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Synchrotron X-ray nanoprobe and correlative electron microscopy reveal the role of surface chemistry of self-assembling peptides in calcium phosphate nucleation†

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A biomimetic peptide (P_{11-4}), which is predominantly negatively-charged, facilitates the nucleation of hydroxyapatite (HAP). P_{11-4} self-assembles into fibrils via β -sheet formation, creating a 3D-gel-network. Here, X-ray nanoimaging and correlative scanning electron microscopy (SEM) investigated P_{11-4} 's surface chemistry and its ability to nucleate HAP in the absence of the 3D-gel-network. P_{11-4} was deposited on silicon nitride (SiN) windows, which were immersed in a mineralising solution (MS) and then mapped using nano-X-ray fluorescence (n-XRF) and differential phase contrast imaging at the hard X-ray nanoprobe beamline (I14) at Diamond Light Source. Elemental calcium and phosphorus maps were extracted using n-XRF, and compared with and without P_{11-4} . The windows were subsequently mapped using SEM and Energy Dispersive Spectroscopy (EDS) to confirm the morphology and elemental compositions of the formed structures. The calcium:phosphorus ratios were calculated to identify the phases formed. P_{11-4} increased the calcium and phosphorus signals with time in MS compared to the control (without P_{11-4}). After 12 hours in MS, calcium ions accumulated on the deposited β -sheets, attracting phosphorus ions at later time points. From the morphology in the images and EDS analysis, the spherical calcium phosphate (CaP) structures appeared to be amorphous, indicating the formation of precursors, likely amorphous CaP, at early time points. In the presence of P_{11-4} , these structures grew and fused into larger CaP formations over time, unlike in the control. Nano-imaging techniques highlighted that P_{11-4} 's surface chemistry accelerates the kinetics and controls the initial CaP crystallisation process, resulting in an amorphous CaP phase.

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It has been suggested that P₁₁₋₄ forms HAP directly by chelating calcium ions (Ca²⁺) on negatively-charged glutamic acid residues, spaced with the same geometry as the Ca²⁺ ions found in HAP.¹³ However, this suggestion was based on *in silico* modelling and lacks experimental data for validation. P₁₁₋₄'s structure was reported to result in *de novo* HAP nucleation, however the peptide's precise mechanism, its nucleation route (*i.e.* classical or non-classical nucleation including precursor phase formation) and early kinetics of action remain unclear. Specifically, the role of surface chemistry in the hydroxyapatite nucleation process is poorly understood. Studying and understanding the process of calcium

phosphate nucleation in the presence of promising biomimetic materials are critical for their development for tissue engineering applications to treat various pathological conditions that affect hard tissues in the human body.

Characterising biomaterials to understand how they affect calcium phosphate mineralisation to elucidate their role in biomimetic mineralisation requires the use of cutting-edge tools to achieve nanoscale resolutions. Formed mineral structures at early time points can be too small to be detected using the conventional characterisation methods in the lab, necessitating the application of high-resolution imaging techniques, such as synchrotron-based X-ray nanoprobe imaging to better understand these processes. To complement the X-ray nanoprobe imaging, lab-based electron microscopy techniques can be used to study mineralisation at different length scales to build a full picture on how biomaterials interact with minerals to enhance their development. At the time of writing, no reported studies have made use of X-ray nanoprobe techniques to understand how biomimetic materials influence the nucleation of calcium phosphate phases.

The secondary conformation and the resulting surface chemistry of the peptide can affect the nucleation pathways. Therefore, this study aims to apply synchrotron X-ray nanoprobe techniques on the I14 beamline at Diamond Light Source (DLS),¹⁴ along with correlative scanning electron microscopy, to investigate these effects in the absence of 3D confinement on the hydroxyapatite nucleation process by depositing the peptide on a substrate. This is to enhance our understanding of crystal growth, in the presence of P₁₁-4, at the nano- and microscale levels and to elucidate the peptide's role in biomimetic mineralisation by addressing the question: does the peptide form hydroxyapatite through a similar pathway to natural enamel biomineralisation? These techniques can be applied to characterise biomimetic peptides and proteins of biological significance. In this study, P₁₁-4 is employed as a model system for characterisation.

2. Experimental

For all the experiments, a 10 mg mL⁻¹ P₁₁-4 (Credentis AG, Switzerland) solution was prepared and adjusted to pH 7 using 1% ammonia. At pH 7, the solution appeared as a pre-gel, viscous liquid containing fibrillar assemblies.¹¹

2.1 Optimising P₁₁-4 β -sheet deposition on silicon nitride windows

For X-ray nanoprobe imaging, the P₁₁-4 solution was deposited on 5 × 5 mm silicon nitride (SiN) windows before immersion in mineralising solution (MS) for different durations. Prior to nano-X-ray fluorescence (n-XRF) and differential phase contrast (DPC) imaging,¹⁵ an assessment by Fourier Transform Infrared Spectroscopy (FTIR) was conducted to establish the avidity of the fibrils to the SiN windows after immersion in MS and establish a deposition protocol that results in the most prominent signal at the 1620 cm⁻¹ wave number, indicating the presence of β -sheets, to investigate how they affect nucleation of mineral.

2.1.1 Deposition method. To produce samples that are appropriate for n-XRF and DPC mapping on the I14 beamline at DLS, a thin film of fibrillar P₁₁-4 is required to be deposited on the SiN windows. Different deposition methods can result in different structures, therefore three deposition methods¹⁶ were tested to



determine which method resulted in the most significant signal at the 1620 cm^{-1} wave number to indicate the presence of β -sheets. The methods are as follows:

- (1) **Method 1 – Drop-coating:** one 6 μL drop of P_{11-4} solution was placed onto the center of a SiN window and allowed to dry.
- (2) **Method 2 – Blotting:** one 6 μL drop of P_{11-4} solution was placed onto the center of a silicon nitride window, excess peptide solution was then blotted with tissue paper. It was then left to dry.
- (3) **Method 3 – Immersion:** SiN windows were dipped in P_{11-4} solution, removed and re-dipped, and then left to dry.

