Liquid Transmission Electron Microscopy Imaging of Organic-Inorganic Interfaces: Exploring Hydrated Collagen Mineralization Processes

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Collagen biomineralization is essential to the formation and maintenance of hard tissues like bone and teeth. A calcium phosphate (CaP) mineral is the principal inorganic constituent of collagenous mineralized connective tissues, whereby the amount and distribution of CaP contribute to the mechanical properties of these tissues [1]. Thus, understanding collagen mineralization offers several advantages for medical research, such as improving mineralized tissue regeneration and eliciting new treatment pathways for hard-tissue diseases. Traditional and cryo- transmission electron microscopy (TEM) techniques have been used to elicit crucial theories on collagen mineralization within the last few decades [2-5]. However, while studied extensively, collagen mineralization is not fully understood. Notably, the aforementioned techniques only provide time-stamp views in static, dehydrated or frozen conditions, non-representative of the dynamic liquid-state environment where reactions occur in real-time. Recent manufacturing advances in thin-film microchips are pushing imaging boundaries through the advent of a new emerging characterization technique: *liquid-TEM*. The room-temperature parallel technique to cryo-TEM, liquid-TEM provides a high-resolution opportunity to explore reactions in real-time through the encapsulation of a liquid sample between microchips [6]. Combined with conventional TEM characterization workflows, liquid-TEM offers a dynamic liquid correlative approach to aid in validating mineralization theories, offering unparalleled characterization insight into organic-inorganic interfaces. Despite this potential, limited researchers have used these methods to study organic-inorganic interfaces. To our knowledge in literature, none have studied native, hydrated collagen mineralization using liquid-TEM.

Herein, this research aims to get a better insight into collagen biomineralization pathways through the exploration of a new, facile liquid-TEM method. A hybrid thin-film enclosure is adapted from works by DiCecco et al. [6] for this collagen system, where a liquid solution is sandwiched between a silicon nitride (SiN_x) microchip with a window membrane thickness of 10 nm (SiMPore Inc., NY, USA) and a 400-mesh Au TEM grid with a 5 nm thin carbon layer (Electron Microscopy Sciences, PA, USA) (Fig. 1). This enclosure is quick and easy to put together and requires limited specialized equipment, providing a far less expensive manner to perform liquid-TEM than commercially available options. Bright field TEM is used for characterization with a Talos 200C (ThermoFisher Scientific, MA, USA) operated at 200 kV with a CETA camera (ThermoFisher Scientific, MA, USA) and a Direct-View direct electron detector (Direct Electron, CA, USA), where electron dosage varied between 40-55 e/Å^2 per acquisition. Automated acquisition using SerialEM [7] (University of Colorado, CO, USA) improved imaging efficiency by

increasing acquisition throughput with automation and facilitating sample screening through low-magnification mapping, which aided to reduce electron beam exposure during screening.

An established rat-tail tendon reconstituted collagen mineralization model was used as a mimic to type I collagen found in hard tissues within the human body [8,9]. Collagen is mineralized in biomimetic conditions with exposure to CaP and polyaspartic acid (pAsp). pAsp is a common additive in *in vitro* collagen biomineralization models which acts as a mimic to soluble non-collagenous proteins found in natural mineralized collagenous tissues [8]. As a nucleation inhibitor, pAsp stabilizes CaP systems and delays extrafibrillar mineralization events, which have been found to promote intrafibrillar collagen mineralization [8]. The final aqueous-based mineralization solution consisted of collagen fibrils (~0.1 mg/ml), pAsp (25 μg/ml) (Alamanda Polymers, Mw 14 kDa), 125 mM NaCl, 1.7 mM, $CaCl_2$, and 9 mM Na_2HPO_4, buffered with 50 mM Tris (pH 7.4, 37°C). Reactions were performed *in vitro* at 37°C in a water bath mixed at 100 rpm, where time points of interest were studied from the bulk solution for liquid-TEM.

