

RESEARCH ARTICLE OPEN ACCESS

Dynamic Observation of Lacy Pattern Formation in a Bone Tissue Model Using Liquid Phase Scanning Transmission Electron Microscopy

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Received: 11 August 2026 | **Revised:** 11 September 2026 | **Accepted:** 17 September 2026

Keywords: biomineralization | in vitro bone model | inpainting | liquid phase electron microscopy | low-dose STEM | sparse imaging | sub-sampling

ABSTRACT

Bone is a hierarchical composite in which carbonated hydroxyapatite platelets form a continuous mineral network within and between collagen fibrils. Although the nanoscale organization of mature bone is well characterized, how mineral precursors transform into this architecture remains unresolved because conventional electron microscopy provides only static images. Here, a low-dose liquid-phase scanning transmission electron microscopy (LP-STEM) workflow is established to visualize collagen mineralization dynamically in hydrated bone tissue. Sub-sampling 20% of the pixels in the image and inpainting-based reconstruction reduce the electron dose five-fold while preserving approximately 4 nm spatial resolution. Time-resolved imaging reveals the deposition and growth of poorly-defined mineral clusters, their development into spherulitic structures, and their subsequent reorganization into crystalline branches separated by unmineralized regions. Our observations demonstrate that the characteristic unmineralized areas in the lacy mineral pattern emerge during crystallization rather than through mineral deposition around a pre-existing organic template. The workflow enables nanoscale investigation of beam-sensitive hydrated biological materials and provides a platform for studying physiological and pathological mineralization processes.

1 | Introduction

Bones are primarily composed of collagen type I, carbonated hydroxyapatite minerals, and non-collagenous proteins. As part of the human skeleton, bones provide structural support, protect internal organs, and assist in movement. To fulfill these roles, both the composition and the hierarchical structure of the material are important, necessitating precise control over the formation process [1]. In bone, the mineral is found inside and

between collagen fibrils, i.e., intra-fibrillar [2] and inter-fibrillar [3] crystals, respectively. These mineral types were demonstrated to be chemically distinct [4], while both contribute to a continuous cross-fibrillar mineral network [5]. To relate form with function, many high-resolution electron microscopy studies have been devoted to discerning the three-dimensional microstructure of bone [5]. In these, the observed features depend on the orientation of the collagen fibrils relative to the imaging plane. When viewed perpendicular to the long axis of collagen fibrils, the

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characteristic 67 nm D-banding pattern of collagen and aligned mineral plates are observed [6]. While when viewed parallel to the collagen fibrils, no D-banding pattern is evident, and the mineral is observed as a so-called “lacy pattern” [5].

Despite the well-defined microstructure [5], details of the mineralization process itself remain ambiguous due to a lack of dynamic nanoscale imaging capabilities. Investigations of partially calcified turkey tendon, zebrafish bone, and human bone suggest that matrix mineralization is cross-fibrillar [7, 8], initiating from an amorphous precursor phase [9] and proceeding in radial (spherulitic) growth patterns. Eventually, these spherulites (also called ellipsoids [10, 11] or tesselles [12]) merge and form a continuous network [12]. However, the reliance on static images has led to different interpretations on how the transformations between states actually occur. In particular, discussion continues on how the lacy pattern forms and what fills the unmineralized spaces in it [7, 12]. From studies on turkey tendon, Macías-Sánchez et al. suggest these spaces are filled with intra-fibrillar molecular complexes that impact the arrangement of the crystals in the fibril [7], while Micheletti et al. suggest that in human bone these spaces are collagen fibrils that template mineral growth [8].

To obtain a full picture of the mineralization process, we must observe it in real time at the nanoscale. Over the past decade, liquid phase electron microscopy (LP-EM) has been established as a high-resolution technique to study dynamic, real-time processes of inorganic materials in aqueous media within electron-transparent enclosures [13]. The method has also shown promise for the observation of soft-matter and biological materials despite the inherent beam sensitivity and low contrast of organic samples [14–19]. Nanometer spatial resolution (4 nm) was achieved in LP-EM imaging of eukaryotic cells using a high electron dose ($740 \text{ e}^- \text{ \AA}^{-2}$) that is significantly above the damage threshold for biological systems [20, 21]. More recently, we used highly electron-transparent graphene liquid cells (GLCs) to support high-resolution (2 nm) imaging at low electron doses ($0.1 \text{ e}^- \text{ \AA}^{-2}$) needed for imaging biological processes [22, 23]. However, the small volumes of these enclosures prevent the observation of processes in their native environment (e.g. in a cell, matrix, tissue).

In the current work, we demonstrate a low-dose LP-EM workflow, allowing the first dynamic nanoscale observation of in vitro collagen mineralization in a bone tissue model. Using sparse imaging, we achieve a five-fold reduction of the electron dose without significant loss of image quality or the creation of visible beam-induced artifacts. Dynamic imaging at 4 nm resolution and an electron dose of $0.3 \text{ e}^- \text{ \AA}^{-2}$ confirms that in our model, collagen mineralization proceeds via deposition of a disordered precursor phase and shows for the first time how the characteristic lacy pattern is formed.

