interesting phenotypes become apparent
 - Preserving structure may also be important
- Fixation to chemically crosslink native structure prior to mineral removal
 - Quantitative analyses of native structure in bone?

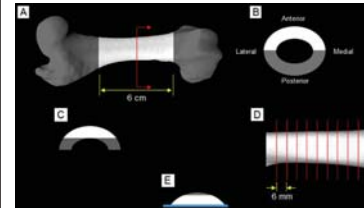
HYPOTHESIS

Fixation of bone prior to demineralization will maintain collagen structure in an undisturbed state

MATERIALS AND METHODS

Animals and Sample Preparation

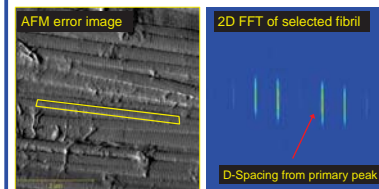
- Cortical bone from porcine femur
 - Ten 6 mm pieces mounted and polished
 - Randomly assigned to control / fix groups



- Control:** 20 min EDTA + 5 min sonication repeated 3 times, final 10 min EDTA
- Fixed:** 0.8% PFA, 0.2% GA in PBS at pH 7.4, 4°C for 24 hours
 - Same demineralization cycles as control bones
 - EDTA had 0.2% PFA and 0.05% GA
- Imaged five 3.5 μm² locations, 60-65 individual fibrils from each sample
- Tail tendon fascicles, 5 female C57BL/6 mice
 - Control:** Imaged wet, then dried and imaged again
 - Fixed:** Same fixation as bone
 - Imaged wet, then dried and imaged again
 - 2-3 location in 2-3 fascicles per tail
 - 5 μm² locations, 55-60 individual fibrils from each sample

Atomic Force Microscopy analysis

- Peak Force Tapping - error images analyzed
- 2D FFT on rectangular region of interest
- D-period determined from the first harmonic peak in the power spectrum



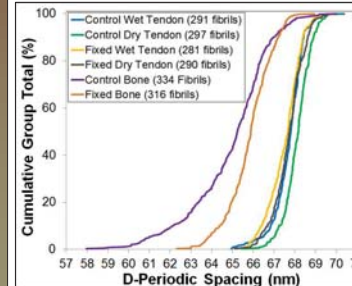
Statistical Analysis

- Bone Analysis
 - Sample values averaged; control vs. fix compared (n=5) with Mann Whitney U-test (since not independent samples)
 - Population distributions compared using Kolmogorov-Smirnov (KS) test
- Tendon Analysis
 - Sample values averaged (n=5 per group)
 - RMANOVA (before and after drying being the repeated measure)
 - Population distributions compared using KS tests

RESULTS

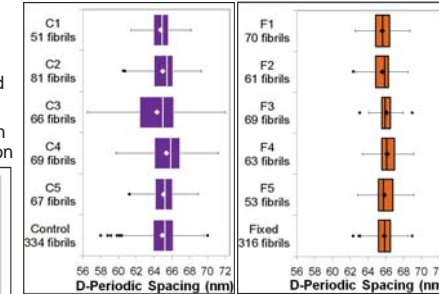
Fixation Effects in Bone

- Fixation ↑ mean D-spacing in bone (n=5, p=0.008)
- D-spacing range: Control 12.0 nm, Fixed 6.6 nm
- Fixed population shifted ↑ (p<0.001) with steep climb indicative of sharp distribution

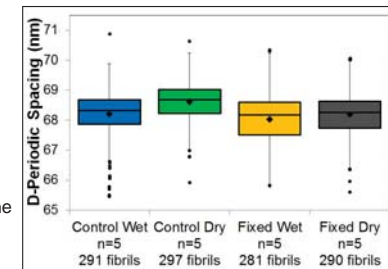


Fixation and Dehydration in Tendon

- Dehydration ↑ mean D-spacing in tendon (n=5, p=0.03)
- All 4 groups had narrower distributions vs. bone
- Unfixed: drying shifts D-spacing ↑ (p<0.001)
- Fixed: no Δ with dehydration (p=0.079)
- Wet Samples: significant effect of fixation (p=0.003)
- Dry Samples: fixation effect exacerbated (p<0.001)
 - driven by shift in unfixed samples upon drying
- Unfixed wet vs. fixed dry: no difference (p=0.181)



Control Bone: 64.9 ± 0.4 nm Fixed Bone: 65.8 ± 0.2 nm



Con Wet Tendon: 67.7 ± 0.2 nm
 Con Dry Tendon: 68.1 ± 0.3 nm
 Fixed Wet Tendon: 67.5 ± 0.3 nm
 Fixed Dry Tendon: 67.7 ± 0.4 nm

DISCUSSION

To image collagen in bone using AFM, removal of water and mineral is necessary

- To date, not possible to study D-spacing in hydrated bone - fibrils fail to exhibit D-spacing
- Mineralized bone contains little exposed collagen and requires demineralization
- Mild fixation to preserve collagen's structure - crosslinks lock structure in place
- ↑ shift in D-spacing similar to previously noted shift in diabetic tissues (effect of AGEs?)

Mild fixation allows for a study of baseline, undisturbed bone

- Tail tendon is a surrogate to define an undisturbed or native state
- Fixation mitigated the effects of dehydration
- D-spacing from fixed and dried tendon indistinguishable from wet unfixed fibrils - fixing and drying leaves collagen near its undisturbed and hydrated native state.
- In bone, mineral may alter the native state but data support that fixed and dried samples are an appropriate approximation of the native state.

Mineralized collagen in bone is under tensile pre-stress, has increased D-spacing

- Contradictory to studies suggesting compressive pre-stress and ↓ D-spacing
- D-spacing measured in individual fibrils, may capture aspects of ultrastructure that are lost using other techniques which average over a larger scale
- Water and mineral are not fully removed here as opposed to other studies

Potential Limitations

- Single bone from single animal - choice made to ↓ biological variability and ↑ chance of detecting the effect of interest.
- Important next step: comparing effects in group where D-spacing change is expected

Fixation in bone prior to demineralization decreases variability in collagen D-spacing which might enhance the chances of detecting changes in collagen due to disease or external stimuli.