S Habelitz
PK DenBesten
SJ Marshall
GW Marshall
W Li

Amelogenin control over apatite crystal growth is affected by the pH and degree of ionic saturation

Authors' affiliations:
Stefan Habelitz, Sally J. Marshall, Grayson W. Marshall, Department of Preventive and Restorative Dental Sciences, University of California, San Francisco, CA, USA
Pamela K. DenBesten, Wu Li, Department of Growth and Development, University of California, San Francisco, CA, USA

Correspondence to:
Stefan Habelitz
Department of Preventive and Restorative Dental Sciences
University of California
707 Parnassus Avenue
D-2260
San Francisco
CA 94143-0758
USA
Tel.: 415 514 0818
Fax: 415 476 0358
E-mail: shabeli@itsa.ucsf.edu

Dates:
Accepted 10 August 2005

To cite this article:
Orthod Craniofacial Res **8**, 2005; 232–238
Habelitz S, DenBesten PK, Marshall SJ, Marshall GW, Li W:
Amelogenin control over apatite crystal growth is affected by the pH and degree of ionic saturation

Structured Abstract

Authors – Habelitz S, DenBesten PK, Marshall SJ, Marshall GW, Li W

Objective – To study the mechanisms which promote the interactions of amelogenin proteins with the forming mineral to establish suitable conditions for the biomimetic synthesis of enamel *in vitro*.

Design – Saturated calcium phosphate solutions were used in conjunction with recombinant amelogenin proteins to induce mineral formation on glass-ceramics substrates containing oriented fluoroapatite crystals (FAP). The height of mineral layers formed on these substrates within 24 h was measured by atomic force microscopy (AFM).

Experimental Variables – The effect of protein concentration, pH and degree of saturation (DS) on the growth of apatite mineral was evaluated. Mineralization experiments were performed at 0, 0.4 and 1.6 mg/ml amelogenin concentrations. Mineralization solutions were used at pH values of 6.5, 7.4, 8.0 and 8.8 and DS of calcium and phosphate between 9 and 13.

Outcome Measure – Height and morphology of mineralized layer formed on glass-ceramic substrates as determined from AFM measurements.

Results – Homogeneous nucleation and crystal growth of thin layers on the FAP were observed, when calcium and phosphate ions were added. The height of these layers grown on (001) planes of FAP was strongly dependent on the protein concentration and pH. At concentrations of 0 and 0.4 mg/ml crystal grew 5–15 nm on the FAP, while they grew approximately to 200 nm at 1.6 mg/ml. The enhanced crystal growth was observed only at pH 6.5, 7.4 and 8.0, while layers only 20 nm thick were obtained at pH 8.8. An increase in DS resulted in uncontrolled growth of calcium phosphate mineral covering large areas of the substrate.

Conclusions – Protein concentration, pH and the saturation of the mineralizing solution need to be considered carefully to

provide suitable conditions for amelogenin-guided growth of apatite crystals.

Key words: amelogenin; apatite; atomic force microscopy; enamel; mineralization

Introduction

The extracellular matrix of developing enamel is unlike other matrices of mineralized tissues. For bone, dentin and cementum mineralization arises from a fibrous collageneous matrix that reinforces itself through the incorporation of apatite nanocrystals, while enamel maturation is generated quite differently (1). Formation of a viscous gel directs the growth of fibrous nanocrystals at the same time as it is degraded to facilitate the transformation into a tissue consisting of 95% mineral (2).

