Assembly of Amorphous Clusters under Floating Monolayers: A Comparison of \textit{in\ Situ} and \textit{ex\ Situ} Techniques

Ahmet Uysal,*,‡,§ Benjamin Stripe,†,|| Binhua Lin,‡ Mati Meron,‡ and Pulak Dutta*‡

†Department of Physics and Astronomy, Northwestern University, Evanston, Illinois 60208, United States
‡Center for Advanced Radiation Sources, University of Chicago, Chicago, Illinois 60637, United States

ABSTRACT: We report synchrotron X-ray scattering studies of biomimetic crystallization of hydroxyapatite (the primary constituent of bone), using monolayers of fatty acid molecules floating on simulated body fluid (SBF) as well as aqueous solutions of calcium phosphate. A ∼10 Å thick film of amorphous material is observed to form immediately at the molecular monolayer, consistent with the proposed formation of “Posner clusters”. This layer becomes denser but not significantly thicker as the subphase concentration and the temperature approach physiological conditions. The amorphous films do not crystallize within 24 h, in contrast to prior reports of more rapid crystallization using electron microscopy on \textit{ex\ Situ} samples. However, crystallization occurs almost immediately after our films are transferred onto solid substrates. These results illustrate the importance of \textit{in\ Situ} measurements for model biomineralization experiments.

1. INTRODUCTION

Vertebrate bone is perhaps the most complex and impressive biominal.* Its hierarchical structure is a good model of what today’s nanomaterials research aims to achieve: controlling nanoscale order to obtain advanced macroscopic properties. For example, the oriented growth of hydroxyapatite (HA) nanoplatelets with their c-axis parallel to the collagen fibrils, and the further microscale self-assembly of mineralized collagen fibrils, cause the Young’s modulus of mammalian leg bones to be much higher in the vertical direction. Therefore, significantly less material is used, compared to an isotropic material, to support the same body weight. It is widely accepted that the growth of bone apatite crystals (or, indeed, any biominal) is controlled by organic molecules present in the body fluid. Understanding how these organic molecules interact with the apatite crystals at the molecular level during the nucleation and the growth is not only crucial to implant and bone substitute development but also beneficial to the other applications of HA such as drug delivery.

Studying the nucleation and growth of HA at the molecular level \textit{in\ vivo} is not practical for most systems. Therefore, computer simulations and \textit{in\ vitro} experiments, mimicking relevant properties of biological systems are important. In particular, floating monolayers of amphiphilic molecules on aqueous subphases, i.e., Langmuir monolayers, provide a unique opportunity to study interactions of organic molecules with the nucleating crystals. Since they are at the air/water interface, Langmuir monolayers are easily accessible to \textit{in\ situ} experimental probes such as Brewster angle microscopy, infrared spectroscopy, and X-ray scattering. Also, crystals grown biomimetically under Langmuir monolayers can easily be transferred on solid substrates for \textit{ex\ Situ} microscopy, spectroscopy, and diffraction studies.

Because we have only mentioned the publications that are most relevant to our studies, they represent a tiny fraction of the studies in the calcium phosphate biomineralization field. We recommend recent reviews for more comprehensive overview of the literature.

Although the exact roles of collagen and noncollagenous proteins are still under debate, it is generally accepted that acidic residues on these organic molecules are responsible for their interactions with HA. Therefore, fatty acids are widely used as templates for model HA biomineralization experiments. However, monolayers with different head groups as well as more complex molecules, such as block copolymers, have also been used for the same purpose. One of the most important results of model HA biomineralization experiments under fatty acid monolayers is the ability to grow HA crystals with their c-axes parallel to the organic monolayer, which implies that acid-rich residues are responsible for the oriented growth of HA. However, the real mechanism causing the oriented growth remains unclear.