After the windows were dry, they were assessed by FTIR (Nicolet iS50 FT-IR) to determine which method resulted in the highest proportion of β -sheets by assessing the intensity of the peak at 1620 cm^{-1} wave number. A bespoke 3D-printed sample holder was designed to hold the sample securely in place during conformational analysis. The following parameters were set for the FTIR scans: number of scans = 32, resolution = 16, final format was absorbance and no correction was specified. From the collected spectra, the amide I region (1720 and 1580 cm^{-1}) was evaluated to indicate the presence of P₁₁₋₄ on the window.

2.1.2 Adherence of P₁₁₋₄ to the windows. To ascertain the peptide's persistence on the SiN windows, each coated substrate was immersed in 700 μ L of MS (120 mM NaCl, 22 mM NaHCO₃, 3.75 mM CaCl₂, 1.67 mM Na₂HPO₄)⁸ for approximately 4–5 hours. Following immersion, they were evaluated using FTIR to ascertain the presence of the β -sheet conformation.

2.2 Mapping of calcium phosphate phases – hard X-ray nanoprobe

2.2.1 Sample preparation. For n-XRF and DPC mapping, all the SiN windows were prepared using method 1 (drop-coating) as described above as it resulted in the most prominent signal at the 1620 cm^{-1} wave number (as shown in Fig. 1), indicating the presence of β -sheet conformation. The coated SiN windows were incubated in 2 mL MS for: 6 hours, 12 hours, 16 hours, 24 hours, 48 hours, and 336 hours (2 weeks). Controls were prepared for each time point by immersing SiN windows in MS only (without prior P₁₁₋₄ deposition).

2.2.2 X-ray nanoprobe imaging. After sample preparation, the SiN windows were mounted on holders and placed on the I14 beamline for n-XRF and DPC imaging. Samples were scanned with a 50 nm focused X-ray beam, at 8 keV. n-XRF spectra were collected using a four-element silicon drift detector, and simultaneous DPC data was collected using a quad Merlin detector ($4 \times$ Medipix3, 55 μm pitch, 512×512 pixels, Quantum Detectors, UK) at a 2 m sample-detector distance. Different sized maps at different spatial resolutions (400 $\times$ 400 μm map at 1 μm step-size and 15 $\times$ 15 μm map at 50 nm step-size) were collected.

2.2.3 Data analysis. The calcium and phosphorus elemental maps were extracted from the n-XRF spectra by either integrating the total intensity in a ± 100 eV window around the Ca K α and P K α fluorescence lines or by fitting in PyMca.¹⁷ Phase images were extracted from the DPC maps using the method described by Quinn *et al.*¹⁵ The scale bars were added to all the images using ImageJ (National Institutes of Health, Bethesda, MD, USA). The mean total signal intensity of calcium and phosphorus was calculated for the all the time points in MS. The $400\ \mu\text{m} \times 400\ \mu\text{m}$ map was split into eight regions, from which the total calcium and phosphorus signal intensity (counts) was calculated. This was

conducted to calculate the mean total signal intensity value (the mean of the total counts per region). The error bars indicate the standard deviation of values from the eight regions. The mean calcium-to-phosphorus signal intensity ratio against time in the presence and absence of P₁₁₋₄ was plotted by calculating the ratio of the mean calcium and mean phosphorus signal intensity values for the different time points. Note that without calibration, the calcium and phosphorous signal intensities and ratios calculated from the n-XRF maps are not equivalent to quantitative concentrations.

2.3 Complementary scanning electron microscopy (SEM) and energy dispersive spectrometry (EDS)

The samples mapped on the beamline were subsequently mapped using SEM (JEOL JSM-6610LV) and EDS using Aztec (Oxford Instruments) for quantitative analysis. The secondary electron images (SEI) mode was selected, and the voltage was set to 20 kV. The working distance (WD) was 11 mm and the spot size was set to 60 nm. The images were taken at various magnifications. The calcium-phosphorus ratio was calculated from the atomic compositions.

3. Results

3.1 Optimising P₁₁₋₄ β -sheet deposition on silicon nitride windows

Three deposition methods, drop-coating, immersion, and blotting, were tested for depositing P₁₁₋₄ onto SiN windows to determine which method yielded the highest deposition of β -sheets to subsequently carry out n-XRF and DPC mapping of mineralisation events on the I14 beamline. The amide I region (1720–1580 cm⁻¹) was analysed to confirm P₁₁₋₄'s presence, with a peak at 1620 cm⁻¹

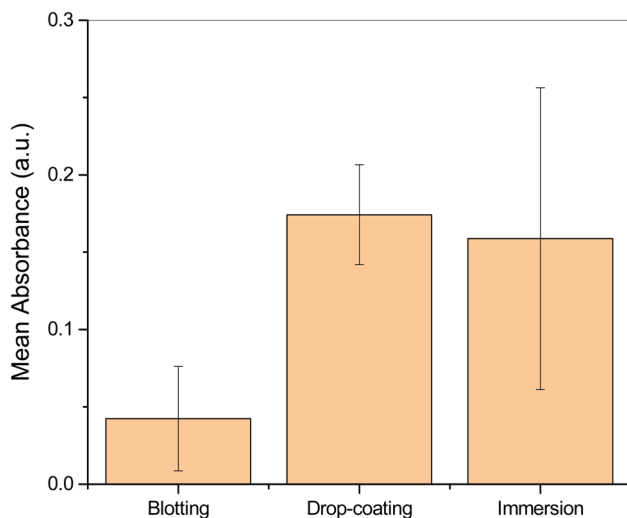


Fig. 1 Mean absorbance signal at 1620 cm^{-1} (wave number), indicative of the presence of the β -sheet conformation, assessed by Fourier Transform Infrared Spectroscopy (FTIR) using three different deposition methods: blotting, drop-coating, and immersion. The error bars represent the standard deviation (SD).

indicating β -sheets. While all methods facilitated some β -sheet deposition, blotting yielded the least amount as shown in Fig. 1 (full spectra in Fig. D in the ESI†). The drop-coating method resulted in the highest mean absorbance signal, and most consistent result, indicating substantial and reproducible β -sheet formation compared to the two other methods (Fig. 1). Consequently, drop-coating was chosen for further sample preparation. To evaluate the stability of the P₁₁-4 adsorbed-layer on SiN windows, P₁₁-4 was deposited using the drop-coating method and immersed in 700 μ L of MS for 4–5 hours. The FTIR spectra (see Fig. E in the ESI†) after 4–5 hours in MS exhibited a persistent peak at 1620 cm^{-1} , with a calculated mean absorbance signal of 0.128 ± 0.004 arbitrary units ($n = 3$).