Amelogenin proteins are the main compound of the developing enamel matrix and have been associated with the formation of long fibrous and aligned apatite crystals in dental enamel. Amelogenin is highly hydrophobic and assembles *in vitro* into nanospheres of about 15–30 nm diameter and into a more heterogeneous distribution between 10 and 200 nm *in vivo* (3). Amelogenin has been shown to act as a signaling molecule and cell-adhesion protein (4,5). Amelogenin also binds to specific sites on the long axis of enamel crystallites (6) and several functions related to protein–mineral interaction have been postulated. These include: 1) amelogenin acts as a spacer between growing crystals and prevents early crystal fusion; 2) amelogenin prevents crystal growth perpendicular to the *c*-axis of apatite, but may enhance growth rates parallel to the *c*-axis and 3) amelogenin promotes the formation of thin, (diameter 50 nm) long (several 100 μm) and aligned apatite crystals packed into enamel rods. *In vitro* experiments confirmed an effect of amelogenin on crystal morphology (3). At concentrations above 50 μg/ml of recombinant amelogenin, the formation of significantly more elongated apatite crystals compared with protein-free solutions was observed (7).

Amelogenin proteins have a low solubility limit in aqueous solutions and tend to precipitate at concentrations of 0.7 mg/ml at physiological pH (8). In a recent study, we reported that the growth rate of the (001) plane of apatite crystals as well as the binding specificity to apatite increases by about 20 times if amelogenin proteins were present at concentrations above the solubility limit (9).

The ionic conditions in which the enamel matrix matures are strictly regulated by a layer of ameloblast cells. Calcium levels (0.5 mM) are unusually low compared to the surrounding tissues or blood plasma. The pH of the fluid space under the ameloblasts varies at different stages of the amelogenesis, between approximately 5.8 and 7.4 (10). This suggests that the physical–chemical driving force for crystallization, e.g. the degree of saturation (DS), is kept low to facilitate the transfer of control over crystal nucleation and growth to the matrix protein. In this study, we tested the hypothesis that only low DS of the mineralizing solution and a narrow pH range will enable amelogenin proteins to interact with the forming apatite crystal and control their texture and morphology.

Materials and methods

A glass-ceramic containing rod-like fluoroapatite (FAP) crystals (1 μm wide, 3–6 μm long) was used to immobilize proteins and study the protein–mineral interactions in calcium phosphate solutions (11,12). These substrates are unique because of their uniaxial alignment of FAP manufactured by high-temperature extrusion. The application of this glass-ceramic for *in vitro* mineralization studies has been described recently (9,13). Briefly, FAP substrates were cut perpendicular to the extrusion axis into 2.5 mm thick discs and ground through a series of silica papers and polished with diamond paste (1, 0.25 and 0.1 μm). Due to the crystal texture, samples exposed predominantly hexagonal (001) planes of FAP on the surface. Topographic images by atomic force microscopy (AFM) (Fig. 1) revealed very flat substrates facilitating the imaging of assembled proteins and their interaction with apatite crystals (6, 14). Furthermore, the initial height difference between glass matrix and the FAP

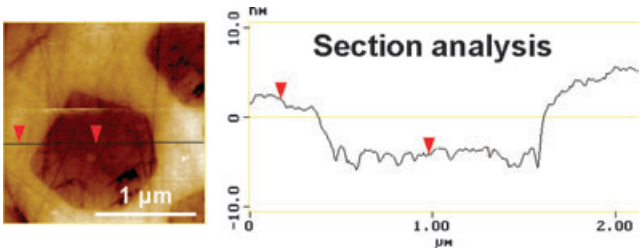

Fig. 1. AFM-image of glass-ceramic substrate after polishing and before immersion into mineralizing solutions. Section analysis shows that the surfaces of FAP crystals (dark hexagon) are about 3–6 nm below the level of the surrounding glass matrix (bright color).

crystals of about 5 nm served as a reference to measure precisely the crystal growth on FAP.

The recombinant full-length human amelogenin, rH174, was synthesized by *Escherichia coli* bacteria as described previously (15). The protein was purified using C4 beads and stored in 0.1% trifluoroacetic acid (TFA) at concentration between 1.5 and 2.5 mg/ml, as determined by UV-photospectroscopy.