2 | Results

2.1 | Workflow Design

In order to study in vitro collagen mineralization, bone tissue was demineralized (Figure 1a) and snap-frozen in liquid nitrogen.

Cryogenic ultramicrotomy was used to prepare thin tissue sections (200 nm, Figure 1b) that were placed on either transmission electron microscopy (TEM) grids or on the SiN_x chip windows of an LP-EM flow cell (Figure 1c). This sample preparation strategy preserves the native physical arrangement of a multitude of collagen fibrils and their non-collagenous proteins, and was shown previously to replicate both spatial control of mineral deposition and the matrix-mineral relationships of natively mineralized collagen fibrils [25–27]. Mineralization was carried out using a biomimetic solution containing poly(aspartic acid) as the crystallization control agent, resulting in the reproducible reconstitution of the complete three-dimensional structure of bone [28, 29] in line with our and others' earlier work (Figures S1 and S2) [6, 27, 30, 31].

Scanning transmission electron microscopy (STEM) tomography on the mineralized sections confirms the reconstitution of the two previously described mineral patterns in bone: [5] the filamentous and lacy patterns (Video S1), visible for different orientations of the matrix structure with respect to the electron beam. This reaffirms mineralization of the extracellular matrix at the nanometer length scale is driven by physiochemical factors and can be achieved using a biomimetic bone tissue model (Figures S1 and S2) [27]. The samples on TEM grids were used to establish imaging parameters, determine the expected duration of mineralization, benchmark the use of sparse imaging for these samples and low electron doses, and confirm the expected microstructure with tomography as mineralization progresses.

Bulging of the SiN_x windows in the LP-chips often produces liquid layers approaching 1 μm in thickness (Text S1, Figure S3, Table S1), favoring STEM that can image micrometer-thick samples in contrast to TEM [19, 32, 33]. We perform the mineralization during liquid flow (i.e., in a relatively large volume of liquid $\sim 1 \mu\text{m}$, Text S1), but purge the flow cell before recording each image using the back pump of the liquid flow system. This practice leaves a thin $\sim 40 \text{ nm}$ hydration layer (Text S1) around the tissue section, reducing the effect of the liquid layer thickness on resolution (Figure S3) while maintaining hydrated conditions.

Imaging a process requires the repeated exposure of the sample to the electron beam, thereby dictating extremely low electron doses per image to maintain an accumulative dose below the visual damage threshold ($1.5 \text{ e}^- \text{ \AA}^{-2}$, Figure 2a–d) [21]. To minimize the electron dose while maintaining a high enough signal-to-noise ratio, we apply sub-sampling and inpainting to achieve a total dose of $\sim 1 \text{ e}^- \text{ \AA}^{-2}$ [34]. With this method, only part of the pixels in a field of view are imaged, after which the complete image is reconstructed using a beta-process factor analysis algorithm [24]. This algorithm approximates a dictionary of latent features learned from a sub-sampled image and reconstructs the complete image using sparse coding [24]. Sub-sampling can also mitigate the beam damage by increasing the distance between the imaged pixels, reducing beam overlap and diffusion of beam damage (e.g. due to knock-on damage and radiolysis) [35]. Using dry, on-bench mineralized thin sections of bone tissue placed on TEM grids, we determined and validated the STEM imaging parameters (Text S2 and Table S2) required to image individual hydroxyapatite mineral platelets in hydrate bone samples ($< 4 \text{ nm}$ spatial resolution and $> 800 \text{ nm}$ depth of focus) (Figure S3) [32].