3.2 Mapping of calcium phosphate phases – hard X-ray nanoprobe

In the control sample, fewer structures were observed in the mapped areas using DPC compared to the SiN windows with deposited P₁₁-4 as shown in Fig. 2. These structures were confirmed to be calcium phosphate, as evidenced by Fig. 3, which presents the elemental calcium and phosphorus maps from the same mapped areas as in Fig. 2 for the equivalent time points.

Additionally, Fig. 2 shows that P₁₁-4 induces the formation of spherical structures, which increase in number with time, and appear to migrate and fuse together over time, forming the biggest calcium phosphate deposits at the longest time point (2 weeks of mineralisation) (Fig. 2). The spherical structures observed in Fig. 2 (in the presence of P₁₁-4 and in the control after 2 weeks of mineralisation) can be observed in the calcium and phosphorus maps in Fig. 3 at the early time points (12, 16 and 24 hours) in the presence of P₁₁-4, confirming that these structures are a calcium phosphate phase. These spherical structures were absent in the control SiN windows imaged using DPC during the early time points as shown in Fig. 2. In addition, the control elemental maps (Fig. 3) do not manifest similar spherical structures as observed in the presence of P₁₁-4. Moreover, the calcium phosphate structures formed in the absence of P₁₁-4 (*i.e.* the control) are smaller, and do not appear to form an interconnected network with neighbouring calcium phosphate structures as manifested in the presence of P₁₁-4 at the early time points. However, the morphology of the mineralised structures formed after 2 weeks in the absence of P₁₁-4 (control sample) is similar to that in the presence of P₁₁-4 as shown in Fig. 2. At 2 weeks, larger calcium phosphate structures form in the absence of P₁₁-4 (control), and appear to be networking with neighbouring structures. From our results, the needle-like nanostructural morphology characteristic of HAp could not be observed, even after 2 weeks in mineralising solution.

In Fig. 3, calcium phosphate can be observed at 6 hours in the presence of P₁₁-4, indicating the presence of small calcium phosphate structures that are approximately 50 nm in diameter. With time in mineralising solution, the calcium phosphate structures grow in the presence of P₁₁-4 compared to the control.

Looking at the distribution of structures over a relatively larger area (400 $\mu\text{m} \times 400 \mu\text{m}$), fewer calcium phosphate structures were observed on the control SiN windows (Fig. 4 and 5) at the early time points compared to the windows with P₁₁-4. For the control, the distribution of calcium phosphate appears to be more random compared to the SiN windows with deposited P₁₁-4 as shown in Fig. 4 and



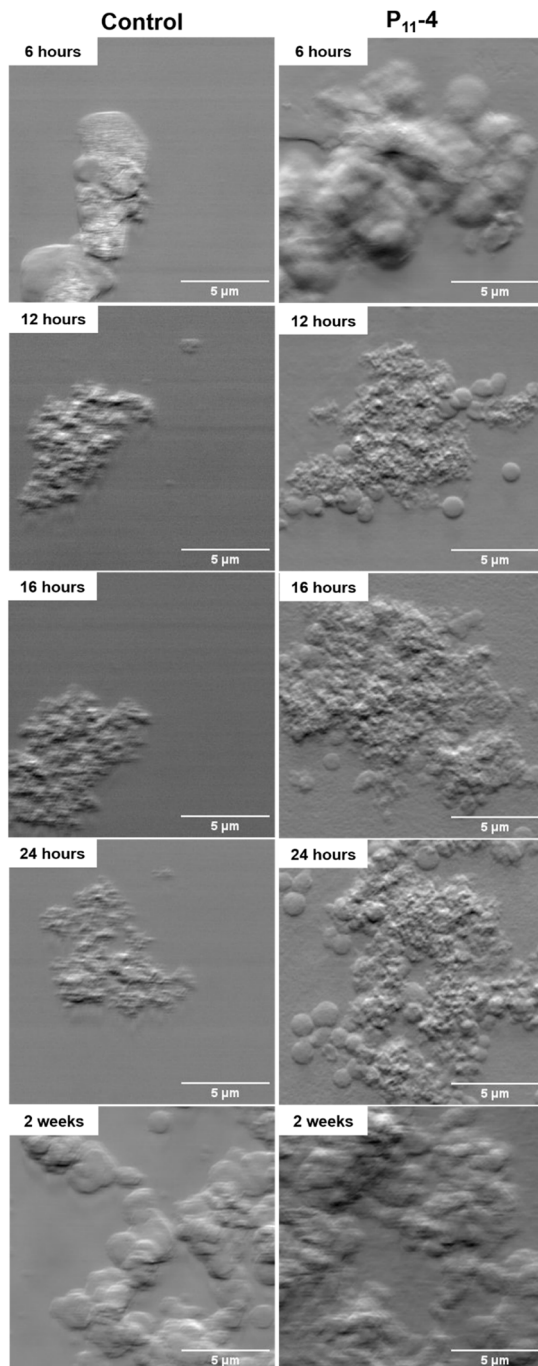


Fig. 2 Typical Differential Phase Contrast (DPC) images of SiN windows with deposited P₁₁₋₄ and without (control) mapped on the I14 beamline at different time points, depicting the morphology of the crystals formed after immersion in mineralising solution. The area shown is 15 μm × 15 μm, with a step size of 50 nm.



