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Exploring Calcium Phosphate Biomineralization Systems Using *In Situ* Liquid Phase Electron Microscopy

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Classically, the biomineralization field has been dominated by traditional and cryogenic electron microscopy (EM) means of characterization. While these methods work well for hypothesizing theories, observing dynamic interactions in biomimetic systems are necessary for validation. Recently, nucleation and growth processes of calcium phosphate (CaP) were revealed dynamically using novel liquid phase EM (LP-EM) [1-3]. However, limited real-time LP-EM research has considered the influence of additional biomolecules relevant to the study of hard tissues such as teeth and bone, where CaP is the primary inorganic constituent. Polyaspartic acid (pAsp) is a synthetic mimic of the soluble non-collagenous proteins found in natural mineralized collagenous tissues and is used extensively in *in vitro* models of collagen mineralization [4]. pAsp acts as a solution nucleation inhibitor, stabilizing CaP systems to delay solution mineralization events, thus promoting intrafibrillar collagen mineralization [4]. Other biomolecules of interest include DNA nanostructures, which can be used as templates for CaP mineralization and provide controllable self-assembled 1D, 2D, and 3D nanostructures [5].

In this work, novel *in situ* LP-EM means are used to expand our understanding of these CaP systems to highlight first-time dynamic observations of CaP mineralization with exposure to pAsp and to shed light on how DNA nanostructures mineralize in CaP systems. For *in situ* trials, the Poseidon Select holder (Protochips) in a silicon nitride (SiN) based microwell configuration was used in a Talos 200X (Thermo Fisher Scientific) operated at 200kV in bright-field (BF) mode with an electron dose rate varying between 0.40-0.75 $e^-/Å^2s$ (Fig. 1). Correlative mineralization *ex situ* was characterized after deposition onto holey or thin-filmed carbon-coated 200 mesh Cu grids. *In situ* observations captured dynamic mineralization interactions and the formation of nucleation clusters (Fig. 1C-2). For the pAsp sample, the solution remained stable *in situ* throughout the study duration (6 hours). Mineralization correlated well with *ex situ* observations where selected area electron diffraction (SAED) paired with electron energy loss spectroscopy (EELS) confirmed amorphous-CaP products were formed that are calcium-based (Fig 2). Here the solution consisted of pAsp (25 mg ml^{-1}), 125 mM NaCl, 1.7 mM, $CaCl_2$, and 9 mM Na_2HPO_4, buffered with 50 mM Tris (pH 7.4, 37°C). Preliminary observations of DNA nanostructures functionalized with peptides in CaP solution *in situ* feature nanoparticle clusters aggregating around DNA templates (Fig. 1C-1). SAED indicated crystalline nanostructures *post situ* corresponded well to structures observed *ex situ*. The DNA mineralization solution consisted of pAsp-incorporated DNA nanostructures (0.03 mg ml^{-1}) buffered with 1.25 mM Ca solution (pH 8) and a 10 mM phosphate solution (pH 7.4).

While this work showcases first-time insights provided by LP-EM by successfully capturing real-time biomineralization dynamics, some drawbacks to the technique remain. These include difficulties in optimizing and translating experimental parameters from large *ex situ* to small *in situ* volumes as well as electron beam effects. Recently published research highlights improved automated acquisition and data collection methods that offers insightful new avenues to address these shortcomings [6]. This work lays the foundation for probing real-time mineralization of CaP systems, notably of collagen under the influence of pAsp and DNA biomineralized nanostructures [7].