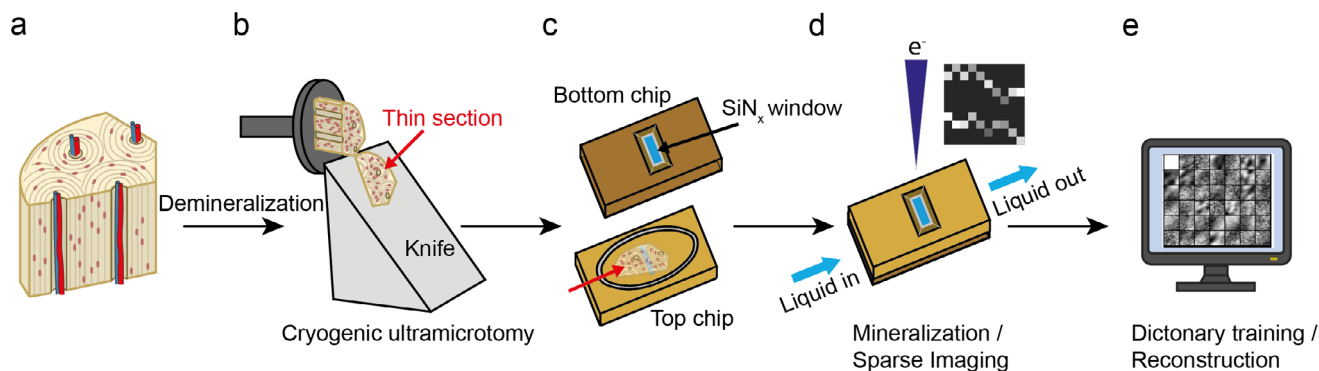


FIGURE 1 | A schematic of the low-dose liquid phase scanning electron microscopy (LP-STEM) workflow using sub-sampling and reconstruction. (a) A sample of bone is demineralized in a solution of ethylenediaminetetraacetic acid (EDTA), and (b) cut into 200 nm thin sections (red arrow) using a cryogenic ultramicrotome. (c) The sections (red arrow) are placed on the SiN_x window of the top LP-chip, and the chip is sealed. (d) Biomimetic mineralization is flowed through the chip using a dedicated flow system. Sub-sampled images are acquired in STEM mode with the beam scanned in a “linehop” regime [24]. Lastly, (e) the complete image is reconstructed by inpainting.

2.2 | Establishing a Sparse Imaging Workflow

An overview of the entire SiN_x window imaged at low magnification (13.9 nm/pixel; 0.003 e⁻ Å⁻²/image) shows regions with different collagen orientations in the hydrated section (Figure 2a and Figure S4). For areas where the *c*-axis of the collagen (i.e., the long axis of the fibril) was perpendicular to the electron beam, the characteristic 67 nm banding pattern of collagen was visible at higher magnification (1.2 nm/pixel) in both the fully (Figure 2b, 1.5 e⁻ Å⁻²/image) and 20% sub-sampled (Figure 2c, 0.3 e⁻ Å⁻²/image) images. Full sampling at 1.5 e⁻ Å⁻² visibly damaged the organic matrix (Figure 2d white bracket), whereas 20% sub-sampling, a five-fold reduction of the electron dose, did not show any visible evidence of beam damage (Figure 2d white bracket). We found that reconstructions of sub-sampled images with less than 20% of pixels sampled had lower spatial resolution than required to resolve single mineral plates (4 nm), and therefore we chose to proceed with 20% sub-sampling (Figure 2e and Figure S5). We note that in studies where 10 nm resolution is sufficient, 10% sub-sampling (Text S3 and Figure S5a) could provide additional temporal resolution or the study of longer processes.

To validate the sub-sampling approach, reconstructions were compared with fully sampled images and also with denoised (Gaussian blur, $\sigma = 2.0$) images as inpainting is inherently also a denoising method [34]. We combined image quality metrics, each of which reflects a different aspect of the image (for details see Text S4, Figures S6 and S7, and Table S3) and visualized the results using custom radar plots. The quality of a reconstructed image is represented by a polygon and its area (*A*) (Figure 2f_{I-IV}). The reconstructions (Figure 2f_{II}) had comparable quality (*A*) with their fully sampled denoised counterparts (Figure 2f_{IV,f_V}). More specifically, the radar plots show that reconstructions had more noise and lower spatial resolution, but higher structural fidelity with the observed pixels in the sub-sampled image (Figure 2f_I). To exclude hallucinations or structural bias introduced by AI-based inpainting and to further validate its use in electron microscopy, we show that reconstructions generated from the same image using different sampling masks exhibit

high fidelity with one another and to the fully sampled image (Figure S7).

2.3 | Dynamic Visualization of Collagen Mineralization

After establishing the sparse imaging STEM workflow, we apply it to the dynamic imaging of collagen remineralization of demineralized bone tissue. We note that mineral precipitation is a stochastic process, therefore, the timeline of mineralization was not uniform over the entire tissue section (Figure S1). Overview bright-field (BF) detector images (Figure 3a) show the growth and densification of the mineral phase, while the dissimilarity analysis (Figure 3b) indicates where the mineralization progresses (green/yellow areas). Initially, small cluster (<500 nm) randomly appear at $t = 120$ min (Figure S8). In one region (Figure 3c), after 180 min, mineral is deposited as poorly-defined spherulitic mineral clusters (Figure S9), which span over relatively large areas (>1 μm²) (Figure 3c_I). 20 min later, we observe the formation of mineral branches with non-mineralized spaces between them (Figure 3c_{II} red arrows). These non-mineralized spaces developed in the exact same area that was previously fully covered by the poorly-defined clusters (Figure 3c_I). The spherulitic mineral growth patterns indicate that collagen in this location is orientated parallel to the electron beam (transverse cross-section, Figures S4 and S9) [7], which explains the absence of the 67 nm banding pattern. The presence of a collagenous matrix was confirmed by a combination of optical imaging and Raman microspectroscopy (Text S5 and Figure S10).