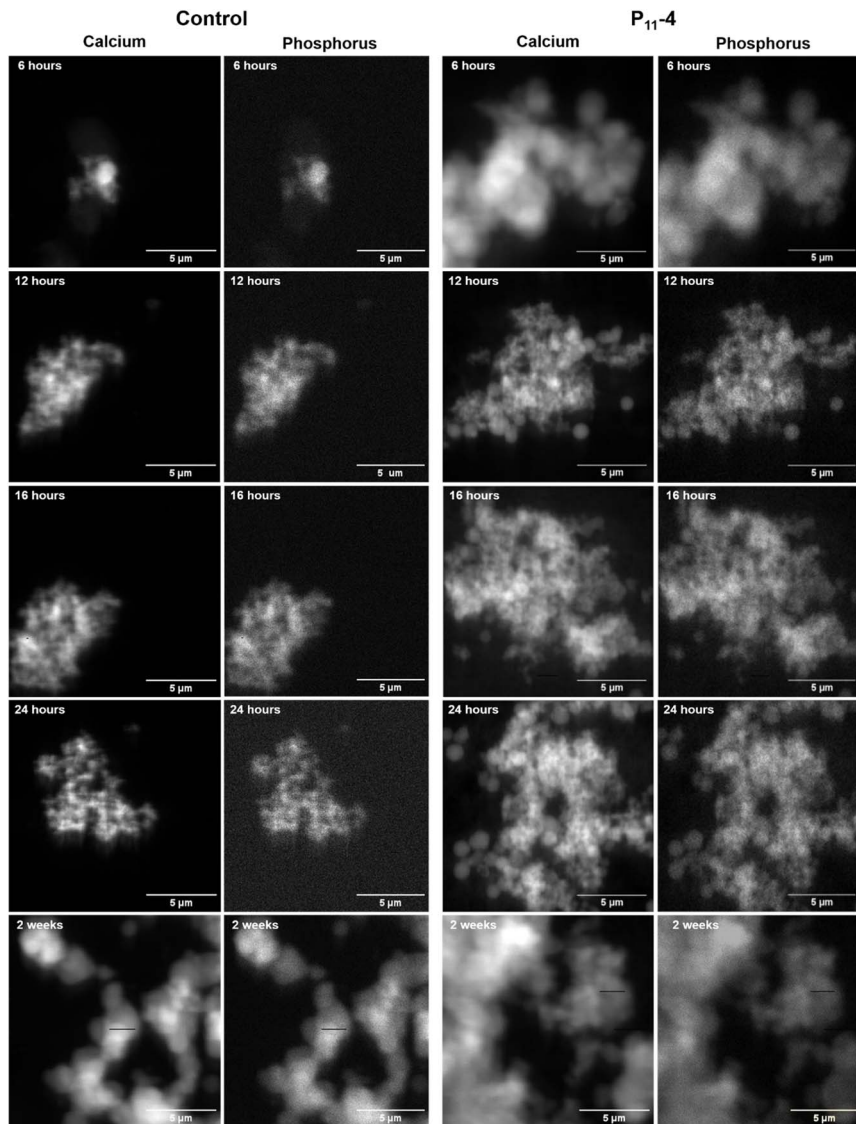


Fig. 3 Typical calcium and phosphorus elemental maps at different time points (6 hours, 12 hours, 16 hours, 24 hours and 2 weeks) comparing the control to the mapped SiN windows with deposited P_{11-4} , extracted using n-XRF mapping on the I14 beamline. These maps depict the morphology of the formed calcium phosphate structures. The area shown is $15\ \mu\text{m} \times 15\ \mu\text{m}$, with a step size of $50\ \text{nm}$.

5. In Fig. 4, at 12 hours, the calcium signal, indicated by the red colour, appears to be higher than the phosphorus signal in the presence of P_{11-4} . After 12 hours of mineralisation, the phosphorus signal increases to indicate phosphorus ion attraction at later time points compared to the calcium ions.

As shown in Fig. 6, the mean calcium and phosphorus intensities generally increased with time in mineralising solution in the presence of P_{11-4} . The mean