FAP substrates were immersed for 24 h into various protein and/or calcium phosphate solutions designed to study the influence of protein concentration, pH and DS on interactions between amelogenin and apatite mineral. Mineralizing solutions were prepared from $Ca(NO_3)_2$, KH_2PO_4, KCl, dissolved in 0.1% TFA and double de-ionized water. We used three solutions with different ionic concentrations of calcium and phosphate containing 150 mM KCl. The reference solution 1 was adapted from concentrations determined in the secretory stage of enamel fluid with DS of 9.4 at pH 7.4 (10). This solution was also used at pH 6.5, 8.0 and 8.8. Solutions 2 and 3 have higher calcium levels and DS. The ion concentrations, pH and DS are listed in Table 1. The DS ($pK_a - pI$) to hydroxyapatite (pK_a (HAP) = 58.6) was calculated according to Larsen (16). All solutions were buffered with 50 mM Tris and the pH adjusted with HCl. Solutions were prepared for the mineralization experiments by dilution from the eightfolded separate calcium and phosphate stock solutions, as described earlier (9). The experiments were performed by placing a FAP substrate into a 1.5 ml siliconized test tube and adding each compound of corresponding solutions to a total volume of 400 μl. The immersed substrates were kept in an incubator at 37°C on a horizontal shaker, removed from solution after 24 h, immediately rinsed with de-ionized water and gently dried with dust-free air. Each experiment was repeated at least three times. All substrates were imaged before and after the experiment using an AFM (Nanoscope III, Digital Instrument, USA) in tapping mode with high aspect-ratio Si-tips operating at approximately 300 kHz resonance frequency. The height of the FAP-crystal with respect to the surrounding glass was determined as an average from three randomly selected points on the crystal. The overall average for each sample was determined from the measurements on five crystals. Student's *t*-test was applied to test for differences between groups with a confidence level of $p \leq 0.05$.

Table 1. **Ion concentrations of solutions used**

Solution no.	$[Ca^{2+}]$ (mmol/l)	$[PO_4^{3-}]$ (mmol/l)	pH	DS
1	0.5	2.45	7.4	9.4
2	2.1	1.27	7.4	11.6
3	3.1	1.86	7.4	12.9

Degree of saturation (DS) was calculated with respect to hydroxyapatite ($pK_a = 58.6$). pH measured at 37°C. All solutions contained 150 mM KCl and 50 mM Tris-buffer.

Results

The surface of a polished FAP glass-ceramic is shown in the AFM image of Fig. 1. The characteristic hexagonal crystal habits of the (001) planes of apatite was revealed and was about 3–6 nm below the surface of the surrounding glass layer as shown in the section analysis in Fig. 1. These substrates were immersed into solutions containing 0.4 mg/ml amelogenin at different pH. Amelogenin assembled into nanospheres that adhered to the glass-ceramic surfaces at pH 6.5, 7.4 and 8.0 (Fig. 2a). However, at pH 8.8, no protein adhesion was observed (Fig. 2b). Solutions that contained 1.6 mg/ml amelogenin precipitated a gel-like matrix as soon as the pH was raised above 6. FAP substrates immersed into these protein solutions did not reveal the characteristic 20 nm amelogenin nanospheres. Instead, larger spheres (~100–200 nm) were observed at all pH values; however, the number of spheres per surface area was lower at pH 8.8 (Fig. 2c).

In a second series of experiments, FAP substrates were immersed into solutions containing calcium and phosphate at different pH values without proteins. No mineral precipitation was observed at pH 6.5 by AFM (Fig. 3a). With increasing pH, the amount of mineral

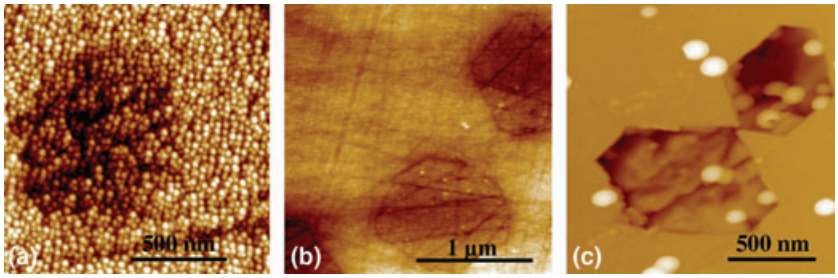

Fig. 2. AFM-images of substrates after immersion for 24 h in amelogenin protein solutions: (a) at pH 7.4 and 0.4 mg/ml; (b) at pH 8.8 and 0.4 mg/ml and (c) at pH 7.4 and 1.6 mg/ml amelogenin.

