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Synchrotron Chemical and Structural Analysis of *Tyrannosaurus rex* Blood Vessels: The Contribution of Collagen Hypercrosslinking to Tissue Longevity

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Recent descriptions of blood vessels recovered from dinosaur bones have raised many questions regarding tissue diagenesis and the associated chemical pathways that led to preservation. Previous analyses have identified preserved elastin and collagen proteins in a variety of specimens [1][2]. In particular, the mechanical, chemical, and thermal susceptibility of fibrillar collagen is partially dependent on the degree of intermolecular crosslinking. While collagen crosslinking can be either enzymatically or non-enzymatically driven in life, in death, only non-enzymatic pathways are available. Hence, non-enzymatic intermolecular crosslinking of fibrillar collagen supermolecular networks in fossil blood vessels has been suggested as a possible contributor to tissue longevity.

In the preliminary stages of this work, we investigated *Tyrannosaurus rex* (MOR 555) blood vessel tissue (Fig. 1a.) using a combination of synchrotron chemical and structural analyses. Preliminary findings with small-angle x-ray scattering (SAXS) suggested the retention of structural order: fibrillar type I collagen is explicitly identified by a 67 nm characteristic spacing, which was observed in both the *Tyrannosaurus rex* tissue and in demineralized modern chicken collagen, used as a control. Initial Fourier-transform infrared spectroscopy (FTIR) findings suggested the presence of hypercrosslinked collagen within the fossil tissue. Further chemical analyses also demonstrated the extensive and intimate association of nanocrystalline goethite (FeO(OH)) with the fossil vessels.

From these results, and based on similar findings in the literature [3], we identified two likely non-enzymatic intermolecular crosslinking mechanisms, Fenton's reaction and glycation, both of which rely on the oxidation potential of iron and the formation of a highly reducing agent (*e.g.*, radical or sugar). To investigate the possible roles of these mechanisms, we incubated fresh, demineralized chicken bone according to either of two corresponding treatments to induce fibrillar collagen crosslinking. Conversely, *Tyrannosaurus rex* tissue was treated with a reducing agent ($NaBH_4$) capable of cleaving low-order intermolecular crosslinks. All tissues were further investigated with the same set of synchrotron analyses, in addition to synchrotron micro-scanning x-ray fluorescence (μ-XRF) mapping and x-ray absorption near-edge structure (μ-XANES) spectroscopy. Additional images were collected using optical and electron microscopy techniques.

Demineralized chicken tissues incubated according to Fenton's reaction and glycation yielded products consistent with induced hypercrosslinking, as noted by shifting of the Amide I peak to higher energies. Both treated chicken tissues developed small ester peaks (approximately 1732 cm^{-1}); in comparison, a prominent ester peak was observed in all FTIR spectra collected for *Tyrannosaurus rex.* Fossil vessels

treated with the reducing agent yielded no significant changes in FTIR analysis, suggesting that the non-enzymatic crosslinks formed in this tissue are irreducible. Such bond formations occur between three or more peptide strands, and as such, tend to be highly resistant to reductive cleavage.

Iron maps produced by synchrotron μ-XRF presented visual evidence of the intimate association of iron-based nanocrystals with the *Tyrannosaurus rex* vessels. Additional analysis with μ-XANES demonstrated that the iron speciation was FeO(OH), or goethite, which is a commonly occurring mineral. The presence of this mineral form likely contributes to the red-brown hue that is typical of fossil vessel structures, including those studied here. Scanning electron micrographs (Fig. 1b.) generated using secondary electrons demonstrated the presence of three unique types of preserved vessel structures in this fossil specimen *(i.e.,* two types of mineral casts and preserved soft tissue). The surfaces of the soft-tissue vessels exhibited distinct striations with fiber dimensions consistent with the size of type I collagen fibrils. Transmission electron micrographs (Fig. 1c.) captured from resin-embedded thin sections of *Tyrannosaurus rex* vessels illustrated the multi-walled nature of these tissues, likely due to subsequent deposition events that occurred during early diagenesis. The external surfaces of the vessels also boast electron-dense nanocrystalline coatings, interpreted to be FeO(OH).

The results of this study yield three significant outcomes. One, a new set of characterization techniques (e.g., synchrotron SAXS, FTIR) was successfully applied to ancient soft tissues recovered from *Tyrannosaurus rex.* Two, hypercrosslinked type I collagen is present in these fossil vessel tissues and has likely contributed to tissue longevity (>65 million years). Three, for the first time, two possible Fe-catalyzed, non-enzymatic crosslink pathways were explicitly identified and tested on modern, demineralized chicken type I collagen; subsequent analyses of the treated tissues indicated that both pathways could have contributed to vessel preservation in *Tyrannosaurus rex,* or other species.

References:

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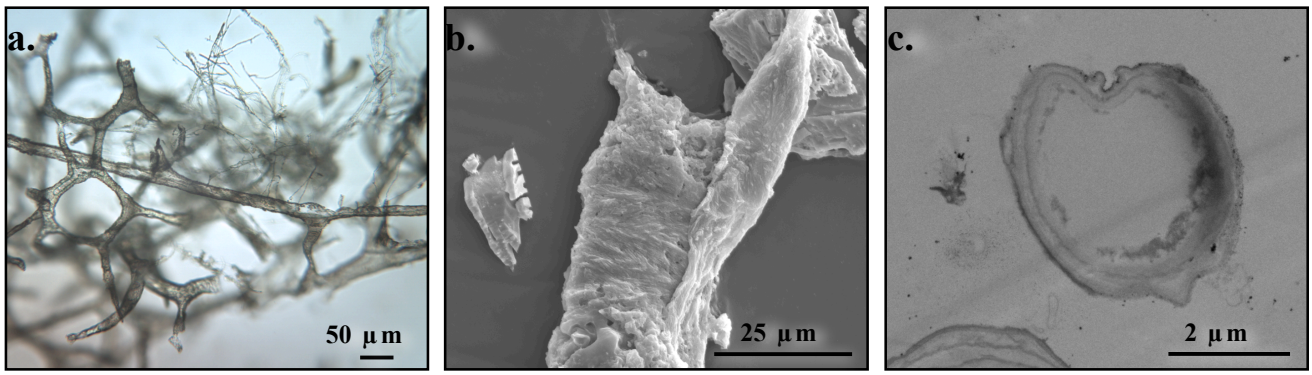

Figure 1a. Optical micrograph of *Tyrannosaurus rex* blood vessels recovered from demineralized bone, **b.** Scanning electron micrograph of an individual vessel structure, detailing fine striations on surface. Feature dimensions are consistent with the size of collagen fibrils. **c.** Transmission electron micrograph of a small resin-embedded vessel in cross-section. A fine layer of electron-dense nanocrystals is present on the vessel surface, interpreted to be the FeO(OH) observed using synchrotron analyses.

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