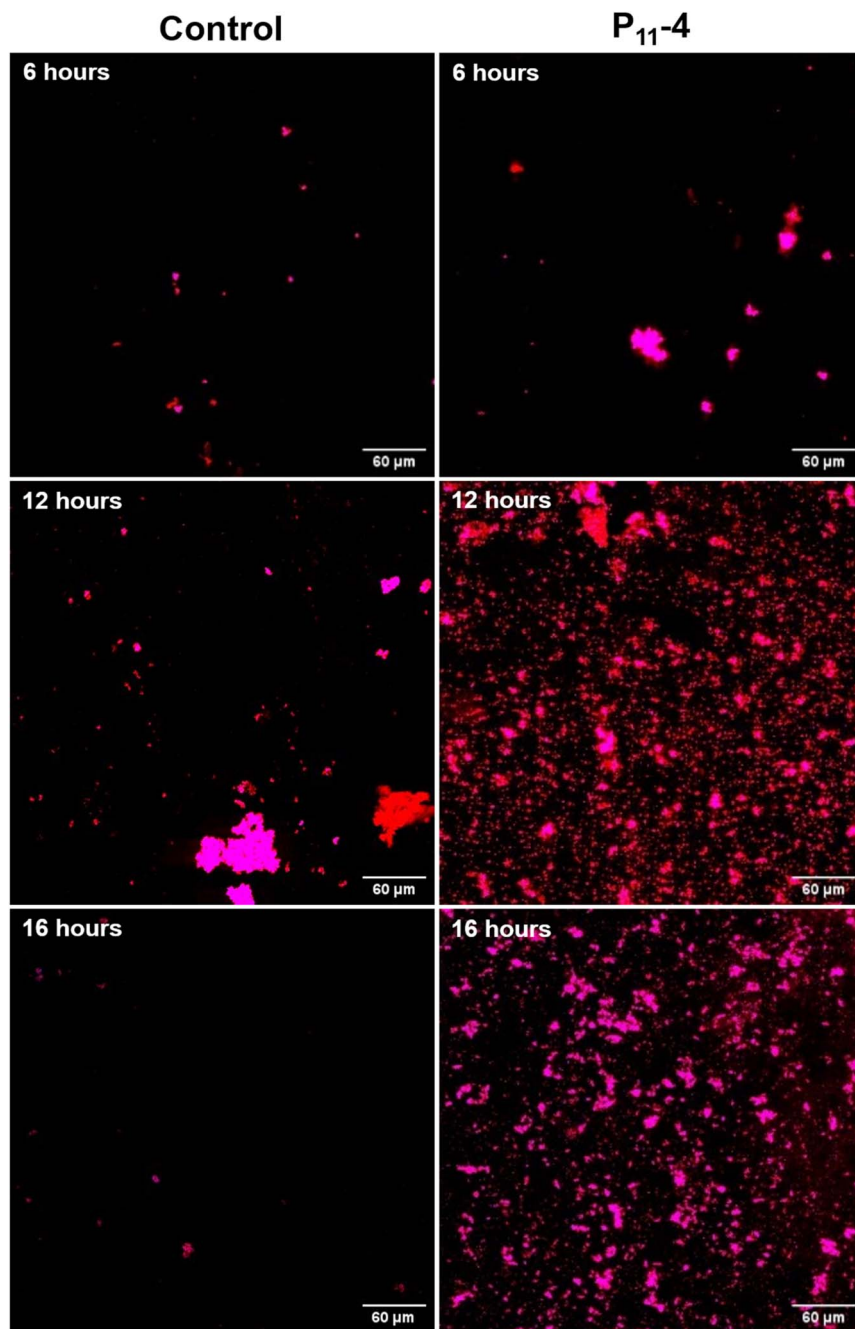


Fig. 4 Combined calcium and phosphorus elemental maps to detect the calcium phosphate distribution across the mapped areas (using n-XRF on the I14 beamline) at different time points under 24 hours, comparing the control to the mapped SiN windows with deposited P₁₁-4. Red = calcium signal, blue = phosphorus signal and pink = both. The area shown is 400 μm × 400 μm with a step size of 1000 nm.



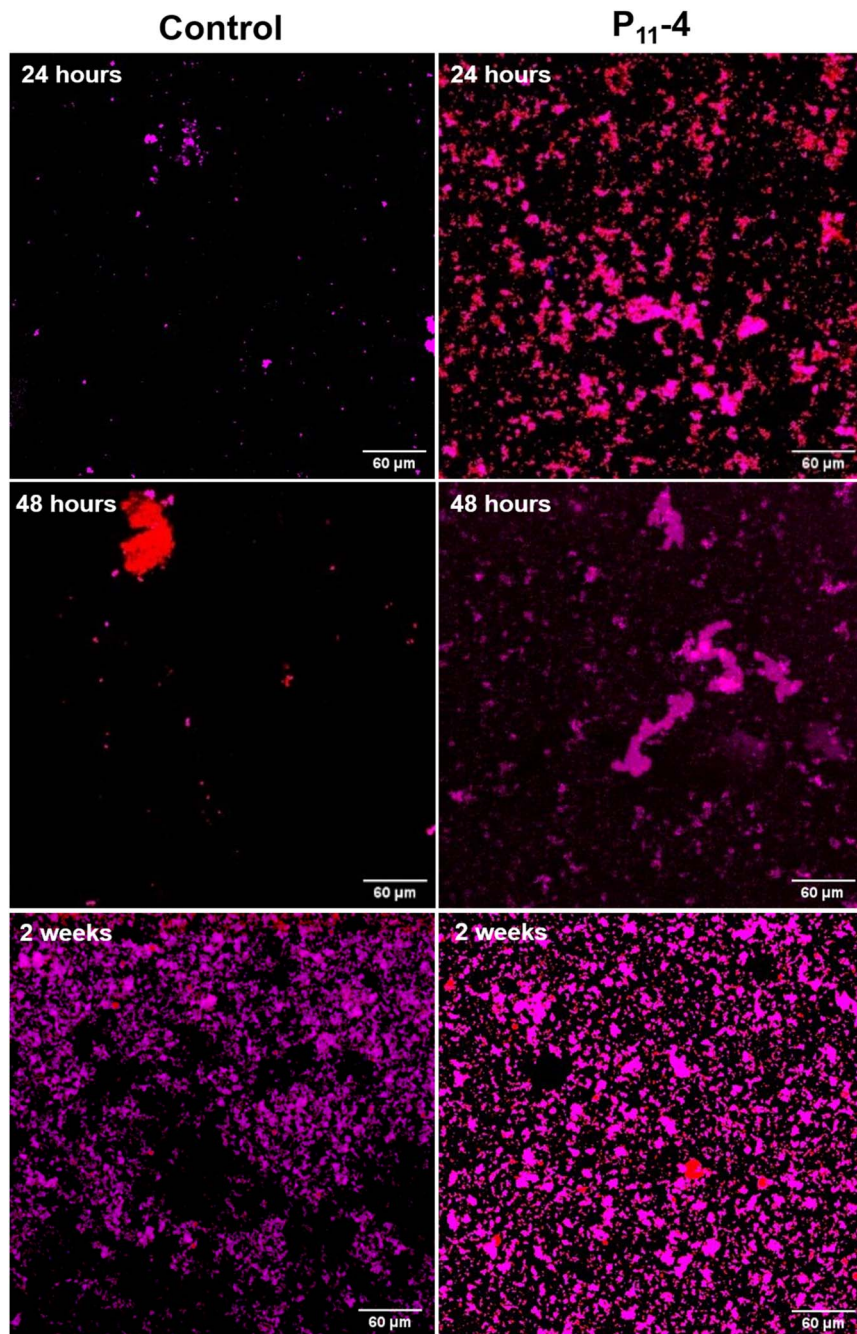


Fig. 5 Combined calcium and phosphorus elemental maps to detect the calcium phosphate distribution across the mapped areas (using n-XRF on the I14 beamline) at different time points over 24 hours, comparing the control to the mapped SiN windows with deposited P₁₁₋₄. Red = calcium signal, blue = phosphorus signal and pink = both. The area shown is 400 μm × 400 μm with a step size of 1000 nm.



