In-situ ellipsometric study of calcium phosphate biomineralisation on organic thin films

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Abstract: In this study, in-situ ellipsometry and scanning electron microscopy (SEM) were used to follow the growth kinetics of calcium phosphate (CaP) films on zein and hexadecanoic acid (HDA) layers self-assembled at the air-liquid interface. The duration of the mineral film induction period was heavily dependent on the organic template used, presence or absence of Mg²⁺, solution pH and concentration. In a standard simulated body fluid (SBF)
solution at 20°C and pH 7.4, CaP film growth occurred after an induction period of 40 min for HDA and 100 min for zein. In the absence of Mg²⁺ the mineralisation induction period was reduced, for only the zein protein, to 50 min. At pH 6, HDA and zein-induced CaP films started forming after 100 min and 180 min, respectively. No induction period was observed when the SBF concentration was doubled, with immediate CaP growth observed for both HDA and zein. This study shows the effect of biomimetic conditions on the growth of CaP with the organic template playing an important role. Knowledge of the factors that influence mineral growth processes is of importance in areas of biomimetic and controlled crystal growth.

**Keywords:** biomimeticisation; zein protein; calcium phosphate; organic template; air-water interface.


**Biographical notes:** Rayomand Shahlori is a second-year PhD student having previously graduated with a BSc (Hons.) in September 2013, under the supervision of Dr. Geoffrey Waterhouse. He is currently undertaking his PhD studies supervised by Dr. Duncan J. McGillivray. His research involves structural and kinetic characterisation biomimetic films.

Geoffrey I.N. Waterhouse is a Senior Lecturer in the School of Chemical Sciences at the University of Auckland, and a principal investigator in the MacDiarmid Institute of Advanced Materials and Nanotechnology. He is also a Chair Professor in the School of Materials Science and Engineering at the South China University of Technology in Guangzhou. His research explores fundamental relationships between the chemical, structural, electronic and optical properties of solids and their function, especially photonic band gap materials and semiconductor photocatalysts.

Andrew R.J. Nelson is an Instrument Scientist for the PLATYPUS neutron reflectometer. His main interests lie in the soft condensed matter area, specifically the interactions of polymers and surfactants with interfaces/colloidal systems – as studied by Neutron Reflectometry and Small-Angle Neutron Scattering.

Duncan J. McGillivray is a Senior Lecturer and Researcher at the University of Auckland who trained in neutron and X-ray scattering in the UK and USA, before returning to set-up a research group in New Zealand. His research involves looking at the surface structures of biological systems using surface sensitive methods. His current research is focussed on understanding the physical bases of biological interactions at surfaces, particularly through investigating the structure of complex non-crystalline protein systems, including membrane proteins and protein colloids.

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1 Introduction

Biomineralisation is a natural process in which living organisms use organic matrices to control the size, morphology and crystal structure of inorganic materials. A common example is mammalian bones and teeth, which are made from nanostructured calcium phosphate. The high strength and lightweight properties of these hard tissues arise from interactions between the organic template and the inorganic precursor [1]. Proteins are typically responsible for nucleating biominerals, with carboxylic acid containing amino acids being vital for initial mineral growth [2–5]. Biomimetic mineral growth is an important area of research as replicating natural strategies could result in the development of more efficient and environmentally benign nanoparticle synthesis procedures. In addition, novel biomimetics would possess more compatibility towards biological systems [6] allowing for new materials to be applied as mineral coatings [7–9], adsorbents/release agents [10–12] and as new composite materials. However, in order to achieve this, the organic template used should be inexpensive, readily available, non-toxic and sustainably sourced. Zein protein provides these traits and has been shown to induce calcium phosphate mineralisation [13–15]. Zein is an amphiphilic, water-insoluble protein that self-assembles into nanostructures upon increasing the hydrophilicity of the surrounding environment [16–18]. We have shown that zein and hexadecanoic acid (HDA) influence calcium phosphate mineral film morphology at the air-water interface [19].