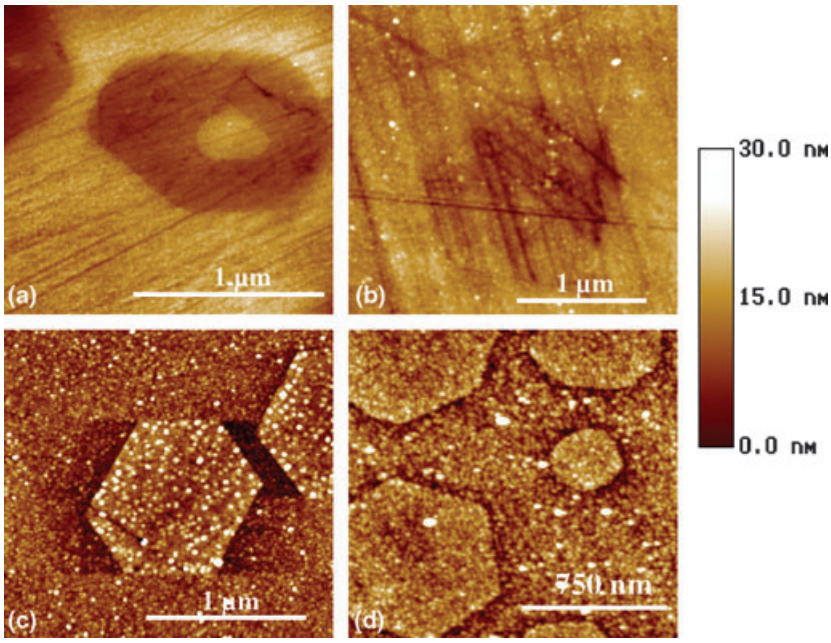

Fig. 3. AFM images of substrates after immersion for 24 h into calcium phosphate solutions with different pHs: (a) pH 6.5; (b) pH 7.4; (c) pH 8.0 and (d) pH 8.8.

precipitate increased (Fig. 3b–d). This precipitate was spherical with diameters of about 10–15 nm. Furthermore, we observed that the height of the FAP crystals increased with respect to the glass surface when exposed to mineralizing solutions at pH 7.4 and above. As shown in the AFM image of Fig. 3b, the step between the FAP and the surrounding glass disappeared, indicating that a mineralized layer of about 3–6 nm formed on the FAP. This layer grew slightly thicker at pH 8.0 and 8.8, with heights of about 5 and 10 nm above the glass layer (Fig. 3c, d).

In a third series, FAP substrates were immersed into solutions containing calcium and phosphate and amelogenin at concentrations of 1.6 mg/ml at different pH values. At pH values of 6.5, 7.4 and 8.0, mineralized layers of about 150–250 nm formed on the (001) planes of FAP. The mineral layer did not consist of one monolithic crystal grown on a single-crystal FAP, but was composed of smaller, elongated crystals that nucleated specifically on the FAP surface. As shown in Fig. 4a–c, the hexagonal shape of the FAP crystals was maintained after superficial mineralization occurred; however, the edges of the crystal now appeared irregular. Crystal growth was only observed on the FAP crystals, while the surrounding glass matrix did not exhibit significant amounts of mineral precipitates or adhering protein nanospheres. In contrast, at pH 8.8 the layer height did not exceed 20 nm (Fig. 4d). At this pH, FAP crystals showed spherical precipitate of about 15–40 nm attributed to protein and/or mineral. Furthermore, larger spheres (100 nm) adhered to both glass and FAP and were attributed to protein assemblies as seen in Fig. 1d. There was no statistical difference found in the height of the layers formed on FAP at pH 6.5, 7.4 and 8.0. The layer height at pH 8.8 was significantly lower as illustrated graphically in Fig. 5.