In another region (Figure 3d–f), initial evidence of mineralization appears after 220 min. Higher magnification images show initial mineral clusters with poorly-defined microstructures (Figure 3d_I). These clusters grow and merge (Figure 3d_{II-III}), eventually forming spherulitic patterns (Figure S8e). After 310 min, we start to resolve needle-like features in the mineral layer (Figure 3e_{I-III}, f_{I-III}; red arrows). The mineralization was stopped after 340 min, and fully sampled images were taken on

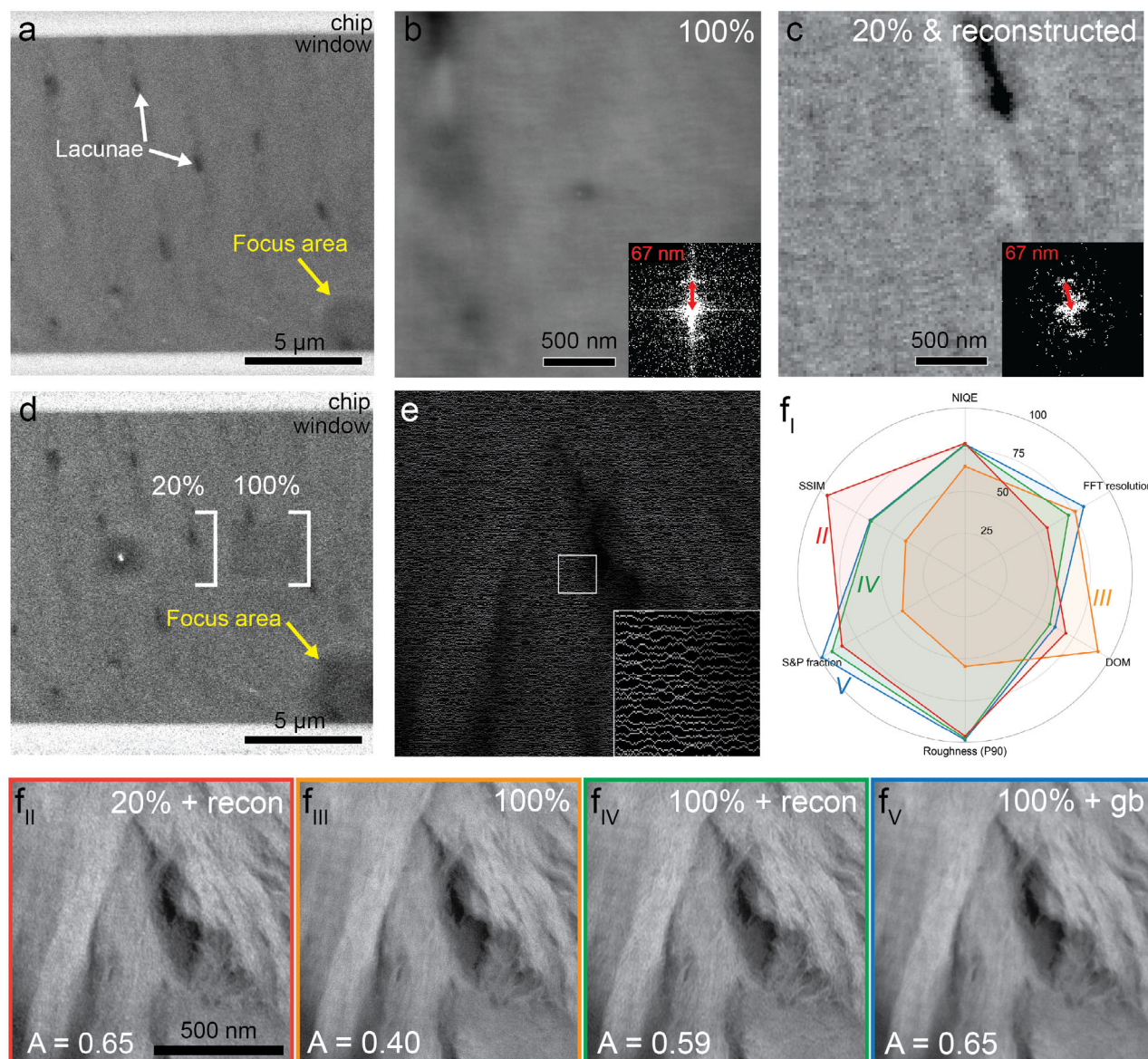


FIGURE 2 | Validating the image quality of sub-sampled and inpainted images. (a) High-angle annular dark-field (HAADF) STEM overview image of demineralized bone tissue with characteristic lacunae (white arrow) in the LP-EM cell. The hydrated collagen is orientated perpendicular to the electron beam. The right bottom corner shows beam damage in an area sacrificed for focusing (yellow arrow). (b) Fully sampled HAADF image of the perpendicularly orientated collagen showing the characteristic collagen 67 nm banding pattern. Inset: the fast Fourier transform (FFT) confirming the 67 nm periodicity. (c) 20% sub-sampled and inpainted HAADF image showing the same characteristic periodicity as (b). (d) HAADF overview image of the same area as in (a) after recording the images presented in (b,c). The locations of the fully (b) and sub-sampled (c) high-magnification images are indicated with brackets. There is visible damage for the fully sampled region in (c). (e) 20% sub-sampled image on a 1h remineralized and subsequently dried tissue section. Inset: high-magnification image illustrating the “linehop” scanning pattern. (f) Radar plots representing different aspects of image quality for (f_I) 20% sub-sampled and reconstructed (red) (f_{II}), fully sampled (orange) (f_{III}), fully sampled and reconstructed (green) (f_{IV}), and fully sampled and Gaussian blurred (blue) (f_V) images. The empirical value ‘A’ indicates the normalized area of the radar plot polygon for each image (color coded) to assess overall image quality. Larger polygon areas correspond to higher overall image quality such that the normalized area of the polygon (A) can be used as a figure of merit. Electron dose: a,d; 0.003 e⁻ Å⁻² / image, b; 1.5 e⁻ Å⁻²/image. c,e,f_{II-V}; 0.3 e⁻ Å⁻²/image.

the open chip (dried), showing more defined mineral structures typical for the lacy pattern observed in bone (Figure S11).

3 | Discussion

Here we present the first dynamic nanoscale observation of collagen mineralization using LP-STEM. Although previous work

imaged hydrated (mineralized) collagen fibrils between a TEM grid and SiN_x microchip [37], until now capturing the dynamics of in vitro mineralization had not been achieved. Recently, we showed the dynamic complexation of fetuin (i.e., a protein) and calcium phosphate using highly electron-transparent GLCs, achieving high contrast and resolution [22, 23]. However, these and other [38, 39] static enclosures have fixed, relatively small volumes approximately 10 000 times too small to sup-