Similar questions have been asked about the oriented growth of other biominalers, such as calcium carbonate and calcium oxalate monohydrate (COM), for a long time. Our group’s \textit{in\ situ} X-ray diffraction studies have shown that in some cases a
1:1 lattice match is responsible for the oriented growth of crystals,21 while in some cases only a larger supercell is shared by the organic and the inorganic surface lattices.20,22 We have also recently showed that even if no lattice match can be found between the surface structures, changing the monolayer lattice spacing can result in the selection of different orientations of calcite.18

In recent years, both theoretical and experimental evidence have shown that the nucleation of many crystals proceeds via amorphous precursors.14,15 Four decades ago, on the basis of their X-ray diffraction studies, Posner et al. suggested11 that amorphous calcium phosphate (ACP) is made up from spherical ionic clusters with 9.5 Å diameter. The suggested chemical formula for these clusters was Ca$_{10}$(PO$_4$)$_6$(OH), and it nicely explains how ACP can transform into HA. Interestingly, the size and composition of these clusters do not depend on the supersaturation level of the solution or on the Ca/P ratio. Indeed, Dey et al. used cryo-TEM of collected samples to study the nucleation and growth of HA crystals under floating stearic acid monolayers15 and observed the formation of distributed Posner clusters in the simulated body fluid (SBF) almost immediately, in either the presence or absence of the monolayer. However, Posner clusters accumulated only at the organic template surface and formed amorphous calcium phosphate particles. These amorphous particles later transformed into HA nanoplatelets. Dey et al. also report that this mineralization happens only at mammalian body temperature, 37 °C. Their experiments at room temperature did not show densification of Posner clusters at the monolayer surface.

In spite of the vast number of bulk HA nucleation studies, there are few in situ studies at the molecule–mineral interface. Sato16 used infrared (IR) external reflection spectroscopy to probe the arachidic acid (CH$_2$$_n$)(CH$_2$)$_{18}$COOH) monolayer as HA crystals nucleate and grow under them. IR spectroscopy cannot determine monolayer surface lattice directly, but it is possible to infer whether the molecules are tilted or not from the interactions between the hydrocarbon chains. This study suggested that arachidic acid monolayers tilt in the early stages of crystallization and then become untitled. We have recently reported20 a similar structural change in the organic template during biomimetic COM crystallization. Both COM and HA crystallize in the bodies of vertebrates. Therefore, there may be a common mechanism to explain the reorganization of the organic monolayer in both experiments.

More recently, Habraken et al.14 have used a variety of in situ and ex situ studies to probe the nucleation of calcium phosphate, both homogeneously and heterogeneously on collagen molecules. They report that the nanometer-sized clusters are calcium triphosphate, which aggregate and take up calcium ions to form amorphous calcium phosphate (ACP) and ultimately hydroxyapatite. They argue that these results can be used to reconcile the observed dynamics of the process with classical crystallization theories.

Grazing incidence X-ray diffraction (GID) is a unique experimental tool that can provide in situ information about both organic template and inorganic crystal structures for biomimetic crystallization experiments under Langmuir monolayers.19,20,22,23 Therefore, in situ GID is the most reliable way to determine the epitaxy between the surface lattices and the tilt of the organic molecules. Besides, the scattering data are collected from a macroscopic area (~1 cm$^2$), which provides statistically significant averages and eliminates the small-sample problem that exists in both microscopy and spectroscopy studies.

However, if the inorganic material nucleating is not crystalline, it becomes invisible to GID. In such cases, complementary methods are available to see the electron density profile normal to the water surface. Specular X-ray reflectivity (XRR) is the most common method to study electron density profile of layered organic structures, but the time required for data collection with XRR is usually around 15–30 min. Since the amorphous precursors of biominerals are sometimes very sensitive to X-ray radiation, such long X-ray exposures are not desirable. Grazing incidence X-ray off-specular scattering (GIXOS) measurements have been shown35,36 to be a good alternative to XRR measurements; these two types of scans yield the same information, but GIXOS scans can be performed in the GID total-external-reflection geometry (and thus almost simultaneously with GID scans). Utilizing off-specular scattering measurements as an alternative to XRR was first suggested by Mora et al.33 Since then, GIXOS has been used to study properties of Langmuir monolayers,35,36 nanoparticle adsorption at organic templates,37 nanorod self-assembly at liquid surface,38 and gold nanoparticle thin films on solid substrates.39