Early (~0-6 hours) to more mature (~6-18 hours) collagen fibril mineralization periods were successfully probed for characterization using liquid-TEM. At early mineralization, the majority of fibrils appeared unmineralized and had diffuse contrast within their surroundings, where small particulate matter could be observed in solution and adjacent to fibrils (Fig. 2A). Signs of mineralization were noted among select fibril regions at early time points, where fine mineral platelets could be visualized aligned in parallel along the long axis of a small minority of collagen fibrils (Fig. 2). Selective electron area diffraction of mineralized collagen regions highlighted fine diffraction (002) and (004) arcs, characteristic of the formation of CaP apatite crystals formed preferentially along the fibril long axis. In more mature collagen mineralization periods past the ~ 6-hour mineralization period, most of the fibrils present appeared to show signs of mineralization, gaining higher contrast in solution with the presence of CaP apatite crystals. Observations made in liquid-TEM were in good agreement with correlative imaging used involving dry conventional TEM preparation methods. This work provides an innovative approach for studying CaP collagen mineralization systems using liquid-TEM, laying a foundation for studying inorganic-organic interfaces in native, hydrated conditions and for the study of dynamic liquid experiments in future [10].

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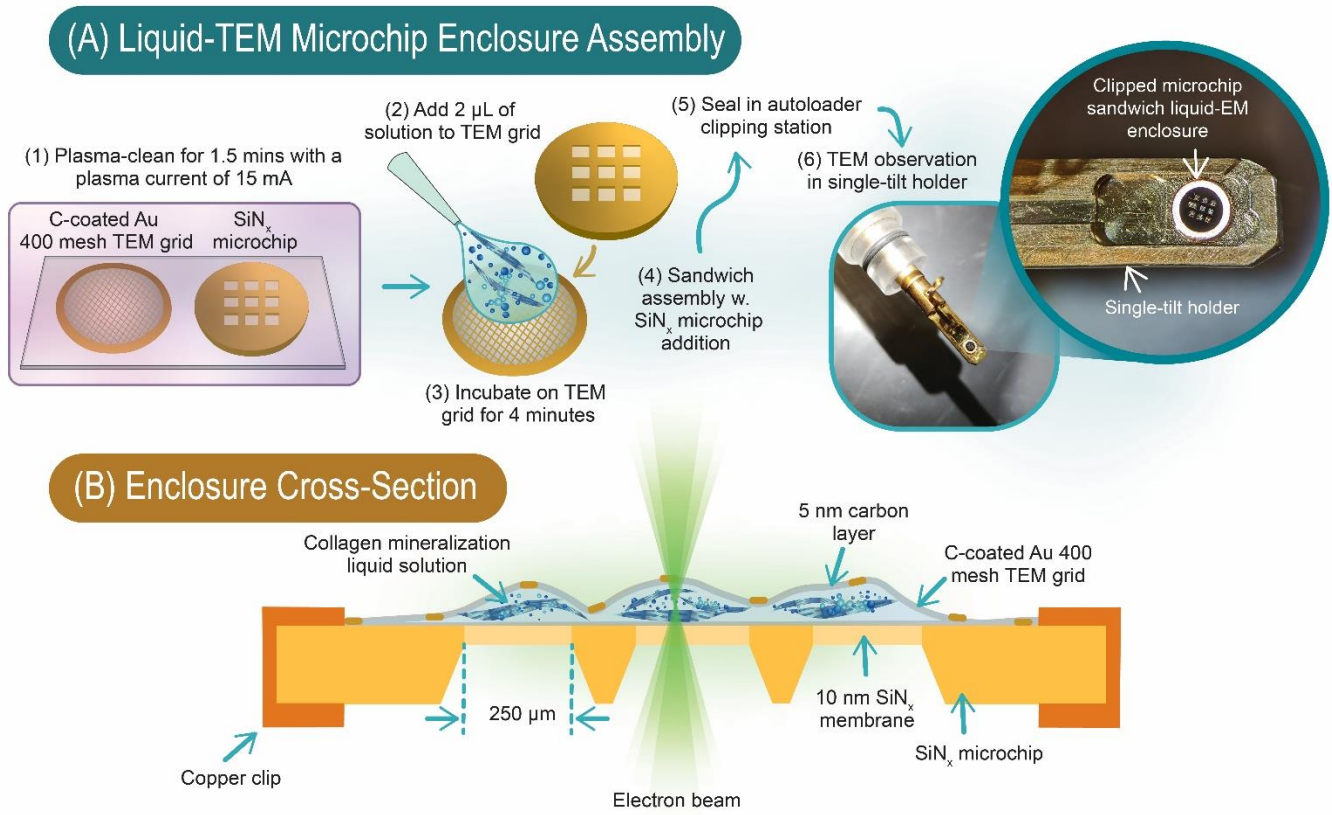