The effects of biomineralisation solution conditions is crucial for understanding natural mineral growth processes, as living organisms are known to regulate such conditions to ensure controlled structures are reproducibly formed [20,21]. In the case of seashell formation by molluscs, the presence of Mg$^{2+}$ ions and pH of sea-water is known to influence calcium carbonate formation [22]. The cause of Mg$^{2+}$ inhibition of biomineral growth is often attributed to its affinity for Ca$^{2+}$ binding sites [23]. The pH of the system is also very critical for biomineralisation. Acidic environments (pH < 6.5) increase the solubility of biominerals resulting in slower growth during mineralisation. In this work we investigate the effect of Mg$^{2+}$ and pH on calcium phosphate mineralisation at the air-water interface.

The air-liquid interface is a suitable model for studying biomimetic mineralisation as natural mineralisation occurs on surfaces or gel phases [13,24–28]. The flat surface provided at the air-liquid interface allows application of reflection-based techniques to obtain structural information from a system [29]. Ellipsometry is an example of such a technique, which uses polarisation changes of incident light upon interaction with a surface to infer physical properties such as film thickness. Here we use in situ ellipsometry for studying CaP mineralisation on different organic thin films, this technique is supported by scanning electron microscopy (SEM). We evaluate the effect of Mg$^{2+}$ ions, pH and concentration of the simulated body fluid (SBF) on mineralisation kinetics using two organic templates (zein and HDA). We have shown in a related study that the calcium phosphate mineral films formed using both zein and HDA were found to be an amorphous hydroxyapatite, using XRD and FT-IR analysis [19].
2 Methods and materials

Zein protein (CAS 9010-66-6) and hexadecanoic acid (Palmitic Acid, CAS 57-10-3, P0500) were obtained from Sigma Aldrich and used without further purification. Ethanol-water (80:20 by volume) solutions of zein were prepared at a concentration of 1 mg mL$^{-1}$. HDA was dissolved in chloroform to make a 0.5 mg mL$^{-1}$ solution. A supersaturated calcium phosphate solution was prepared using an ionic composition and concentration similar to human blood plasma. This solution is a SBF and was prepared by dissolving various salts in Milli-Q H$_2$O, following a similar recipe to that of Tas and Bhaduri [9]. Table 1 shows concentration of ions within the SBF solution, with an ionic strength of 193 mM and a pH of 7.4. A 50 × 50 mm Teflon well was used to hold 10 mL of SBF solution, and either 50 µL of zein protein solution or 10 µL of the HDA solution was spread on the surface to induce mineralisation.

Table 1 Concentration of ions in SBF solution

<table>
<thead>
<tr>
<th>Ion</th>
<th>Na$^+$</th>
<th>K$^+$</th>
<th>Ca$^{2+}$</th>
<th>Mg$^{2+}$</th>
<th>Cl$^-$</th>
<th>HPO$_4^{2-}$</th>
<th>CO$_3^{2-}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>mmol L$^{-1}$</td>
<td>160</td>
<td>5</td>
<td>3</td>
<td>1</td>
<td>152</td>
<td>1</td>
<td>10</td>
</tr>
</tbody>
</table>

Solution pH = 7.4, Total ionic strength = 193 mmol L$^{-1}$.

2.1 Ellipsometry

Measurements were taken on a Beaglehole Instruments Imaging Ellipsometer [30] at variable angles (40° $< \theta < 60^\circ$) using a quartz halogen light source with a wavelength of 632 nm selected by an interference filter. Data was fitted to a two slab model using the software Thin Film Companion [31] with the Levenberg-Marquardt algorithm used to minimise least squares. A fixed refractive index of 1.433 was used for modelling the amorphous calcium phosphate [32] layer, and 1.450 for the zein protein [33]. Measurements were taken at 20°C every 5 min during mineralisation and plotted to show the mineral film growth over time.

2.2 Scanning electron microscopy

Images were taken using a Philips XL-30S field emission gun scanning electron microscope (FEGSEM) operated at an electron accelerated voltage of 5 kV in high vacuum. Prior to analysis, specimens were mounted on black carbon tape and sputter coated with platinum for 60 s with a Quorum Q150RS to reduce the specimen charging. Mineral films were rinsed with Milli-Q H$_2$O to remove dissolved salts before being placed onto a glass slide substrate for imaging.

