# Nanomechanical Assessment Of Osteogenesis Imperfecta Collagen Fibrils In Indentation And Tension

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INTRODUCTION: The collagen superfamily is a diverse group of proteins constituting the main structural protein in human bodies. It features a complex hierarchical structure with striking flexibility in supramolecular organization while collagen type I being most abundant. At molecular level of type I collagen, two α1(I) chains and one α2(I) chain tightly pack into a heterotrimeric triple helix of 1.5 nm diameter and 330 nm length. On the next hierarchical level, collagen molecules stagger into the smallest microscopically discernible structural element of collagenous tissues, the collagen fibril (CF), which is about 50 nm to 300 nm in diameter. Due to the staggered formation of molecules, CFs feature a characteristic periodic D-banding repeating every 67 nm. In osteogenesis imperfecta (OI), or brittle bone disease, this structural order is adversely affected by mutations at the molecular level. While being well investigated on whole tissue level especially with bone tissue, little is known about implications on supramolecular assemblies like the CF.
In this study, we used a novel custom nano-tensile instrument, enabling high-throughput testing of CFs, and atomic force microscopy (AFM) to study structural and mechanical properties of CFs in a B6C3Fe-a/aCol1a2$^{oim/oim}$ model of severe OI. In the studied OI model, the α2(I) chain is substituted by a third α1(I) chain leading to defective supramolecular organization and homotrimeric type I tropocollagen molecules.

METHODS: A collagen fascicle was separated from a tail of each a wild-type B6C3Fe-a/a +/+ (WT) and a homozygous B6C3Fe-a/aCol1a2$^{oim/oim}$ mouse (model of severe OI), both 14 weeks old. Fascicles were opened up by use of sharp tweezers and smeared over a microscopy slide while maintaining hydration. This exposes single CFs. After drying in a desiccator, 50 µm magnetic beads made from NdFeB alloy were glued onto exposed CFs using a custom-made magnetic tweezer. Magnetic beads were picked up with the activated on magnetic tweezer, dipped into epoxy resin and placed on single CFs. About 100 µm along their long axis, the same CFs were glued onto the microscopy slide by a further droplet of epoxy resin. After curing, CFs were imaged with AFM in dry conditions to assess D-banding and CF dry diameter. Subsequently, CFs were submerged into 10x phosphate buffer saline solution (PBS). AFM based nano-indentation was performed to assess CF wet diameter and indentation modulus by applying the Oliver-Pharr method (PNP-DB cantilever, nominal force constant 0.48 N/m, NanoWorld). Without dehydrating, samples were then transferred to a custom tensile testing instrument. Tensile force is measured by an interferometric cantilever based force probe (force constant 4.73 N/m, Optics11) that is actuated by a 100 µm piezo lever stage (Physikinstrumente). The force probe's cantilever is equipped with a 3D-printed micro gripper about 150 µm x 100 µm x 50 µm (l x w x h) in size. For tensile testing, the end of the CF attached to the magnetic sphere is disengaged from the microscopy slide and lifted by applying a magnetic field with the magnetic tweezer. The now levitating magnetic sphere is picked up with the micro gripper. After pick up, tensile tests were performed to fracture at 5 % strain-rate.
Mice were maintained under standard laboratory conditions and experiments were conducted in compliance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines for reporting. Briefly, mice were housed up to 5 per cage in polypropylene cages with wood chip and paper bedding and provided purine rodent maintenance diet and water ad libitum throughout the study. All procedures complied with the US IACUC procedures and were reviewed and approved by IACUC local ethics committee of The City College of New York (New York, US).

RESULTS SECTION: In total, five WT and two OI CFs were so far assessed in this study. Due to low numbers, properties were not statistically compared. OI CF diameter in dried and hydrated state are well below WT diameter (72.2±3.6 nm vs. 132.8±5.3 nm, 133.1±9.3 nm vs. 249.4±10.6 nm respectively). Concurrently, swelling ratio (1.9 vs 1.9), being the ratio of hydrated over dry diameter, as well as indentation modulus (1.5±0.2 MPa vs. 1.4±0.3 MPa) seems similar for OI and WT CFs. In contrast, tensile testing results show vast differences between both groups. OI CFs rupture at 5-fold higher ultimate stress (13.7±2.8 GPa vs. 2.4±1.0 GPa) and ultimate strain (65.9±15.4 % vs. 15.3±3.7 %) compared to WT CF. Results are depicted in Table 1.

DISCUSSION: Vastly diverging ultimate stress and ultimate strain of OI CFs from WT CFs indicate a defective supramolecular organisation that has its origin in the substitution of the α2(I) chain. Generally, increased ultimate stress and ultimate strain correlates to higher density of intermolecular cross-links within CFs. Potentially, OI mice upregulate Lysyl oxidase activity to compensate for loss of structural organisation. Furthermore, decreased CF diameter of OI CFs may be explained by both, defective fibrillogenesis due to altered molecular structure and lower expression of the COL1A1 gene. It seems surprising that OI CFs are stronger and stiffer compared to WT CFs, however, this may be necessary to overcome putatively smaller overall amounts of collagen in the fascicles studied. Finally, how these properties propagate to mineralized fibrils is unknown.

SIGNIFICANCE/CLINICAL RELEVANCE: Understanding fundamental implications of osteogenesis imperfecta on supramolecular organization could enable the design of clinical treatments helping to improve life expectancy and quality of patients. High throughput tensile testing of CFs might become a potent tool to test effects of treatments on supramolecular level that is not possible with current methods in literature.

IMAGES AND TABLES:
Table 1: Grouped results of nano-indentation and tensile testing experiments. Physical properties are depicted in mean (standard deviation).

| group | # of fibrils tested | fibril diameter dry (nm) | fibril diameter hydrated (nm) | swelling ratio |
|---|---|---|---|---|
| WT | 5 | 132.8 (5.2) | 249.4 (10.6) | 1.9 |
| OIM | 2 | 72.3 (3.6) | 133.1 (9.3) | 1.9 |

| group | indentation modulus (MPa) | ultimate stress (GPa) | ultimate strain (%) |
|---|---|---|---|
| WT | 1.4 (0.3) | 2.4 (1.0) | 15.2 (3.7) |
| OIM | 1.5 (0.2) | 13.7 (2.8) | 65.9 (15.4) |

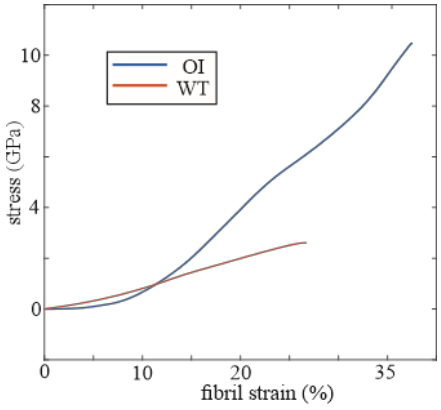

Figure 1: Exemplary stress-strain diagram of one OI collagen fibril and one WT collagen fibril.