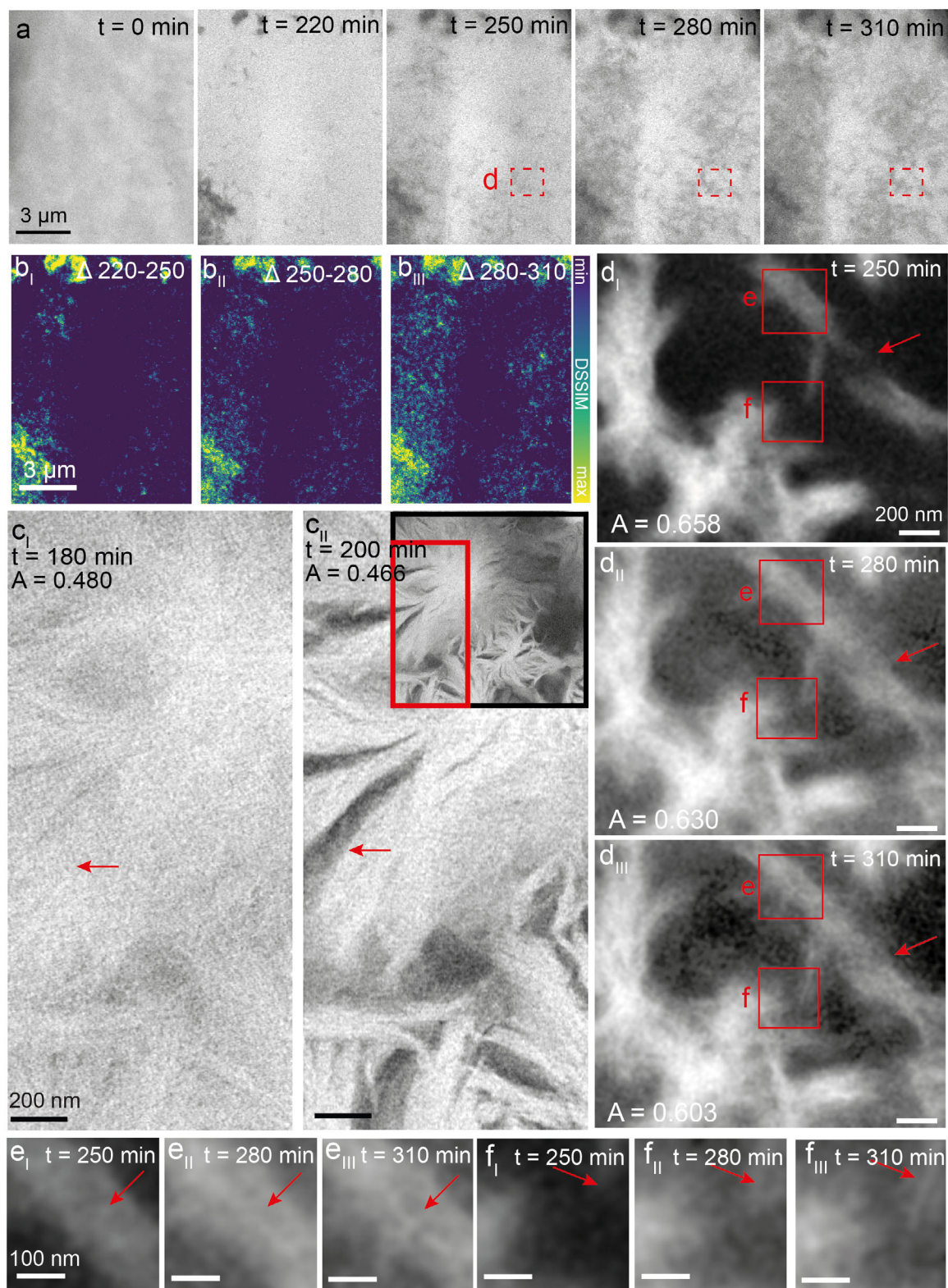


FIGURE 3 | Dynamic visualization of collagen mineralization. (a) Time-resolved overview BF images of a mineralizing collagen section in an LP-EM chip. Red rectangles indicate the area of higher-magnification sparse imaging in (d). (b) Structural dissimilarity (DSSIM) [36] analysis of the indicated timepoints showing the locations where the mineralization is progressing. (c) 20% sub-sampled and reconstructed HAADF STEM image showing how the dense mineral phase transforms into branches of mineral with low-contrast areas in between. The brightness of the raw images was corrected to account for the difference in liquid layer thickness calculated from intensity changes between the overview images. An overview image of this area can be found in Figure S9. (d) 20% sub-sampled and reconstructed HAADF time series of collagen mineralization at higher magnification of the area marked in (a) from $t = 250$ to 310 min. (e, f) Zoom-in of the area indicated with the red box in (d) showing the growth of a single mineral plate. Electron dose: a; $0.003 \text{ e}^- \text{ \AA}^{-2}/\text{image}$, (c–f) $0.31 \text{ e}^- \text{ \AA}^{-2}/\text{image}$. Accumulative dose in liquid: $1.25 \text{ e}^- \text{ \AA}^{-2}$.

ply sufficient physiological calcium solution (1.6 mM Ca^{2+}) to mineralize even a single 200 nm collagen fibril (Table S4). This effectively excludes the use of GLCs and similar non-flow cells for dynamic study of collagen mineralization, meaning it can only be performed in a liquid flow cell. On the other hand, commercially available liquid flow cell systems have SiN_x windows, which are significantly less electron transparent than their 2D material counterparts [40]. Moreover, their bulging in the vacuum of the electron microscope generally leads to a total specimen thickness approaching 1 micrometer (Text S1). This, together with the low contrast and beam sensitivity of organic materials, makes low dose imaging in such set-ups a challenge. By optimizing imaging parameters and applying sub-sampling in LP-STEM, we maintain sufficient signal-to-noise ratio and reduce the electron dose approximately 100-fold compared to recent LP-EM [37] and cryo-STEM approaches [41]. This approach enabled time-resolved observation of collagen mineralization at nanoscale resolution (~ 4 nm) using a liquid flow system.