In this paper we report in situ X-ray scattering studies of HA nucleation under Langmuir monolayers. We used a heneicosanoic acid (CH$_2$$_n$)(CH$_2$)$_{20}$COOH) monolayer as the template. We used calcium phosphate solutions with various concentrations for concentration-dependent experiments and SBF for temperature-dependent experiments. We conducted GIXOS and GID experiments simultaneously to determine the organic and inorganic structures in situ simultaneously at the early stages of the nucleation. We also transferred the thin calcium phosphate films from air/water interface onto solid substrates, and studied them using X-ray diffraction (XRD) and scanning electron microscopy (SEM) in order to see if the system behaves differently under ex situ conditions.

2. MATERIALS AND METHODS

2.1. Subphase and Langmuir Monolayer Preparation. We prepared calcium phosphate solutions according to the method described by Ball et al.13 for the concentration-dependent measurements. Both calcium and phosphate solutions were prepared in TRIS-HCl buffer at a total concentration of 12 mM. Later they were mixed in appropriate amounts to make solutions with 1.0, 1.5, 2.0, and 3.0 mM [Ca$^{2+}$] and [HPO$_4^{2-}$] concentrations. Prepared solutions are filtered through a 0.22 μm membrane.

We also prepared SBF solutions for temperature-dependent studies, in a similar way to that described by Kokubo et al.20 SBF has ion concentrations very similar to the real body fluid. In particular, it has 2.5 mM [Ca$^{2+}$] and 1.0 mM [HPO$_4^{2-}$] which are the same as the concentrations of those ions in human body plasma.

We spread heneicosanoic acid monolayer from chloroform solution over the subphase with a microsyringe. The monolayer then compressed to a surface pressure slightly above zero.

2.2. In Situ X-ray Scattering Experiments. GID and GIXOS experiments were conducted at Sector 15-ID (ChemMatCARS) of Advanced Photon Source by using a custom-made Langmuir trough with temperature control. We used a Pilatus 100 K pixel array area detector, located 0.6 m away from the sample, for quick and high-resolution data collection. The X-ray beam energy was 10 keV ($\lambda$ = 1.24 Å). For both experiments the incoming beam was fixed at grazing incidence geometry, which is slightly below the critical angle ($\alpha_c$ ~ 2
Article

mrad). A typical measurement period was below 4 min for both GID and GIXOS. The sample was translated perpendicular to the beam between each scan to prevent beam damage. Consecutive measurements at a constant spot were also taken to show the absence of beam damage for relevant time scales.

For GID measurements, the scattering intensity was measured as a function of the momentum transfer (\(q\)). The in-plane (\(q_{xy}\)) and out-of-plane (\(q_z\)) components of \(q\) were recorded as a contour intensity map by the area detector. One-dimensional diffraction scans were later derived from these contours by integrating the data over a small \(q_z\) interval. Technical details of these measurements have been reported previously.43

For GIXOS measurements, we moved the detector arm (2θ) slightly away from the direct beam (0,0,0) and measured the diffuse scattering intensity distribution as a function of \(q_0\), without moving the detector. By assuming that \(q_{xy} \approx 0\) and that the film is uniform in the lateral plane; we can write43

\[
I_{\text{diffuse}}(q_{xy}) \approx 0, q_z = B |T(q)|^2 \left| \frac{1}{\rho_0} \frac{d \rho}{dz} q_{xy} d z \right|^2
\]

where \(B\) is a constant which depends on the instrumental parameters and \(T(q)\) is the transmission function of the outgoing beam.43 This expression can be related to the specular XRR signal we would expect from the same system. Therefore, we can describe an “equivalent reflectivity curve” \(R_{eq}(q)\) as

\[
R_{eq}(q) = \frac{R_0(q)}{B |T(q)|^2} I_{\text{diffuse}}(q_{xy} \approx 0, q_z)
\]