In the fourth experimental group, FAP substrates were immersed into calcium phosphate solutions at a physiological pH of 7.4 with a much higher DS. As expected, the amount of mineral formed increased

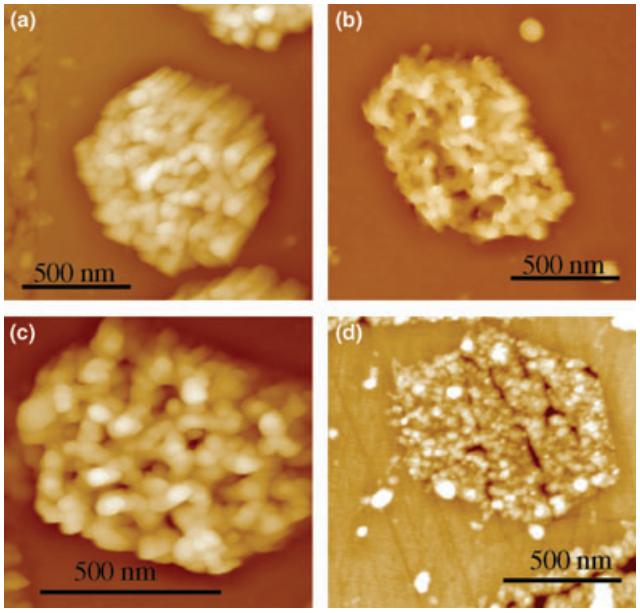

Fig. 4. AFM images of substrates after immersion for 24 h into calcium phosphate solutions containing 1.6 mg/ml amelogenin at different pHs: (a) pH 6.5; (b) pH 7.4; (c) pH 8.0 and (d) pH 8.8.

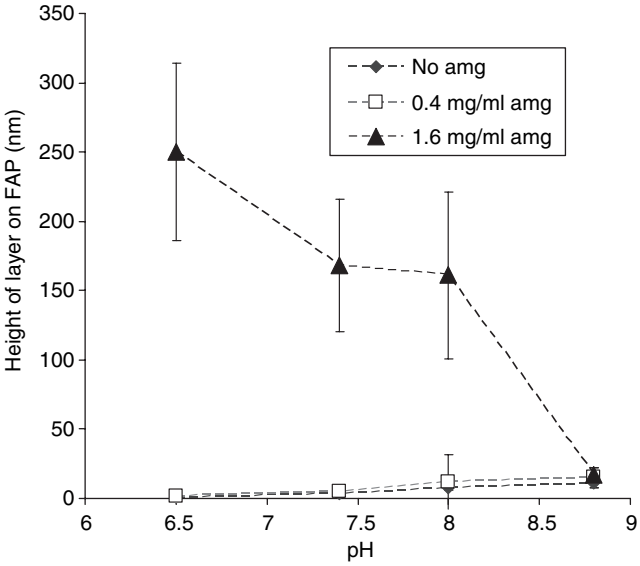

Fig. 5. Graph comparing the height of the mineralized layer formed on FAP crystals at different pHs and protein concentrations. Layer height was greatly increased when 1.6 mg/ml amelogenin was used at pH between 6.5 and 8.0.