3 Results and discussion

The mineralisation of CaP films on organic templates proceeds through an initial induction period, namely a period in which no significant film growth is observed. During this stage the assembled organic template (HDA or zein) at the air-liquid interface induces adsorption of Ca$^{2+}$ and PO$_4^{3-}$ ions, which continues until enough adsorption has occurred such that CaP nuclei form.
In situ ellipsometric study of calcium phosphate biomineralisation

In situ ellipsometry measurements show the effect of Mg\(^{2+}\), pH 6 SBF and 2× SBF on CaP induction and growth, with parameters from these measurements displayed in Table 2. During induction, the mineral thickness does not increase more than 1 nm. CaP film growth began after 100 min for the zein protein and 50 min for the HDA layer at pH 7.4. After the induction stage, there is a gradual increase in the rate of mineral film growth, reaching a maximum growth rate when each film is approximately 40 nm thick. For both films the growth rate decreases above a thickness of 40 nm with mineralisation continuing throughout the 5 h measurement.

**Table 2**  
Kinetic parameters obtained from ellipsometry measurements

<table>
<thead>
<tr>
<th>Mineralisation condition</th>
<th>CaP induction time (min)</th>
<th>Maximum growth rate (nm min(^{-1}))</th>
<th>CaP film thickness after 5 h (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDA SBF pH 7.4</td>
<td>40 ± 3</td>
<td>0.81 ± 0.04</td>
<td>119 ± 6</td>
</tr>
<tr>
<td>Zein SBF pH 7.4</td>
<td>100 ± 5</td>
<td>1.13 ± 0.04</td>
<td>104 ± 4</td>
</tr>
<tr>
<td>HDA 2× SBF pH 7.4</td>
<td>0</td>
<td>3.41 ± 0.06</td>
<td>338 ± 6</td>
</tr>
<tr>
<td>Zein 2× SBF pH 7.4</td>
<td>0</td>
<td>3.19 ± 0.05</td>
<td>242 ± 4</td>
</tr>
<tr>
<td>HDA SBF (without Mg(^{2+}))</td>
<td>40 ± 3</td>
<td>1.12 ± 0.06</td>
<td>120 ± 6</td>
</tr>
<tr>
<td>Zein SBF (without Mg(^{2+}))</td>
<td>50 ± 5</td>
<td>1.23 ± 0.05</td>
<td>135 ± 6</td>
</tr>
<tr>
<td>HDA SBF pH 6.0</td>
<td>100 ± 4</td>
<td>0.58 ± 0.02</td>
<td>78 ± 3</td>
</tr>
<tr>
<td>Zein SBF pH 6.0</td>
<td>180 ± 7</td>
<td>0.49 ± 0.04</td>
<td>42 ± 3</td>
</tr>
</tbody>
</table>

The effect of Mg\(^{2+}\) ions, SBF pH and concentration on both HDA and zein-induced CaP film formation is shown in Figures 1 and 2, respectively. The absence of Mg\(^{2+}\) has resulted in a reduced induction time for zein, however for HDA this effect was not as pronounced. Mineralisation using pH 6 SBF caused the CaP induction period to be extended for both zein and HDA, compared to pH 7.4. After induction, the pH 6 SBF system shows a slower mineral growth rate relative to the rate observed at pH 7.4. When using a 2× SBF there was no induction period even in the presence of Mg\(^{2+}\) ions, with mineralisation occurring immediately and proceeding at a higher rate.