We can treat \(R_{eq}(q)\) just as we treat regular XRR data and fit it using the Parratt formalism.43 A detailed comparison of XRR and GIXOS studies can be found in the publications by Wiegert et al.44 and Dai et al.44

2.3. Ex Situ Experiments. We also transferred the inorganic films onto silicon substrates for ex situ XRD and electron microscopy studies. The silicon substrates were cleaned with piranha solution (30:70 \(H_2O_2:HF\)) before the transfer. A Rigaku ATX-G high-resolution X-ray diffractometer and Hitachi S-3400 were used for XRD and SEM studies, respectively.

3. RESULTS

3.1. In Situ GID Experiments. We studied the in-plane fatty acid monolayer structure and possible mineralization-dependent changes to it by GID. A sample GID contour from the heneicosanoic acid monolayer is shown in Figure 1 (left). There is either a very broad in-plane peak centered at \(q_{xy} \sim 1.50–1.55\) Å\(^{-1}\) or more likely two in-plane peaks at \(q_{xy} \sim 1.5\) Å\(^{-1}\) and \(q_{xy} \sim 1.6\) Å\(^{-1}\). The crucial point is that there are no off-plane peaks (\(q_z \neq 0\)). This is a clear indication that the monolayer molecules are untitled (normal to the water surface).17 The GID data from the organic monolayer do not show any out-of-plane peaks at any point during the experiments; in fact, all GID contours are very similar to the one shown in Figure 1. We studied the vertical Bragg rod (BR) profiles of the peaks to calculate the thickness of the monolayer. The half-width at half-maximum (\(\Delta q_{BR}\)) of a BR is inversely proportional with the layer thickness \(d_{BR} \sim \pi/(\Delta q_{BR})\).45 The Bragg rod derived from Figure 1 (not shown) has a width \(\Delta q_{BR} \sim 0.1\) Å\(^{-1}\), which corresponds to a \(d_{BR} \sim 30\) Å. This thickness is in good agreement with the full length of the heneicosanoic acid molecule.

To observe the changes during mineralization, we derived one-dimensional horizontal diffraction scans (as a function of \(q_z\) at \(q_{xy} \sim 0\)) by integrating the GID contours between \(Q_z = 0.02–0.04\) Å\(^{-1}\) (Figure 1, right). The GID data do not change in the first 3 h. After 5 h the peak intensities drop due to the roughening of the surface. However, the peak positions do not change more than 0.01 Å\(^{-1}\). Formation of an inorganic film is indicated by a slight change in the optical reflectance of the film, visible to the naked eye, after 2–3 h (for 3 mM calcium phosphate solution and SBF). However, at no point did we observe any in situ diffraction peaks from the inorganic film forming under the fatty acid monolayer, not even after 24 h. This indicates that any inorganic material is disordered and that the time scales for in situ ordering are longer than 24 h.

3.2. In Situ GIXOS Experiments. We studied the concentration and temperature dependence of the electron density profile normal to the surface of the fatty acid monolayer at the very early stages of mineralization by GIXOS. Top panel of Figure 2 shows the GIXOS patterns for various subphase concentrations (open circles) and the best fits (solid lines). To obtain the control data (Figure 2a), we spread the monolayer on pure water and compressed it to an untitled phase, so we can use it as a reference to the data collected on the supersaturated subphase (Figures 2b–e and 3b–d). We used a three-box model to derive the electron density profiles from the GIXOS data (Figures 2 and 3, bottom panels). The first two boxes represent the lipids and the headgroup region of the fatty acid molecules, and the third box represents the inorganic film under the monolayer. The data fits were performed by starting with random values of the parameters

![Figure 1](https://example.com/figure1.png)
and then allowing the software to vary them without restrictions on their value until the best fit is found. The fitting parameters for the best fits are listed in Table 1.

