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Time-Resolved *In-Situ* Raman Spectroscopic Observations of a Biomineralization Model System

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The mechanisms underlying the formation of nanocrystalline apatite in vertebrate hard tissues (i.e. bones and teeth) remain nebulous. The long-held view of biomineralization via "classical crystallization theory", or ion-by-ion addition from solution, is challenged by "non-classical" theories in which the pathway proceeds through transient precursor phases [1]. The presence of an amorphous calcium phosphate (ACP) mineral precursor has been observed during the formation of zebrafish fin bones and dental enamel [2, 3]. However, the sample preparation necessary for electron microscopy and ethical considerations inherent to vertebrate samples preclude *in situ* observations of apatite mineralization.

To circumvent these obstacles, researchers have developed both *in vitro* and *ex vivo* models to study biomineralization. Nudelman et al. describes several *in vitro* biomineralization models, several of which have reported ACP in the presence of an organized, fibrillar collagen matrix [4]. The polymer-induced liquid-precursor (PILP) process is one of the first precursor-based *in vitro* model systems that is capable of reproducing a fundamental nanoscale building block of vertebrate hard tissues, the mineralized collagen fibril [5]. Despite a simplification of the complex biological environment, these *in vitro* model systems are subject to sample preparation limitations for reliable *in situ* measurements of ACP. Conversely, at least one group has used an *ex vivo* biomineralization model system to assess the presence, or absence, of transient apatite precursor phases during mineralization of mouse calvaria via Raman spectroscopy [6]. Chatzipanagis et al. recently used a static hydration chamber to collect Raman spectra *in situ* during a surface mediated transformation of citrate-stabilized ACP nanoparticles to hydroxyapatite (HA) in different solvents [7]. To the best of our knowledge, time-resolved *in situ* measurements of ACP-HA transformation in the presence of an organic matrix have not been performed

Here, we present proof-of-concept for a novel temperature-controlled flow cell designed for *in situ* collection of time-resolved Raman spectra to assess the dynamics of precursor based mineral transformations (Figure 1A). To accomplish this, a custom flow-cell, capable of maintaining a temperature of approximately 37 °C, was designed and built to fit within the stage of a Horiba Raman system. A single type-I collagen fiber, obtained from mature Sprague-Dawley rat-tail tendon, was suspended inside the flow-cell mineralization chamber and 60 ml of mineralization solution was injected through the system at a rate of ~5 ml per hour using a digital syringe pump. The mineralization media was a slightly modified PILP solution, namely the synthetic polymer "process-directing agent" was replaced with bovine milk osteopontin (OPN) at a concentration of 50 μg/ml [8, 9]. Time-resolved Raman spectra were generated with a 50X objective using a 532 nm wavelength laser at an output power of 20-25 mW with a spot size of ~ 1 μm. The signal was averaged over 10 scans for 15 seconds per each scan. Transmission electron microscopy images and corresponding selected area diffraction patterns were collected *ex situ* to confirm

the *in-situ* measurements and the presence of apatite. Time-resolved Raman spectra are shown in Figure 1B. The initial Raman spectra (t = 0 hrs) match that of native type-I collagen and show characteristic peaks related to the amide I and III bonds, C-H stretching, and proline side-groups. Around the 3.5 hour timepoint a distinct shoulder appeared at ~957 cm^{-1} which slightly attenuated the intensity of the collagen signal compared to the initial spectra. By the 5.5 hour timepoint the intensity from ν_1PO_4 stretching dominates the spectra, indicating a significant presence of phosphate. After 8.5 hours, the relative intensity of the ν_1PO_4 increased even further and the peak appeared to sharpen and shift to a higher wavenumber (~659 cm^{-1}) which may indicate a transition from ACP to HA.