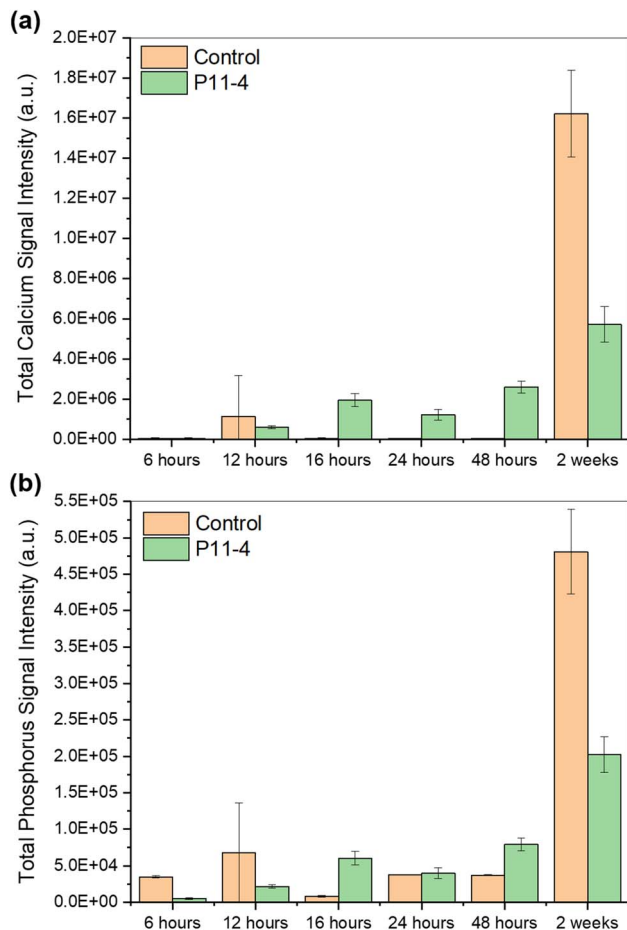


Fig. 6 Mean total signal intensity of (a) calcium and (b) phosphorus with time (6 hours, 12 hours, 16 hours, 24 hours, 48 hours and 2 weeks) comparing the control to the SiN windows with deposited P₁₁-4. The 400 $\mu\text{m} \times 400 \mu\text{m}$ map was split into eight regions, from which the calcium and phosphorus signals intensity was calculated to calculate the mean total signal intensity value of calcium and phosphorus. The error bars indicate the standard deviation of values.

calcium intensity was higher in the presence of P₁₁-4 compared to the control for the early time points, except for the time point at 12 hours. However, after 2 weeks in mineralising solution, the calcium signal was significantly higher in the control, which was also the case for the phosphorus signal. The phosphorus signal was higher in the control compared to the SiN window with deposited P₁₁-4 at 6 hours and 12 hours. At 16 hours, the signal dropped below the level of the SiN treated with P₁₁-4. By 24 hours, the signals, in the presence and absence of P₁₁-4 (control), were nearly equal, however at 48 hours, it was lower again in the control compared to the SiN window with deposited P₁₁-4.

Fig. 7 shows no obvious trend in the control, with the ratio increasing from 6 to 12 hours, decreasing until the 48-hour time point, and then increasing drastically at 2 weeks. On the other hand, the calcium-to-phosphorus ratio with time is



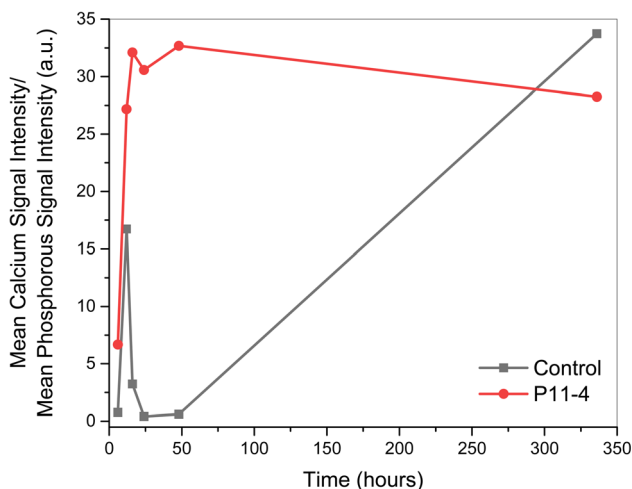


Fig. 7 Mean calcium-to-phosphorus signal intensity ratio against time, comparing the control to the SiN windows with deposited P₁₁-4. The 400 μm $\times$ 400 μm map was split into eight regions, from which the calcium and phosphorus signals intensity was calculated. The mean signal intensity value of calcium and phosphorus was calculated from the eight regions, and the ratio of the values was plotted on the y-axis against time on the x-axis. The dark grey line indicates the values calculated for the control while the red line indicates the values in the presence of P₁₁-4.

shown to have a trend in the presence of P₁₁-4 (where the ratio increases until 16 hours, decreases slightly at the 24-hour time point, and then plateaus) compared to the control. The ratio of the mean intensity values increases to 33.7 after 2 weeks for the control, while the ratio of the mean intensity values after 2 weeks is 28.3 in the presence of P₁₁-4, which is within the range of ratio values after 12–48 hours in the presence of P₁₁-4.

3.3 Complementary scanning electron microscopy (SEM) and energy dispersive spectrometry (EDS)

The samples imaged at the hard X-ray nanoprobe were subsequently imaged using lab-based SEM for complementary analysis of the SiN windows. SEM imaging enabled imaging the whole SiN window for each sample, as shown in Fig. 8. EDS was also conducted on the windows to confirm the elements within certain regions on the SiN windows to confirm and complement the previous findings (Fig. A–C in the ESI†).

Fig. 8 showed reduced amount of calcium phosphate structures on the control SiN windows when compared to the SiN windows with P₁₁-4 for the early time points. EDS spectra of the control SiN windows detected no calcium or phosphorus signals for the early-time-point samples (6–48 hours). However, Fig. 8 shows that mineralisation has occurred in the control after 2 weeks. EDS analysis of the control after 2 weeks in MS showed that the formed structures were a mixture of calcium phosphate and sodium chloride. On the SiN window with deposited peptide, no sodium or chlorine signals were detected.