We used only two boxes for the control experiment because in that case monolayer floats on pure water. The ∼30 Å thickness ($L_{T} + L_{M}$) of the monolayer is in good agreement with the GID Bragg rod calculation (previous section) and the known length of the molecule. Since our GID experiments show that the monolayer is always untilted for all subphase concentrations and temperatures, we used the tail density ($\rho_{T}$) and length ($L_{T}$) values obtained from the control as fixed parameters to fit the rest of the data. However, the qualitative trends in electron density profiles, and hence the conclusions we draw from them, do not change, even if we allow $\rho_{T}$ and $L_{T}$ to vary. When we fit the 1 mM concentration data (Figure 2b) with three boxes, the electron density for the third box goes to the electron density of water. Therefore, we listed results based on a two-box model fitting.

Roughness due to capillary waves contributes to all interfacial roughness parameters. In addition, there is some intrinsic interfacial roughness even if the water surface were perfectly smooth. Since we compressed the monolayer only slightly above the zero pressure, it is reasonable to expect that the monolayer and the very thin inorganic film (i.e., all interfaces) will follow the contours of the water surface. Therefore, we set all interfacial roughness parameters to be equal to each other ($\sigma$). However, we have also tried assigning separate roughness parameters to each interface, and this does not significantly change the results.

We used the same fitting methods for the temperature-dependent experiments with SBF (Figure 3). The same control data (Figure 3a) and electron density profile (Figure 3, bottom) are plotted again in Figure 3 for easier comparison.

Figure 2. Top: GIXOS data (open circles) collected from a heneicosanoic (C21) acid monolayer on calcium phosphate solutions with different concentrations ∼10 min after preparing the sample. The solid lines show the best fit to the data. (a) Heneicosanoic acid monolayer on pure water. (b–e) Heneicosanoic acid monolayer on supersaturated CaP solution with 1.0, 1.5, 2.0, and 3.0 mM [Ca^{2+}], respectively. Bottom: normalized electron density profiles derived from the best fits to the GIXOS data. The cartoon depicts the fatty acid monolayer and the Posner clusters accumulating underneath. The dashed lines indicate the positions of the layers used to model the system.

Figure 3. Top: GIXOS data (open circles) collected from a heneicosanoic (C21) acid monolayer on SBF solutions with different temperatures ∼10 min after preparing the sample. The solid lines show the best fit to the data. (a) Heneicosanoic acid monolayer on pure water and (b–d) heneicosanoic acid monolayer on SBF at 10, 20, and 37 °C, respectively. Bottom: normalized electron density profiles derived from the best fits to the GIXOS data. The cartoon depicts the fatty acid monolayer and the Posner clusters accumulating underneath. The dashed lines indicate the positions of the layers used to model the system.
we assigned to the headgroup \( (L_{\text{H}}) \) shows that the mineral layer actually penetrates into the headgroup region.

We obtain similar results with temperature dependence experiments (Figure 3, bottom). The density of the inorganic mineral layer increases with temperature, while its thickness stays around \( L_{\text{M}} \sim 9 \) Å. We observe the highest density for the mineral layer \( (\rho_{\text{M}}) \) at the highest temperature studied, 37 °C. However, in contrast to the report of Dey et al.,15 we do not observe anything qualitatively different happening at 37 °C. Indeed, there is no reason to expect 37 °C to be special since, for example, fish develop bones in spite of their much lower body temperatures.