Figure 1. Liquid-TEM microchip enclosure overview. (A) Assembly process of the liquid-TEM microchip enclosure used for this research. (B) Enclosure cross-section with key dimensions.

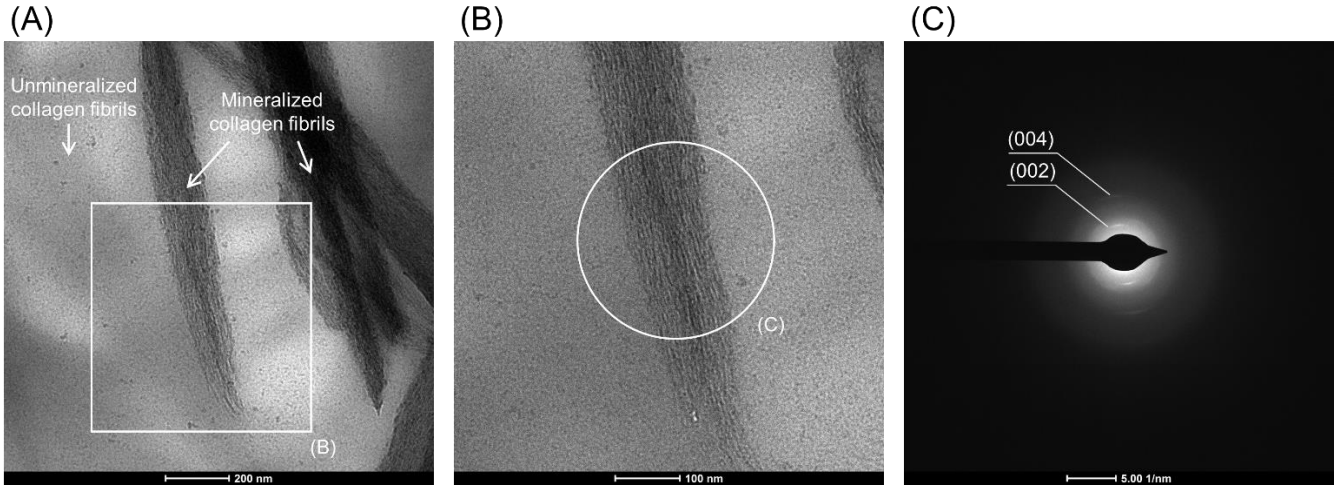

Figure 2. Representative bright field liquid-TEM imaging of collagen fibrils mineralized for approximately 4 hours in the presence of pAsp. (A) Overview of representative region, with callouts highlighting darker collagen fibrils which appear mineralized in comparison to more diffuse regions of unmineralized collagen fibrils in the background. (B) Magnified view of collagen fibril where mineral platelets can be visualized. (C) Selective electron area diffraction pattern of mineralized region enclosed in (B) highlights CaP apatite crystals have formed preferentially along the long-axis of the collagen fibrils, with characteristic (002) and (004) arcs noted.