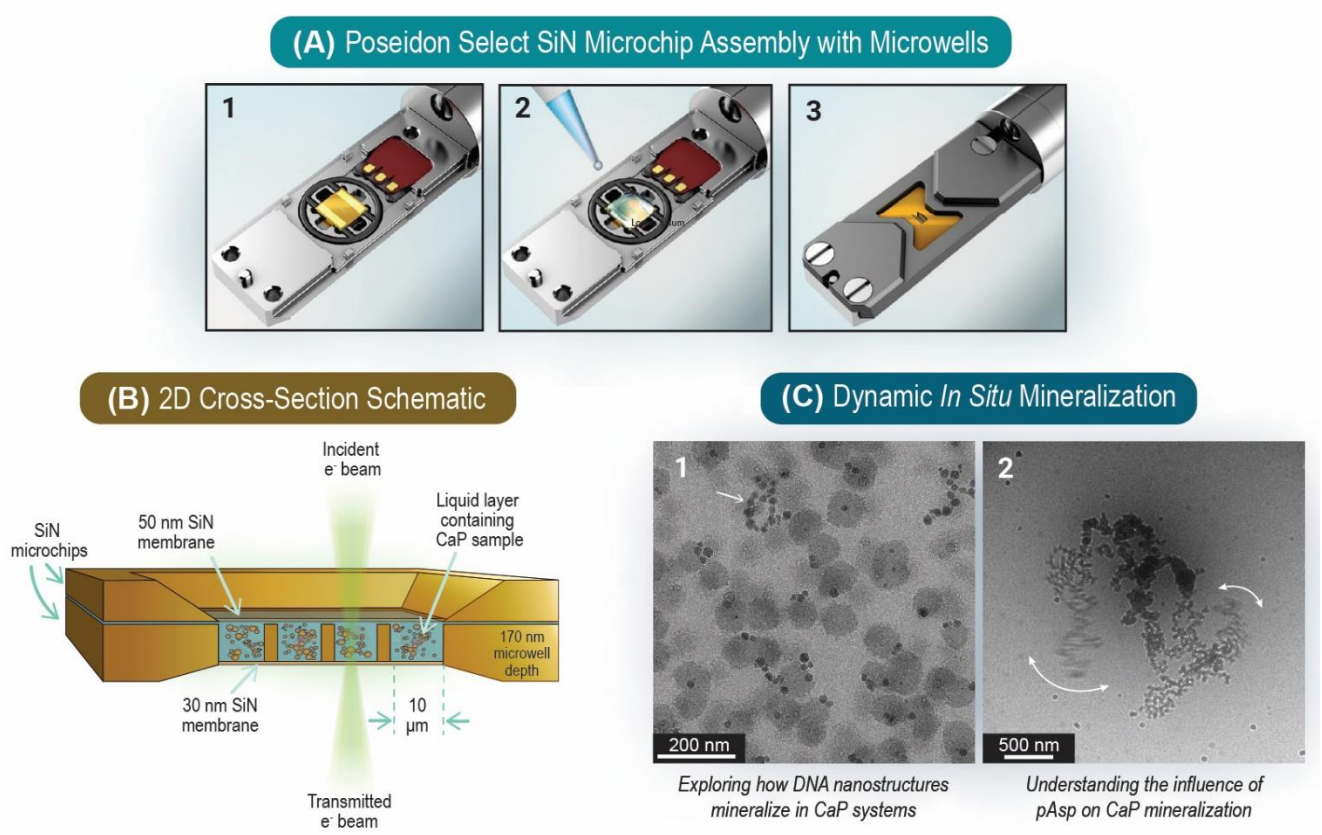

Figure 1. LP-TEM CaP biomineralization system configuration and visualization. (A) Poseidon Select (Protochips) SiN assembly configuration showing (1) bottom EPB-55A-10 microchip (300 x 90 membrane window, 50 nm thick), (2) solution addition varying between 0.25-1 µL, and (3) loading of top EPT-42A1-10 microchip with integrated microwells (array of 8 x 16 wells; 10 µm x 10 µm in viewing size, 30 nm thick). (B) 2D cross-section schematic of the SiN microchip assembly. (C) BF TEM dynamic *in situ* mineralization captured of hydrated CaP systems, with motion highlighted in regions by arrows of (1) DNA CaP nanostructure system and (2) the pAsp CaP mineralization at a 4-hour time point.

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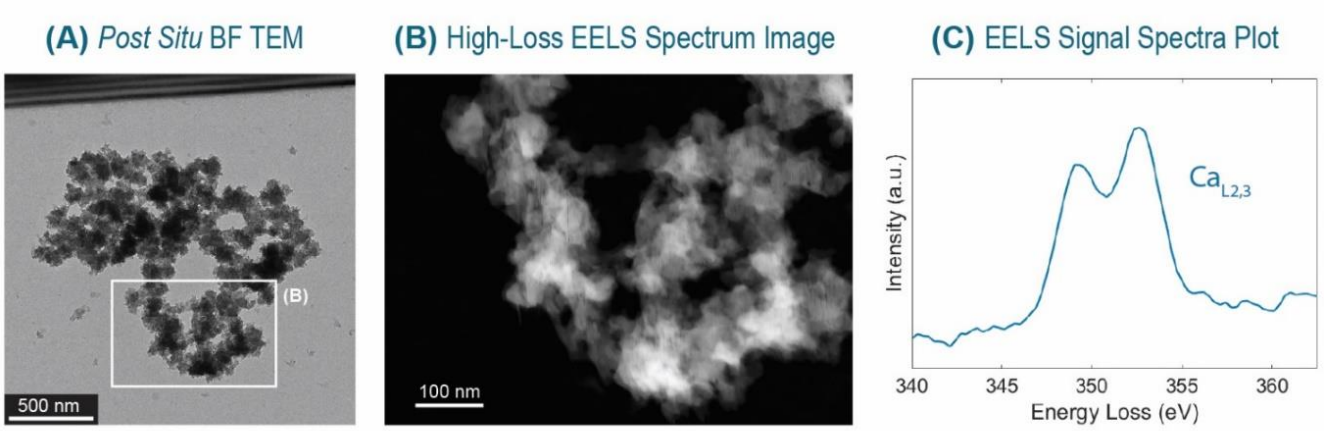

Figure 2. *Post situ* characterization of *in situ* CaP-based mineralization products after 6 hours in the presence of pAsp. (A) BF TEM acquisition of survey region with product adhered to the SiN membrane. (B) High-loss EELS spectrum image collected from the enclosed box in (A). (C). EELs signal spectra plot of the overall region highlighting that the products visualized as are Ca-based where the Ca-$L_{2,3}$ edge is identified.

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