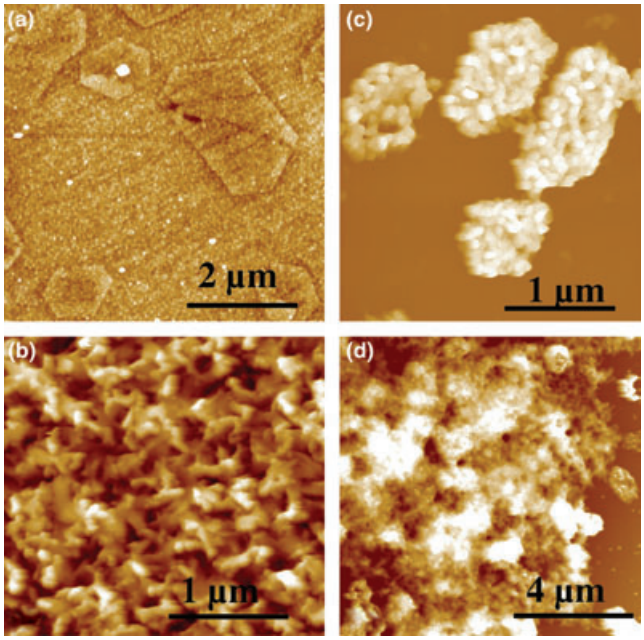

Fig. 6. AFM images of substrates after immersion for 24 h into calcium phosphate solutions with increased degrees of saturation (DS) and with and without amelogenin: (a) DS = 11.6, no amelogenin; (b) DS = 12.8, no amelogenin; (c) DS = 11.6, 1.6 mg/ml amelogenin and (d) DS = 12.8, 1.6 mg/ml amelogenin.

substantially with DS. In the protein-free calcium phosphate solution a dense layer of mineral precipitate and homogenous crystal growth was observed at DS of 11.6. The FAP layer height was about 15 nm (Fig. 6a). When DS was further raised to 12.8, strong precipitation occurred, covering the entire surface of the FAP substrate with a dense mineral layer, as shown in Fig. 6b. This growth pattern changed considerably when 1.6 mg/ml amelogenin protein was added to these calcium phosphate solutions. At DS of 11.6 mineral formation occurred predominantly on FAP crystals (Fig. 6c). The layer height (162 nm) obtained and the microstructure of the layers were comparable to the layers formed at DS of 9.4, as described above (Fig. 4). Compared to the protein-free experiment at DS of 11.6, there was no significant heterogeneous precipitation on the glass matrix observed. At the higher DS of 12.8, homogeneous growth of apatite on FAP occurred. The height of this homogeneous growth was at 145 nm comparable with the samples obtained at lower DS. However, large areas (>50% of total surface area) of the substrates immersed into solutions with DS of 12.8 nm exhibited additional crystallization around the FAP and on the glass matrix (Fig. 6d).

Discussion

The interaction of proteins with inorganic ions and compounds is dependent on a variety of biochemical as well as physical–chemical parameters. In a recent study, we showed that growth rates of apatite crystals along the *c*-axis drastically increased in the presence of

full-length amelogenin proteins (9). The solubility limit of the amelogenin appeared to be the decisive factor in inducing enhanced crystal growth. Protein-guided growth was only observed when the amelogenin concentration exceeded the solubility limit.

In this study, we examined if other factors, pH and DS, affect the ability of amelogenin to promote apatite crystal growth. Amelogenin molecules assembled into nanospheres with a variety of diameters depending on the pH (17). In this study, nanospheres formed and adhered to the glass-ceramic substrate at pH between 6.5 and 8.0. The nanosphere diameters were in the range of 20–30 nm, with a more monodisperse distribution at pH 7.4 and 8.0. At pH 8.8, however, no or few amelogenin nanospheres adhered to the FAP substrates as revealed by AFM. It has been shown that amelogenin still assembles into nanospheres at this pH (14), but according to this study, the assembled proteins no longer adhere to the apatite and glass surfaces provided in the substrate. This lack of protein binding indicates a change in the secondary structure of the molecule, which may have been induced by changes in surface charges, since the isoelectric point of the hydrophilic portion of amelogenin is at approximately pH 8.0 (3). When the protein concentration was raised above the solubility limit, which differs with pH, the characteristic nanosphere formation and adhesion was no longer observed. It has been reported that the size of amelogenin nanospheres, when measured by dynamic light scattering, increased to about 60 nm in diameter at concentrations of 4 mg/ml (18). In this study, the size of amelogenin nanospheres was drastically increased to diameters between 100 and 200 nm once the concentration was raised above the solubility limit. However, only a sparse amount of protein adhered to the substrate at 1.6 mg/ml and hence the majority of the protein was not detected by the AFM analysis.