As shown in Fig. 8, SEM imaging confirmed the gradual formation of calcium phosphate in the presence of P₁₁-4. Comparing the peptide-treated SiN windows



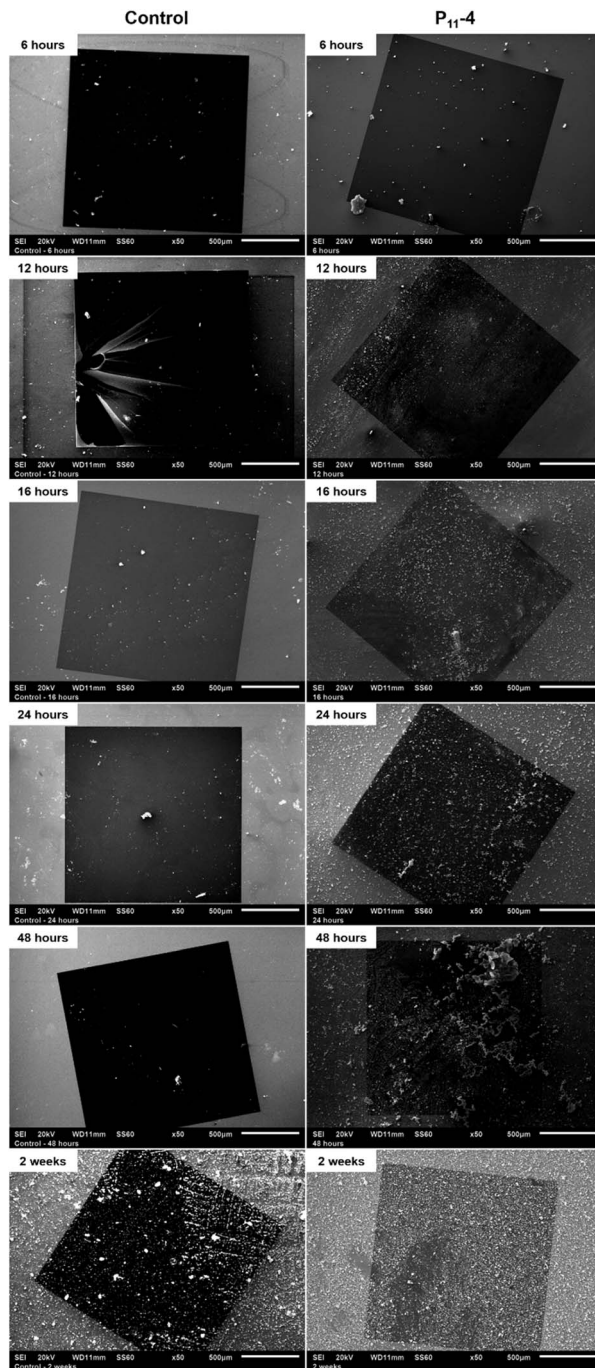


Fig. 8 Scanning electron microscopy (SEM) images of the SiN windows that were imaged on the I14 beamline to show the whole window (magnification = $\times 50$) at the different time points, comparing the control to the mapped SiN windows with deposited P₁₁-4. The secondary electron detector was used at an energy of 20 kV, a working distance of 11 mm and a spot size of 60 nm.



to the control, the peptide is shown to induce the formation of calcium phosphate structures, as confirmed by n-XRF imaging in the previous section, with time for the early time points (6–48 hours). For the 6-hour time point, little crystallisation can be observed on the whole SiN window and the EDS analysis of the window showed no calcium or phosphorus peaks in the spectrum to indicate very little or no calcium or phosphorus signals.

At higher magnification, Fig. 9b shows that after 12 hours in mineralising solution, spherical calcium phosphate structures, as confirmed by the EDS analysis of the region as shown in Fig. A in the ESI,[†] start forming in the presence of P₁₁-4. From Fig. 9, it appears that the calcium phosphate structures begin to grow further at 16 hours and even more at 24 hours, migrating towards each other

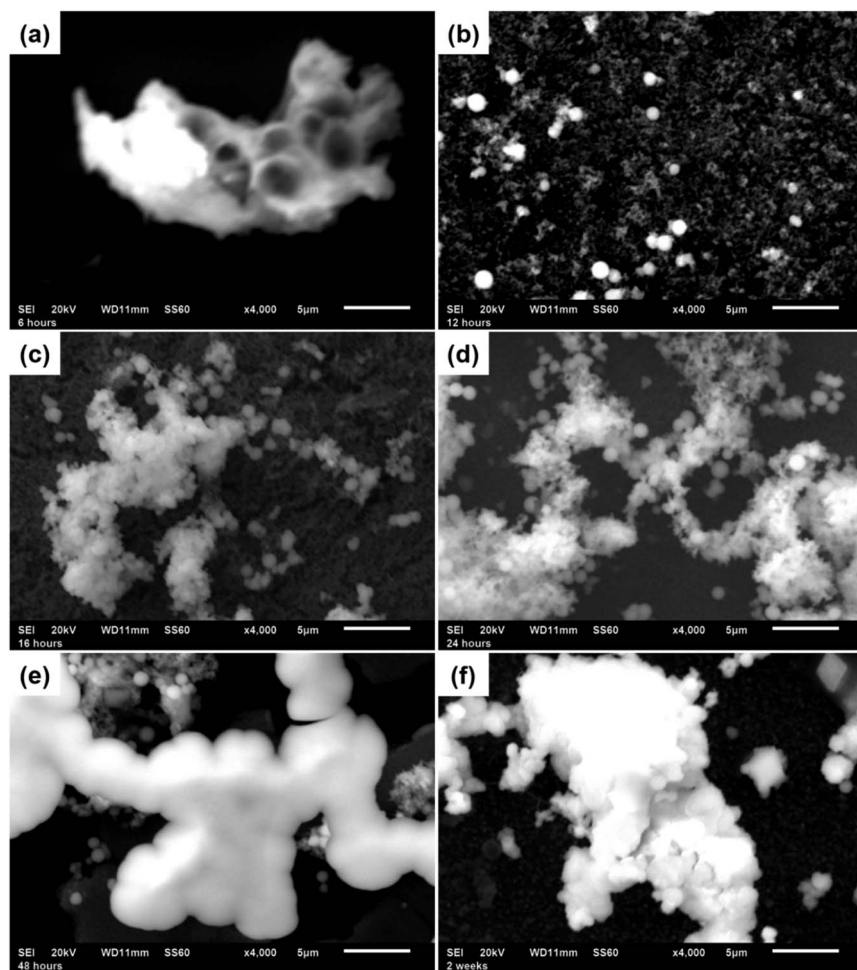


Fig. 9 Typical scanning electron microscopy (SEM) images of the SiN windows with deposited P₁₁-4 that were imaged on the I14 beamline (magnification = $\times 4000$). (a) 6 hours, (b) 12 hours, (c) 16 hours, (d) 24 hours, (e) 48 hours and (f) 2 weeks. The secondary electron detector was used at an energy of 20 kV, a working distance of 11 mm and a spot size of 60 nm.