Our acquired image series shows that regions containing spread, poorly-defined mineral clusters transform into crystalline branches separated by unmineralized (low-contrast) spaces, forming the characteristic lacy pattern of bone. This transformation implies lateral material transport, most likely through dissolution and reprecipitation of the mineral [42]. The mineral branches consist of apatite platelets with their *c*-axis oriented parallel to the electron beam (ED pattern, Figure S12), forming the aforementioned lacy pattern [5, 43]. We confirm mineral growth is cross-fibrillar, propagates in radial growth patterns, and initiates from clusters with poorly-defined (likely amorphous) microstructures. Our time-resolved observations are consistent with a scenario in which the lacy pattern is created by the emergence of unmineralized spaces during the crystallization process, rather than through directed crystal arrangement by a pre-defined collagen template [8], or by non-collagenous biomolecule assemblies [7].

We pose that our findings are not significantly compromised by beam-induced effects or confinement effects, as the final dry in-chip three-dimensional microstructure is consistent with that found for (1) tissue sections mineralized outside the microscope (Figure S1 and Video S1), (2) previous in vitro experiments [27], and (3) native bone [5]. Moreover, in areas where the organic matrix was damaged due to fully sampled imaging (Figure 2d), mineral was randomly precipitated without the observation of a lacy pattern (Figure S11), implying an intact extracellular matrix is required for its formation. These observations also suggest that radiolysis (i.e., generation of reactive chemical species, potentially causing local pH variations and ion supersaturation [44]) of the mineralization solution in the LP-chip did not significantly affect our results.

Still, a limitation in our study is our inability to perform electron diffraction (ED) in situ. Within the current capabilities, acquiring reliable ED patterns requires exposing the sample to electron doses that will damage the collagen matrix. ED performed on the dried chip suggests that despite its poorly-defined microstructure, the mineral at the periphery of the spherulites is not amorphous, but rather made up of nanometric crystallites (Figure S13). Another limitation is a technical limit on the tilt range

for on-chip tomography, which prevents visualization of the three-dimensional collagen and mineral structure.