Substrates that were immersed into calcium phosphate solutions without proteins showed only little or no mineral formation. The amount of precipitate increased with pH and DS (Fig. 3). The mineral precipitation was attributed to heterogeneous nucleation of calcium phosphate that randomly formed on the glass-ceramic surface. With increasing pH, growth of FAP was observed, indicated by the increase in height of the FAP crystals in relation to the surrounding glass matrix. This type of crystal growth was attributed to the homogeneous nucleation of apatite on the FAP crystals of the substrates. The layers grown on FAP reached a maximum height of only 10 nm at the highest pH of 8.8.

In contrast, the layer height increased drastically when 1.6 mg/ml of amelogenin were added to the mineralizing solution with pH 6.5, 7.4 and 8.0, but was almost unchanged at pH 8.8. Hence, the ability of amelogenin to interact and assemble an inorganic phase is dependent on the pH. In conjunction with the above observation that amelogenin nanospheres do not bind to inorganic charged surfaces at high pH (Fig. 1c) we presume that the secondary structure of amelogenin is altered at pH 8.8, reducing its binding affinity and its ability to interact with the forming mineral. In the physiological range of pH and as long as the pH remains below the isoelectric point (8.0) of the hydrophobic portion of amelogenin, the protein is able to bind and promote apatite crystallization. We have confirmed by Raman spectroscopy and previously reported that the crystals formed on FAP are carbonate-containing apatite with organic phases.

In our experimental system that includes calcium phosphate solutions at a DS of 9.4 and an amelogenin concentration of 1.6 mg/ml, the growth rates were about 0.2 μm per day. *In vivo*, dental enamel grows by about 2 μm per day, mineralizing the entire crown within 4 years. With the current approach it would yet take about 40 years to grow the size of a complete enamel crown *in vitro*. We therefore attempted to increase DS in order to provide higher concentrations of calcium and phosphate, assuming it would accelerate the growth of apatite. Nevertheless, the results did not support this hypothesis. Using an amelogenin concentration of 1.6 mg/ml and adding additional calcium and phosphate ions to increase DS from 9.0 to 11.6 resulted only in a comparable height of the layer grown on FAP and continued to preserve the hexagonal shape of the original FAP template, indicating that the reaction was restricted to the FAP surface. A further increase of DS to 12.8 resulted again not only in similar heights of layers grown on FAP, but also caused mineral formation on the surrounding glass. We assume, in this case, that the protein was not able to restrict crystallization to the FAP template. By increasing the calcium and phosphate concentration of the solution, crystallization became controlled by the physical chemistry of the solution and no longer by the activity of the amelogenin protein. This suggests that there is only a

narrow range of supersaturation in calcium and phosphate that can be controlled by amelogenin proteins. If the ratio between the DS in inorganic ions exceeds the concentration of available proteins, crystallization will be controlled by the physical–chemical driving forces and thus result in conventional heterogeneous or homogeneous crystallization. To transfer the control over crystal assembly to the organic phases and macromolecules, saturation in calcium and phosphate must be kept low. Such protein-guided crystal growth *in vitro* is basically analogous to the process of biomineralization *in vivo*, in which extracellular matrix proteins control mineral formation, while cells supply the extracellular space with matrix proteins and monitor specific concentration of inorganic ions through ion pumps. Matrix proteins can now be synthesized by cloning and recombination techniques of modern biotechnology, while micro and nanotechnology allows monitoring of ionic levels with nano-Mol precision. Considering that the usual task of the biological cell can be managed by biochemists and tissue engineers, we conclude that an *in vitro* approach to regenerate a mineralized tissue is feasible.

Acknowledgements: The authors acknowledge Dr Christian Ruessel, Otto-Schott-Institute, University of Jena, for providing the glass-ceramic substrates. This study was supported by NIH/NIDCR P01DE09859, R21DE015416, R01DE015821 and R01-DE13029.