### 3.3. Ex Situ XRD and SEM Experiments

We transferred the calcium phosphate films formed at the air/water interface from 3 mM solutions on to silicon substrates by vertical dipping, after 4 h of growth. The SEM images (Figure 4) show a uniform film made up from crystalline platelets with their thin edges parallel to the organic monolayer. This is very similar to the previous SEM studies of a similar system,4 suggesting the HA platelets grow with their \( c \)-axis parallel to the organic monolayer. We recorded the XRD pattern of the film right after the transfer and one week later (Figure 5). The immediate transfer and one week later (Figure 5). The immediate

**Table 1. Best Fit Parameters for in Situ GIXOS Experiments\(^a\)**

<table>
<thead>
<tr>
<th>[Ca(^{4+})]</th>
<th>( \rho_M/\rho_{\text{W}} )</th>
<th>( \rho_M/\rho_{\text{H}} )</th>
<th>( L_{\text{T}} ) (Å)</th>
<th>( L_{\text{H}} ) (Å)</th>
<th>( L_{\text{M}} ) (Å)</th>
<th>( \sigma ) (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 mM</td>
<td>0.90(^b)</td>
<td>1.12 ± 0.01</td>
<td>23.3(^b)</td>
<td>10.4 ± 0.1</td>
<td>3.6 ± 0.4</td>
<td>1.70 ± 0.02</td>
</tr>
<tr>
<td>1.5 mM</td>
<td>0.90(^b)</td>
<td>1.04 ± 0.30</td>
<td>23.3(^b)</td>
<td>2.1 ± 0.8</td>
<td>9.6 ± 3.1</td>
<td>2.07 ± 0.03</td>
</tr>
<tr>
<td>2.0 mM</td>
<td>0.90(^b)</td>
<td>1.06 ± 0.03</td>
<td>23.3(^b)</td>
<td>4.1 ± 0.3</td>
<td>9.2 ± 0.9</td>
<td>2.39 ± 0.02</td>
</tr>
<tr>
<td>3.0 mM</td>
<td>0.90(^b)</td>
<td>1.02 ± 0.06</td>
<td>23.3(^b)</td>
<td>3.6 ± 0.4</td>
<td>9.8 ± 0.8</td>
<td>2.81 ± 0.02</td>
</tr>
</tbody>
</table>

\(^a\) \( \rho_T, \rho_{\text{H}}, \rho_M \), and \( \rho_{\text{W}} \): electron density of the tail group, the headgroup, the mineral layer, and water, respectively. \( L_{\text{T}}, L_{\text{H}}, \) and \( L_{\text{M}} \): thickness of the tail group, headgroup, and the mineral layer, respectively. \( \sigma \): interfacial roughness between the layers. The electron density of the subphase and superphase are fixed to 1 and 0, respectively, in all cases. \(^b\) Length and density parameters for tail groups are fixed to the values obtained from the control sample on pure water.

**Figure 4.** SEM image of calcium phosphate film transferred on a silicon substrate after 4 h of mineralization under heneicosanoic acid monolayers from a 3 mM calcium phosphate supersaturated solution. The inset shows a higher magnification image of the same sample. Thin platelets of crystals are mainly aligned perpendicular to the surface as observed in previous HA\(^4\) and OCP\(^4\) crystallization experiments.

**Figure 5.** X-ray diffraction data for a calcium phosphate film transferred onto a silicon substrate. These data were obtained using a laboratory X-ray source. Patterns are shifted vertically for clarity. (a) The diffraction pattern of the film 2 h after the transfer shows that the film is mainly OCP. (b) The diffraction pattern from the same film 1 week after the transfer shows that OCP was transformed into HA.

measurement shows that the film is initially octacalcium phosphate (OCP) (Figure 5a). OCP \( [(\text{Ca}_{10}\text{H}_{2}\text{(PO}_{4}\text{)}_{6}\cdot 5\text{H}_{2}\text{O})] \) is a well-known precursor for HA\(^{1,11,12,14,46-48}\). Indeed, their crystal structures are so close to each other that it is not possible to distinguish a few peaks. However, OCP has a unique peak at low \( q \) (0.34 Å\(^{-1}\)) which makes it easy to identify (ICDD PDF#: 00-026-1056).