4 | Conclusion

Here, we present a low-dose LP-STEM workflow that enabled the first dynamic nanoscale observation of collagen mineralization in hydrated bone tissue sections. By combining a liquid flow system with sub-sampling and inpainting we were able to capture the transformation of a poorly-defined mineral phase into the crystalline mineral branches, forming the lacy pattern. We confirm that the collagen templates the mineralization process, and show it is not directly responsible for creating the unmineralized areas in the lacy pattern i.e., their location is not pre-determined, but a product of the crystallization process. Our direct observations provide confirmation and insights that no other single method can currently provide. In the future, this methodology may be advanced with additional imaging modalities (integrated differential phase contrast (iDPC)-STEM and 4D-STEM), as well as new reconstruction algorithms, which together may further reduce the required electron dose and improve spatial resolution (Text S6). The described low-dose LP-STEM workflow will be a powerful tool for understanding the dynamics of biological mineralization processes and has great potential for future nanoscale studies of biological material formation processes in their native context.

5 | Experimental

5.1 | Human Bone Sample

Whole human femurs were retrieved from an anonymous healthy male donor in compliance with European regulations for human cells and tissues (directives 2004/23EC, 2006/17EC, and 2006/86EC) and were kindly donated by HCM Medical. Femurs were freshly frozen at -20°C and serologically tested to be free of major pathogens (HIV, hepatitis B/C, and HTLV) and microbiological contamination (Clostridium, Streptococcus, fungi). Demineralization of bone blocks was carried out in a solution of 9.5% EDTA in PBS at pH 7.4 at room temperature for 12 days. Each block was submerged in 5 mL of demineralization solution. The solution was renewed every workday. From the demineralized bone cubes, slices of 200 μm thickness were cut using a Leica VT1000S vibratome using a steel blade for the cutting. Slicing was performed with the speed and vibration frequency set to 3.5 and 3, respectively.

5.2 | Cryo-Ultramicrotomy

The 200 μm bone slices were infiltrated overnight with 2.3 M sucrose solution as a cryoprotection. Subsequently, they were positioned on a mounting pin and plunged into liquid nitrogen. The frozen specimens were then sectioned into 200 nm thin sections with a Leica Ultracut UCT + EMFCS cryo-microtome (Leica, Wetzlar, GE) and transferred with a loop containing 1.15 M sucrose and 1% methylcellulose to either a Gold 200 mesh continuous carbon TEM grid (Electron Microscopy Sciences, Morgantown, US) or a STREAM top chip (DENSolutions, Delft,

NL). The chip was placed overnight on a droplet of PBS to wash out sucrose.

5.3 | Remineralization

The demineralized ultrathin sections were remineralized with a biomimetic solution prepared by adding 6.8 mM CaCl_2 , 5 mM K_2HPO_4 , 600 $\mu\text{g/mL}$ pAsp (2000–11 000 mw; Merck, Darmstadt, GE) and 3.4 x PBS in a 1:1:1:1 ratio. This results in a final concentrations of 1.7 mM CaCl_2 , 114 mM NaCl, 2 mM KCl, 10 mM PO_4^{3-} , and 0.2 mg/mL pAsp. The biomimetic solution was used directly after addition of the phosphate buffers and replaced every hour for the grids and every half hour for the chips. The solution was kept at 37°C during mineralization.

5.4 | Liquid Phase Transmission Electron Microscopy

The top and bottom chips were assembled in a STREAM holder (DENSsolutions, Delft, NL) directly after loading the sample to prevent drying out. The parallel SiN_x windows were aligned during assembly. The chip holder was connected to the flow system and inserted into the microscope. Mineralization solution was flowed through the system and replaced every half hour. After mineralizing for 20–60 min the chip was purged to create a thin layer of liquid, and images were taken. We note that folds in the tissue section (Figure S4) could affect the liquid flow through the cell, which was also prone to clogging during the experiments.

Images were acquired with a TALOS F200c G2 (Thermo Fisher Scientific, Eindhoven, NL) equipped with a Panther segmented STEM detector (Thermo Fisher Scientific, Eindhoven, NL) using a 200 kV acceleration voltage. Image parameters were determined using a Python script adapted from de Jonge (2018) [32]. The semi-convergence angle was 1.7 mrad, screen current 18 pA, HAADF collection angle 10–117 mrad and BF collection angle 0–12 mrad. Fully sampled overview images with a pixel size of 14 nm and 1 μs dwell time were acquired using Velox (Thermo Fisher Scientific, Eindhoven, NL). Linehop 20% sub-sampling with a pixel size of 1.212 nm and 2 μs dwell time were acquired using a Quantum Detector scan engine and the SenseAI software (SenseAI, Liverpool, UK).

5.5 | Scanning Transmission Electron Microscopy Tomography

Tilt series were recorded using TOMO software (Thermo Fisher Scientific, Eindhoven, NL) and the same acquisition parameters as in 5.4. Images were acquired with a TALOS F200c G2 (Thermo Fisher Scientific, Eindhoven, NL) equipped with a Panther segmented STEM detector using 200 kV acceleration voltage.