References

1. Weiner S, Addadi L, Wagner HD. Materials design in biology. *Mater Sci Eng C – Biomimet Supramol Syst* 2000;**11**:1–8.
2. Robinson C, Brookes SJ, Bonass WA, Shore RC, Kirkham J. Enamel maturation. *Ciba Found Symp* 1997;**205**:156–70.
3. Fincham AG, Moradian-Oldak J, Simmer JP. The structural biology of the developing dental enamel matrix. *J Struct Biol* 1999;**126**:270–99.
4. Hoang AM, Klebe RJ, Steffensen B, Ryu OH, Simmer JP, Cochran DL. Amelogenin is a cell adhesion protein. *J Dent Res* 2002;**81**:497–500.
5. Veis A. Amelogenin gene splice products: potential signaling molecules. *Cell Mol Life Sci* 2003;**60**:38–55.
6. Wallwork ML, Kirkham J, Zhang J, Smith DA, Clarkson BH, Brookes SJ et al. Binding of matrix proteins to developing enamel crystals: an atomic force microscopy study. *Langmuir* 2001;**17**:2508–13.
7. Wen HB, Moradian-Oldak J, Zhong JP, Greenspan DC, Fincham AG. Effects of amelogenin on the transforming surface microstructures of bioglass in a calcifying solution. *J Biomed Mater Res* 2000;**52**:762–73.
8. Tan J, Leung W, Moradian-Oldak J, Zeichner-David M, Fincham AG. Quantitative analysis of amelogenin solubility. *J Dent Res* 1998;**77**:1388–96.
9. Habelitz S, Kullar A, Marshall SJ, DenBesten PK, Balooch M, Marshall GW et al. Amelogenin-guided crystal growth on fluoroapatite glass-ceramics. *J Dent Res* 2004;**83**:698–702.
10. Aoba T, Moreno EC. The enamel fluid in the early secretory stage of porcine amelogenesis: chemical composition and saturation with respect to enamel mineral. *Calcif Tissue Int* 1987;**41**:86–94.
11. Hoche T, Moisescu C, Avramov I, Russel C, Heerdegen WD, Jager C. Microstructure of SiO2-Al2O3-CaO-P2O5-K2O-F-glass ceramics. 2. Time dependence of apatite crystal growth. *Chem Mater* 2001;**13**:1320–5.
12. Moisescu C, Jana C, Russel C. Crystallization of rod-shaped fluoroapatite from glass melts in the system SiO/sub 2/-Al/sub 2/O/sub 3/-CaO-P/sub 2/O/sub 5/-Na/sub 2/O-K/sub 2/O-F/sup. *J Non-Cryst Solids* 1999;**248**:169–75.
13. Habelitz S, Ford D, Marshall SJ, DenBesten PK, Balooch M, Marshall GW et al. Amelogenin induces biomimetic mineralization at specific pH. In: *Materials Research Society Symposium Proceedings* 2003;Vol. O 7.8.
14. Wen HB, Fincham AG, Moradian-Oldak J. Progressive accretion of amelogenin molecules during nanospheres assembly revealed by atomic force microscopy. *Matrix Biol* 2001;**20**:387–95.
15. Li W, Gao C, Yan Y, DenBesten PK. X-linked amelogenesis imperfecta may result from decreased formation of tyrosine rich amelogenin peptide (TRAP). *Arch Oral Biol* 2003;**48**:177–83.
16. Larsen J. *Ion Products of Calcium Phosphate Solutions*. Aarhus, Denmark: Royal Dental College; 2001.
17. Moradian-Oldak J. Amelogenins: assembly, processing and control of crystal morphology. *Matrix Biol* 2001;**20**:293–305.
18. Moradian-Oldak J, Simmer JP, Lau EC, Sarte PE, Slavkin HC, Fincham AG. Detection of monodisperse aggregates of a recombinant amelogenin by dynamic light scattering. *Biopolymers* 1994;**34**:1339–47.