In conclusion, *in situ* Raman spectroscopy facilitates time-resolved measurement of the increase in crystallinity and ordering associated with the transformation of a precursor phase to crystalline mineral [10]. However, to our knowledge, this is the first *in situ* demonstration of the ACP-HA transformation in the presence of a collagen matrix. Raman microscopy is increasing relevant to the biological sciences due the minimal sample preparation and the ability to collect measurements in hydrated environments. As correlative microscopy techniques continue to advance, the incorporation of an isolated, temperature-controlled flow-cell may provide opportunities to study simplified vertebrate biomineralization models or assess environmental effects (e.g. temperature, pH, etc.) on the formation of invertebrate biomineral exoskeletons, such as calcium carbonate-based corals and shells. Future work will employ computational peak decomposition to assess the amorphous and crystalline mineral fractions and determine the kinetics of transformation

References:

[1] S Weiner and L Addadi, Annual Review of Materials Research **41** (2011), p. 21.
[2] J Mahamid et al., Proc Natl Acad Sci U S A **105** (2008), p. 12748.
[3] E Beniash et al., Journal of structural biology **166** (2009), p. 133.
[4] F Nudelman et al., Journal of Structural Biology **183** (2013), p. 258.
[5] MJ Olszta et al., Connect Tissue Res 2003;44 Suppl 1:326-34, p.
[6] NJ Crane et al., Bone **39** (2006), p. 434.
[7] K Chatzipanagis et al., CrystEngComm **18** (2016), p. 3170.
[8] B Wingender et al., Matrix Biology, http://dx.doi.org/10.1016/j.matbio.2016.02.004(2016), p.
[9] B Wingender et al., Materials (Basel, Switzerland) **11** (2018), p.
[10] JA Stammeier et al., MethodsX **5** (2018), p. 1241.

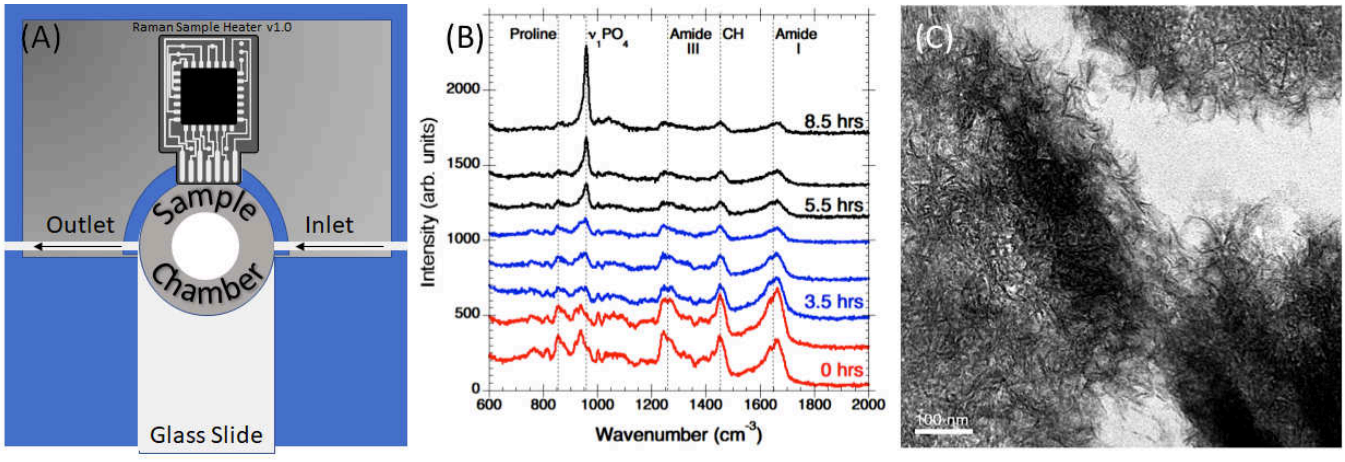

Figure 1. (A) Simple schematic of heated low-cell. (B) Time-resolved Raman spectra of ACP-HA transformation. (C) TEM micrograph of needle-like calcium phosphate precipitates (scale bar 500 nm).