

Biom mineralization of Hydroxyapatite Revealed by *in situ* Electron Microscopy

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Hydroxyapatite (HA) is the principal mineral component of human bones and teeth. Within these mineralized tissues, HA distributes itself in a highly organized arrangement in a fibrous collagen matrix. Although many bone mineralization mechanism theories have been put forward based on *in vitro* models, many questions such as the formation of an amorphous calcium phosphate (ACP) intermediary, and the functions of non-collagenous proteins (NCPs) on crystals nucleation and subsequent growth, still remain due to the lack of real-time experimental evidence [1]. Liquid-phase transmission electron microscopy (LP-TEM), with the ability to record biom mineralization process *in situ* with nanoscaled spatial resolution and sufficient temporal resolution, is a promising technique to unveil bone mineralization mechanisms. The formation of intermediate amorphous phases has been noted *in situ* for simpler mineralized systems, such as calcium carbonate [2]. However, understanding the mechanisms of more complex HA mineralization is critical for both pathological biom mineralization research and biomimetic material synthesis related to humans.

In this work, an HA biom mineralization solution was placed in a static liquid cell inside the TEM to observe HA particle nucleation and growth. A JEOL 2010F was operated at 200 keV in combination with a commercially available liquid cell TEM holder (Poseidon 500, Protochips Inc., USA). A small amount (0.2 μL) of HA biom mineralization solution was sealed between stock microwell Poseidon E-chips with SiN windows. The solution was made up of two parts: 1) a phosphate solution (9.5 mM Na_2HPO_4 in 125 mM NaCl, 50 mM Tris, pH 7.40), and 2) a calcium solution (1.7 mM CaCl_2 in 125 mM NaCl, 50 mM Tris, pH 7.40) [3]. These two solutions were mixed and filtered through a 0.2 μm acrodisc syringe filter right before sealing in the liquid TEM holder. The electron beam dose rate was estimated at $2.198 \times 10^3 \text{ e}/\text{\AA}^2 \text{ s}$. A thinner liquid region near the edge of the microwells was used for imaging. After the *in situ* experiment, the E-chips were removed from the liquid TEM hold and dried for further *ex situ* characterization via X-Ray Photoelectron Spectrometry (XPS) and electron energy loss spectroscopy (EELS).

In scanning-TEM bright-field imaging mode (BF-STEM), tiny particles with an average diameter of around 10 nm appeared after 2 minutes. With increasing time, more particles appeared and these had the ability to move and aggregate with each other to generate clusters instead of growing into bigger particles individually (Figure 1). These particles were confirmed as HA crystals via *in situ* selected area diffraction patterns in TEM mode, and also supported by complementary *ex situ* characterization with EELS (Figure 2) and XPS.

We present the first real-time imaging of HA nucleation and growth with *in situ* LP-TEM. This is the first step in understanding HA biom mineralization in more complex organic–inorganic physiological solutions. This work lays the foundation for further investigation of HA nucleation in the presence of NCPs, and for the exploration of remineralization of extracellular matrices such as collagen *in situ*.

References:

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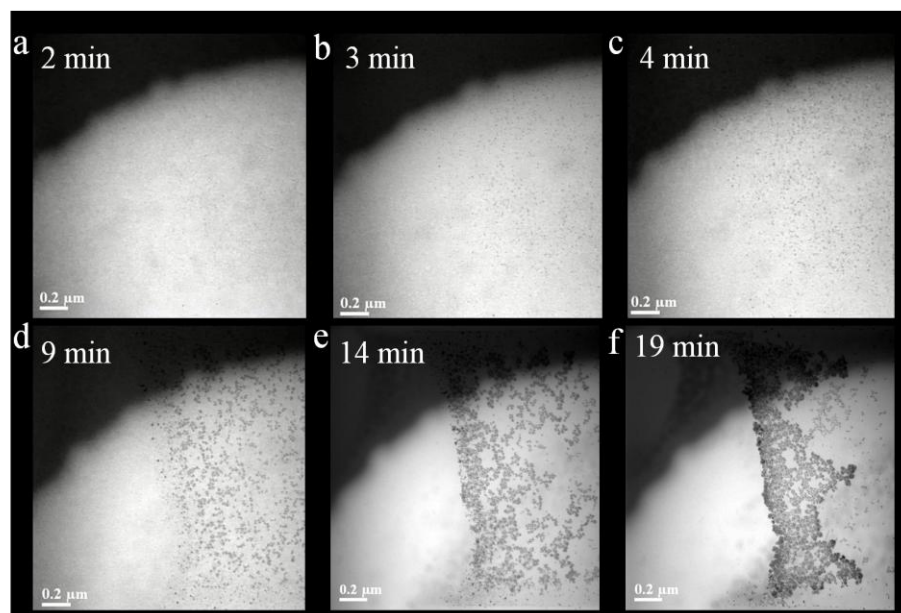


Figure 1. *In situ* BF-STEM images showing initial nucleation and growth of HA particles over 20 min.

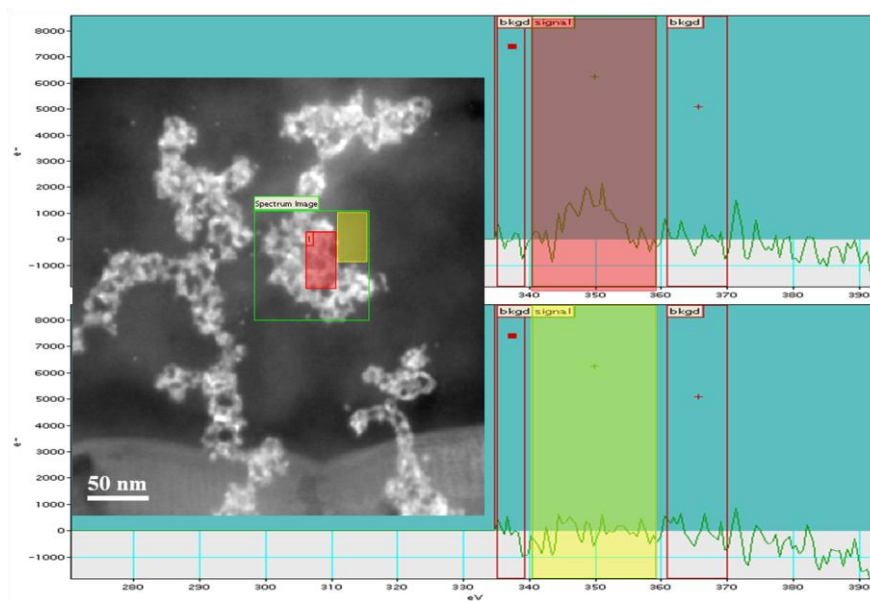


Figure 2. The mineralized product was characterized via EELS. The Ca edge was identified inside mineralized particles (red), and absent in the surrounding regions (yellow). Selected area electron diffraction, and XPS confirmed the chemical structure of these Ca-rich particles to be hydroxyapatite.