When we repeat the same measurement 1 week later, we observe an increase in (010) OCP peak intensity and also HA (100) and (200) and (211) peaks appear (Figure 5b). This suggests that ACP to OCP and OCP to HA transformations happen with time. The comparable intensity of the (211) peak, which is the strongest in powder diffraction, to the (100) and (200) peaks suggests that the HA crystals are oriented with their \( c \)-axis parallel to the substrate, confirming the previous observations\(^6\) and the conclusions from the electron micrographs (Figure 4).

### 4. DISCUSSION

#### 4.1. Crystallinity of the Calcium Phosphate Film

One of the most interesting results of this study is the absence of in situ GID peaks from the calcium phosphate film, even after 24 h, because previous ex situ studies\(^6\) under similar conditions
reported HA crystal formation within that time period. We have previously shown that the in situ GID with high brilliance synchrotron X-rays is very sensitive to crystal formation, and it is possible to detect diffraction peaks even at very early stages. Therefore, it is not possible to miss diffraction peaks if they existed. Moreover, we have transferred the films onto solid substrates only after 4 h of growth (similar to the previously reported experiments) and observed diffraction peaks with an in-home X-ray diffractometer, which has couple of orders of magnitude less intensity than the synchrotron source we have used.

Thus, our observations clearly show that the calcium phosphate film forming under the fatty acid monolayer stays amorphous longer than it has been assumed previously. The transfer process causes film to crystallize much faster than it would do in the solution. Therefore, for ex situ measurements transfer and drying methods and even the storage time before the measurement is very important (Figure 5). Quite generally, ex situ data such as TEM2,19 should be treated more carefully.

4.2. Structure of the Fatty Acid Monolayer. Another interesting result of our experiments is the steady structure of the heneicosanoic acid monolayer during the mineralization. At all concentrations and temperatures the in situ GID data show in plane peak(s) for the organic monolayer from beginning to the end of the experiment. This is in contrast to the mechanism inferred from in situ IR external reflection spectroscopy.4 According to that model, fatty acid molecules tilt during the first 3 h after sample preparation and then become tilted.

As discussed earlier, spectroscopy experiments cannot determine the monolayer structure directly, but the structure is inferred from the relative intensity changes of C=H stretching peaks in the spectrum.4 On the other hand, considering the simple 2-dimensional geometry of the monolayer, GID gives direct information about the monolayer structure. GID is especially very sensitive to the tilting of the molecules. Even a couple of degrees of tilting creates a qualitatively different diffraction pattern.17

Considering this result together with our previous studies of COM20 and gold19 crystallization under floating monolayers shows the importance of amorphous precursors in the mineralization process. In both COM and gold experiments, crystallization starts almost immediately without any precursors. Interestingly, in both cases the organic monolayer structure changes with time to satisfy a lattice match with the growing crystals. On the other hand, amorphous calcium phosphate does not have such an effect on the monolayer.

4.3. Interactions of Posner Clusters with the Fatty Acid Monolayer. The electron density profiles derived from GIXOS data provide in situ evidence of the prenucleation clusters pathway described by the recent experiments.15 The thickness of the mineral layer (Tm, Table 1) is around 9 Å for all conditions (except 1 mM); its density (ρm) increases as the subphase concentration and the temperature increase. It should be noted that the GIXOS data do not contain lateral information and do not show individual clusters. However, the layer thickness remains almost the same under a range of conditions and nicely matches the size of the predicted Posner clusters. Therefore, we can say that the Posner clusters exist in the subphase immediately after preparation and that they accumulate under the fatty acid monolayer.

Note that for the most dilute (1 mM) subphase the ion concentration is probably not high enough to form Posner clusters. We observe only the adsorption of ions around the headgroup region and an increase in the thickness of this layer. If we fit the 1 mM data by using a three-box model, the density of the third box goes to the electron density of water, and the electron density profile does not change at all.