5.6 | Transmission Electron Microscopy

Dried images and diffraction patterns on the open chip were acquired in parallel illumination with a TALOS F200c G2

(Thermo Fisher Scientific, Eindhoven, NL) equipped Ceta-S camera (Thermo Fisher Scientific, Eindhoven, NL) using a 200 kV acceleration voltage.

5.7 | Image Reconstruction

Sub-sampled images were reconstructed using SenseAI software and Python interface (SenseAI, Liverpool, UK). SenseAI allows users to define dictionary parameters (number of atoms/patches, patch size), as well as tune hyperparameters (batch size and learning rate) during dictionary training. We have expanded on the provide Python interface (GitHub-Low-dose-liquid-phase-STEM) by adding live dictionary manipulation during training, including increasing or decreasing the dictionary size, removing user-selected atoms, and resetting user-selected atoms. Additionally, we have added options for offline dictionary manipulation, enabling operations such as combining dictionaries and applying filters to specific atoms. The modified dictionary can then be used in a new or existing training session. We can also track image quality metrics and visualize atom weights (i.e., their prominence) during image reconstruction. Our code keeps track of the best reconstruction and dictionary according to the tracked metric, in addition to the latest reconstruction and dictionary. Finally, we use a post-training overview of parameter changes over time, allowing users to correlate parameter adjustments with changes in any metric. We also include a video demonstrating the use of the Python interface (Video S2).

5.8 | Image Quality Analysis

Reconstructed image quality was assessed using complementary metrics capturing structural fidelity (SSIM), edge sharpness (DOM), no-reference natural image quality (NIQE), salt-and-pepper noise fraction, image roughness, and FFT-based spatial resolution (Table S3). These metrics were visualized using radar plots (Figure 2f and Figures S6 and S7), in which each axis was oriented such that improved image quality was represented toward the periphery. The normalized polygon area (A), defined as the ratio between the measured polygon area and that of an ideal polygon, was used as an overall measure of reconstruction quality, while the polygon shape highlighted trade-offs between individual metrics. As SSIM requires a reference image, we created a masked version that compared only experimentally observed pixels in the sub-sampled image with the corresponding pixels in the reconstruction. Additional details can be found in Text S1, and the Python codes are available on [GitHub-Low-dose-liquid-phase-STEM](#).

5.9 | Additional Image Processing

Tilt series for the tomogram video was reconstructed using IMOD (University of Colorado, Boulder, US). DSSIM analysis was performed in Fiji (standard deviation 2, size 15, per-frame contrast, $\alpha = 1$, $\beta = 0.1$, $\gamma = 0$) [36].

5.10 | Raman Spectroscopy

Confocal Raman Spectroscopy was performed on a Witec Alpha 300R Raman microscope equipped with a 50x objective

(EC Epiplan-Neofluar Disc 50x/0.8 objective, Zeiss, Germany). Single spectra were acquired using 532 nm excitation (20 mW at the sample) and a dwell time of 4 s with 4 times averaging. The spectral resolution was 2–3 cm^{−1} and the Raman spectra were collected in the range from 267 to 2392 cm^{−1} using the 600 g/mm grating. The obtained data was analyzed using the WITec project SIX analysis software.

Author Contributions

Luco Rutten: writing – original draft, writing – review and editing, conceptualization, investigation, methodology, visualization, formal analysis, data curation, software. **Avital Wagner:** writing – original draft, writing – review and editing, conceptualization, investigation, visualization, data curation, formal analysis, software, methodology. **Rona Roverts:** methodology. **Luco Buise:** methodology, software, visualization, investigation. **Ben Joosten:** data curation, methodology. **Anat Akiva:** supervision, funding acquisition. **Elena Macías-sánchez:** funding acquisition, supervision, writing – review and editing, conceptualization. **Nico Sommerdijk:** funding acquisition, supervision, writing – review and editing, conceptualization, resources.

Acknowledgements

The author like to thank Dr. Rob Mesman from the Radboud University for his help with the cryo-ultramicrotomy and SenseAI for the valuable discussions. This project was supported by the European Research Council (ERC) Advanced Investigator grants (H2020-ERC-2017-ADV-788982-COLMIN) and (H2025-ERC-2023-ADV-101141998 -REVALVE) and the SPARQ grant (Thermo Fisher Scientific) to N.S. A.A. was partly supported by a VENI grant from the Netherlands Scientific Organization NWO (VI.Veni.192.094). EMS was supported by the Ramón y Cajal Program (RYC2023-045512-I) funded by MCIN/AEI/10.13039/501100011033 and FSE+ and the project PID2022-141993NA-I00 funded by MICIU/AEI/10.13039/501100011033 and ERDF/UE.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are openly available in Low-dose liquid phase STEM at <https://doi.org/10.5281/zenodo.21874966>.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting File 1: adfm78921-sup-0001-SuppMat.docx.

Supporting File 2: adfm78921-sup-0002-VideoS1.avi.

Supporting File 3: adfm78921-sup-0003-VideoS2.mp4.