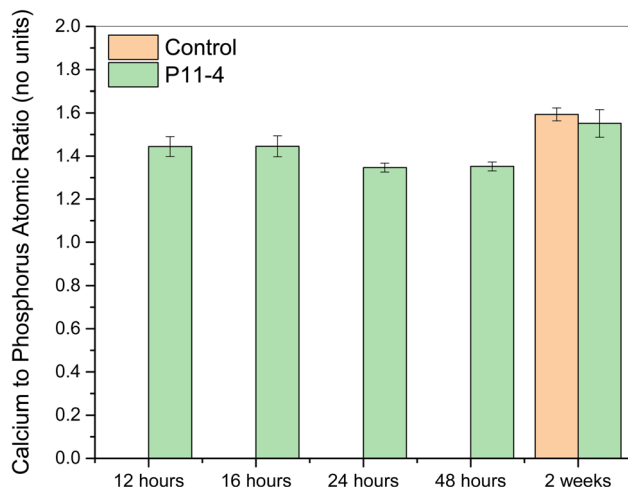


Fig. 10 Calcium/phosphorus atomic ratios, calculated from the atomic compositions, from the Energy Dispersive Spectrometry (EDS) analysis of the structures formed at 12 hours, 16 hours, 24 hours, 48 hours and 2 weeks. No calcium or phosphorus signals were detected for the control at the 12-, 16-, 24-, and 48-hour time points.

to form larger structures to support the results from the synchrotron experiment. These structures appear to be fusing together with time in mineralising solution. These larger structures start to connect with neighbouring structures to form a scaffold-like network as described earlier. In addition, Fig. B in the ESI† confirmed that the formed structures after 16 hours and 24 hours are made of calcium phosphate as the spatial maps showed calcium, phosphorus and oxygen signals.

After 48 hours in mineralising solution, the peptide appears to be inducing the formation of bigger calcium structures that form a larger network as shown in Fig. 9e. In the background (behind the large network of calcium phosphate structures), the spherical rounded structures can be observed, in which they appear to be migrating towards each other. The formed network of structures is confirmed to be a calcium phosphate phase as shown in Fig. C in the ESI.† After 2 weeks in mineralising solution, even bigger calcium phosphate structures start forming (Fig. 9f).

The calcium-to-phosphorus atomic ratio is similar at 12 and 16 hours (see Fig. 10), at around 1.4–1.5. The ratio decreases after 24 hours (to ~1.35) in mineralising solution, and is similar at 48 hours. The ratio increases after 2 weeks to ~1.5–1.6. As no signal was detected for the control samples at earlier time points (12–48 hours), the ratio could not be calculated.

4. Discussion

This study aims to investigate the effect of self-assembling peptide P₁₁₋₄'s fibrillar surface chemistry on calcium phosphate mineralisation in the absence of the 3D-gel network, which has been reported to nucleate hydroxyapatite (HAP).⁸

The results presented in this showed that more calcium phosphate structures were formed on the silicon nitride (SiN) windows at the early time points in the



After 12 hours of mineralisation, the calcium signal appeared to be higher than the phosphorus signal in the presence of P₁₁-4, indicating calcium ion accumulation on the deposited β -sheets. This supports the idea that P₁₁-4 acts as a template to promote mineralisation by providing nucleation sites, mimicking the role of organic matrix proteins (*e.g.* amelogenin) in facilitating nucleation and supporting mineral deposition through the presence of functional domains within their structure.²¹ The results suggest that in the first 12 hours, the calcium ions are attracted to the deposited P₁₁-4 fibrillar assemblies as suggested by *in*

In the study by Kirkham *et al.*,⁸ HAp was reported to form in the presence of a self-assembled, scaffold-like gel. The findings of this study suggest that P₁₁-4's surface chemistry results in the formation of what appears to be ACP rather than hydroxyapatite (HAp). The formation of ACP highlights P₁₁-4's role in biomimetic mineralisation as ACP is a known precursor of HAp during the biomineralisation process of dental enamel.²² During maturation, ACP is thought to transform to HAp in permanent mature enamel and control the crystal formation and growth process during amelogenesis. The transient phase, ACP, was proposed to be stabilised by enamel matrix proteins, like amelogenin, to control the mineralisation process.⁴ This transformation of ACP to HAp might be dependent on the presence of the assembled P₁₁-4 gel network. Longer studies are required to make better conclusions as the time scales for complete biomineralisation could be longer *in vivo*. The SiN windows can serve as substrates for characterising similar materials using the hard X-ray nanoprobe beamline (I14) at DLS.

An easy and robust method to deposit P₁₁-4 β -sheets to characterise the biomaterial at the nanoscale level, has been established. In comparison to the control (no P₁₁-4), P₁₁-4 induces and promotes the formation of amorphous calcium phosphate structures at early time points by providing sites for nucleation (calcium binding sites). After 12 hours in MS, the calcium signal was high on the SiN window showing that the calcium ions were attracted to the fibrils to form

The data for this study was collected as part of a PhD studentship in collaboration with Diamond Light Source (DLS) and is currently archived at DLS under experiment number mg32152. Following the completion of the PhD project, the data will be deposited in a repository. In the interim, the data is available upon request.

Reham Gonnah contributed to conceptualization, data curation, formal analysis, investigation, methodology, project administration, validation, visualization, writing the original draft, reviewing and editing. Julia Parker contributed to conceptualization, formal analysis, funding acquisition, investigation, methodology, resources, supervision, validation, visualization, writing the original draft, reviewing and editing. Robert Davies contributed to conceptualization, formal analysis, funding acquisition, investigation, methodology, resources, supervision, validation, writing the original draft, reviewing and editing. Maisoon Al-Jawad contributed to conceptualization, funding acquisition, methodology, resources, supervision, validation, writing the original draft, reviewing and editing.

There are no conflicts to declare.

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