As with XRR experiments, the electron density profiles generated from GIXOS data are not unique. The profiles depend on the model selected. Therefore, it is necessary to use other measurement methods to limit the possible solutions. In this case, the supplementary information comes from the GID data and the amount of the monolayer we spread on the subphase. Since GID data clearly show that the fatty acid monolayer is untitled from the beginning to the end of the experiment, and we spread the same amount of fatty acid molecules over the same trough area, we know that the thickness (L) and the density (ρ) of the layer we assigned to the tail group should be same for all temperatures and concentrations. Using this information to fix the parameters helps us make comparisons between different conditions more easily. However, we also tried fitting the GIXOS data without fixing these parameters and obtained the same qualitative trend showing the increasing density of Posner clusters with the increasing temperature and the subphase concentration.

Selecting a certain number boxes for the model also needs to be justified. We have found that it is not possible to fit the mineralization data (except 1 mM) by using a one- or two-box model. On the other hand, using four or more boxes can improve the goodness of the fits (as expected, since there are more variable parameters), but matching the parameters with physical reality becomes harder. Also, defining layers much smaller than the spatial resolution (∼5 Å) is not reasonable. Therefore, using three boxes for the tail, headgroup, and mineral layers gives reasonable fits and matches well with our previous information about the system.

5. CONCLUSIONS

We have studied the nucleation and growth of calcium phosphate films under heneicosanoic acid monolayers and compared the in situ and ex situ measurements. While our ex situ experiments showed crystalline OCP formation, which transformed to HA later, the in situ GID measurements strongly suggested that any crystallization observed in time scales less than 24 h are a result of the transfer itself. It has to be noted that all our experiments are conducted with solutions with low supersaturation levels, which do not show any precipitation without the organic monolayer. Therefore, crystallization experiments with different kinetic conditions may have shorter crystallization times. The in situ GID experiments also revealed that the monolayer studied does not change structure during the process. While this result may be specific to the molecules and experimental conditions used, it is in agreement with the fact that the inorganic film is amorphous and therefore cannot force the organic film to change its structure by imposing an external periodicity. This can be contrasted to minerals such as gold and COM, which crystallize immediately and force the monolayer to reorganize so that there is a lattice match.10,20

The GIXOS experiments show the in situ evidence for Posner clusters at the organic—inorganic interface and are generally in good agreement with the ex situ TEM studies.15 However, we find that the adsorption of Posner clusters to the organic monolayer is not limited to mammalian body temperature as suggested in that study. We observed that immediately after spreading the fatty acid monolayer a ∼9 Å thick layer forms under it. As we bring the system closer to the
physiological conditions, in terms of concentration and temperature, the density of this layer increases; however, its thickness does not change, suggesting that the number of clusters in the plane increases, which does not affect the thickness but increases the density.

In summary, we have conducted the first in situ experiment giving direct structural information about the organic–inorganic interface during the nucleation of calcium phosphate under a fatty acid monolayer. Our results stress the importance of in situ measurements and suggest a careful approach to effects of transfer processes in nucleation and crystallization studies.

■ AUTHOR INFORMATION

Corresponding Author
*E-mail: ahmet@anl.gov (A.U.); p dutta@northwestern.edu (P.D.).

Present Addresses
§A.U.: Chemical Sciences and Engineering Division, Argonne National Laboratory, 9700 South Cass Avenue, Argonne, IL 60439.
§R.S.: Advanced Photon Source, Argonne National Laboratory, 9700 South Cass Avenue, Argonne, IL 60439.

Notes
The authors declare no competing financial interest.

■ REFERENCES

(8) Yang, Y.; Cui, Q.; Sahai, N. How does bone sialoprotein promote the nucleation of hydroxyapatite? A molecular dynamics study using model peptides of different conformations. Langmuir 2010, 26 (12), 9848–9859.