Kinetic parameters obtained from the ellipsometry measurements are displayed in Table 2. These results show the effect Mg\(^{2+}\), pH and SBF concentration has on CaP growth. For both HDA and zein-induced CaP mineralisation in 2× SBF, no induction time was observed, the fastest maximum growth rate and the thickest CaP films were obtained. Growth rates of 3.4 nm min\(^{-1}\) and 3.2 nm min\(^{-1}\) were observed for HDA and zein using 2× SBF, respectively, compared to 0.8 nm min\(^{-1}\) and 1.1 nm min\(^{-1}\) for the standard SBF concentration. The mineral films were thicker after 5 h of growth from 2× SBF, CaP films were found to be 338 ± 6 nm and 242 ± 4 nm in thickness for HDA and zein, respectively. This is the result of a higher level of calcium phosphate supersaturation within the 2× SBF. The absence of Mg\(^{2+}\) within the SBF caused a reduction in CaP induction from 100 ± 5 min to 50 ± 5 min on the zein template. However, for HDA this effect was not observed owing to the higher affinity of Ca\(^{2+}\) for the carboxylated surface. Mineralisation performed at SBF pH 6 was found to induce the longest induction time, slowest growth rates and thinnest films. HDA and zein induction times were extended to 100 ± 4 min and 180 ± 7 min, respectively, compared to 40 ± 3 min and 100 ± 5 min at pH 7.4. The slightly acidic environment also reduced
the maximum growth rates to 0.6 nm min$^{-1}$ and 0.5 nm min$^{-1}$ from 0.8 nm min$^{-1}$ and 1.1 nm min$^{-1}$ for HDA and zein, respectively. The CaP film thickness after 5 h of mineralisation at pH 6 was $78 \pm 3$ nm and $42 \pm 3$ nm for HDA and zein, considerably thinner than the SBF pH 7.4 thickness of $119 \pm 6$ nm and $104 \pm 4$ nm. Mineralisation was greatly affected at pH 6 as CaP is known to be more soluble in acidic environments. A shift to pH 6 also results in a net positive charge on the zein protein surface, as its isoelectric point [34] is 6.8, thus zein-induced mineralisation is further hindered owing to the formation of a less favourable surface for Ca$^{2+}$ adsorption.

Figure 1 In situ ellipsometry measurements showing the effect of solution conditions on hexadecanoic acid-induced calcium phosphate film mineralisation (growth rate is shown as dashed lines, axes on right) (see online version for colours)

Figure 2 In situ ellipsometry measurements showing the effect of solution conditions on zein-induced calcium phosphate film mineralisation (growth rate is shown as dashed lines, axes on right) (see online version for colours)
SEM images for CaP mineral films grown from HDA and zein at the air-liquid interface are shown in Figure 3. We have reported the cross-sectional images of mineral films produced from zein and HDA, which reveal distinct morphologies that are dependent on the organic template [19]. The HDA-induced CaP appears to be flat and continuous. This is expected as the densely packed carboxylic acids groups provide a planar, negatively charged surface for calcium phosphate formation. The CaP film formed using zein protein is made up of a collection of quite monodisperse partially-fused mineral hemispheres with a radius of ~150 nm. As further mineralisation occurs, the hemispheres expand and fuse forming a film that is continuous. The morphological distinction is attributed to the localised negative charges on the initial zein protein template [35]. The hydrophilic residues of the protein consist of amine groups (glutamine, lysine) and carboxylic acid groups (glutamic acid, aspartic acid). The negative charge from the carboxylate regions will induce calcium phosphate formation at specific regions of the protein template. The difference between the induction sites for HDA and zein results in the alternative mineral morphologies. Figure 3 also shows that Mg$^{2+}$ has little effect on the morphology of the CaP films. Ellipsometry results reveal that Mg$^{2+}$ hinders mineral growth, this is confirmed using SEM of the mineral films at specific time periods. Images shown in Figure 3 show the film grown without Mg$^{2+}$ appear slightly thicker than those grown with Mg$^{2+}$.

**Figure 3** SEM images showing the calcium phosphate film mineralised using HDA (A, C) and zein (B, D) after 150 min from a 2× SBF. Panels C and D show films formed using 2× SBF without Mg$^{2+}$. Scale bar: 500 nm

**4 Conclusion**

Ellipsometry is a powerful technique for following biomimetic mineralisation on organic films such as zein and HDA. CaP growth on zein protein has a longer induction period than on HDA in SBF solution. Which we postulate is owing to a lower surface charge density. Mg$^{2+}$ ions suppressed CaP growth for zein, which showed a longer induction time. For both organic templates mineralisation at pH 6 SBF also resulted in longer induction periods, owing to the increased solubility of CaP. The 2× SBF resulted in no detectable induction, despite the inhibiting effects of Mg$^{2+}$. Studying the effect of organic templates and solution conditions on biomimmineralisation kinetics may lead to many useful strategies for synthesising mineral coatings, adsorbents and as new composite materials.
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References and Notes

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