Matrices of Acidic β-Sheet Peptides as Templates for Calcium Phosphate Mineralization**

By Shlomit Segman-Magidovich, Haviv Grisaru, Tamar Gitli, Yael Levi-Kalisman, and Hanna Rapaport*

Designed biomaterials that accelerate mineralization under physiological conditions may provide new multifunctional biomimetic scaffolds amenable for bone tissue regeneration. Bone and teeth dentin are composite biomaterials composed of carbonated apatite crystals that are aligned within type-I collagen fibrils of the extracellular matrix.[1–3] Non-collagenous proteins isolated from bone extracellular matrices that are rich in acidic amino acids[4] have been proposed to be involved in the nucleation and growth of carbonated apatite.[5] Despite numerous studies aiming at unravelling the principles of apatite biomineralization, detailed mechanisms that account for the role of acid rich proteins in this process are yet to be elucidated. Addadi and Weiner have proposed that acidic proteins locked in an ordered and rigid conformation present favorable sites for heterogeneous nucleation of calcium minerals, whereas a flexible polypeptide chain in solution may inhibit crystallization.[6,7] Rigid interfaces of biomaterial may induce biomineralization processes through selective electrostatic accumulation of the mineral ionic components and by structural correspondence with specific planes in the crystal. The inhibition of crystal growth along certain directions in solution was demonstrated by tailor-made additives that adsorbed specifically by structural matching to planes of a mature crystal.[8] Biomineralization in general has been linked to acidic proteins in β-sheet conformation. The major acidic protein of tooth enamel, enamelin, has been shown to fold into β-sheet structure during enamel formation.[9] Other acidic, extracellular matrix macromolecules have been found to adopt the β-sheet structure in the presence of calcium salts.[6]

Designed peptides that assume β-sheet structures have been shown to form assemblies with unique structural characteristics at the nanometer scale.[10–14] Several families of amphiphilic peptides that tend to adopt β-sheet structures have been shown to assemble into elongated fibers of nanometer-scale width.[13–15] These β-sheet nanofibers may form three-dimensional entangled networks that produce hydrogels. Assemblies of peptides in predefined secondary structures have been used as templates for crystallization of calcium carbonate phases and other inorganic minerals.[16–19] Recently, anionic β-sheet peptides have been applied to increase mineral gain in dental lesions.[20]

Various previously reported studies have claimed detecting apatite nucleation on different types of molecular templates. Nonetheless, the identification and analysis of the first-formed mineral deposits are difficult, because specimens may be influenced by various undesirable factors, for example, dehydration which may become the main cause for transformation into a different crystalline phase. It is therefore as yet challenging to characterize calcium phosphate nucleation on defined molecular templates in order to advance understanding of the system and, thus, enable the design and synthesis of new composite mineralized biomaterials.

There is an intriguing structural correspondence between the hexagonal unit cell dimensions of crystalline hydroxyapatite,[21] \( a = b = 9.432 \text{ Å} \) and \( c = 6.881 \text{ Å} \), namely the (100) planes, and the repeat dimensions of β-sheet structures, which has been suggested to play a role in apatite crystallization.[22] Hydrogen-bonded strands in β-sheets are ca. 4.7–4.8 Å apart, about half the \( a \) or \( b \) axes of the apatite unit cell, whereas the distance between every second amino acid along a β-strand, ca. 6.9 Å, is essentially identical in length to the apatite \( c \) axis. Nonetheless, there is growing evidence that amorphous granules are prevalent in early stages of many biomineralization systems as well as in bone tissue.[23] Here, we monitored early stages of calcium phosphate mineralization on designed β-sheet peptides that may serve as nucleating templates for hydroxyapatite. We have previously demonstrated that a family of acidic and amphiphilic β-sheet peptides, Pro-X(Y-X)_n-Pro, where \( X = \text{Glu}, Y = \text{Phe}, n = 2–5 \), denoted PFE=−n, may assemble spontaneously into crystalline monomolecular films at interfaces.[24] The alternating sequence of hydrophilic (X) and hydrophobic (Y) amino acids direct the β-strand pleated conformation whereas the two proline termini residues were found to induce the two-dimensional monomolecular organization.[24] The β-strands within the ordered domains were also found deform in a quasi-elastic manner under lateral pressure.[25] Recently, a new calcite crystal habit, nucleated by ordered lipidated β-sheet monolayers, has been linked to the flexibility of the monolayer template and to its
adaptability to the growing crystal. In this study we utilized the peptide Pro-Asp-(Phe-Asp)_5-Pro, PFD-5, in the form of two-dimensional monomolecular templates at interfaces and in three-dimensional fibrilar structures to study the early stages of calcium phosphate mineralization on well-defined β-sheet templates. In order to monitor the mineralization under conditions that may be of biological relevancy, we have utilized a solution composed of the various ions present in the blood serum, in 1.5 mM solution of the peptide Pro-Asp-(Phe-Asp)_5-Pro, PFD-5, in the form of the peptide monolayer rigidifies during the course of incubation owing to continuous adsorption of ions from the SBF solution.

Monomolecular films of PFD-5 peptide on SBF were studied in home-made troughs that were held covered in a humidified chamber at 37 °C. After 5, 10, and 20 days, samples of the interfacial layers were transferred onto various supports for further inspection. Interestingly, peptide films that were incubated for 20 days could be observed by the naked eye. Carbon-coated electron microscopy grids were placed over the interface for an hour to allow the upper face of the peptide film to adhere to the hydrophobic grid. The grids were then picked up and rinsed with water in an attempt to remove any traces of the ionic solution that could lead to quick crystallization owing to evaporation of the SBF solution. Peptide films that were transferred from the interface after 5 or 10 days showed (Fig. 2) either clusters of spherical particulates ca. 30–90 nm in diameter or planar aggregates. Both of these features, which comprised calcium and phosphate ions according to energy-dispersive spectroscopy (EDS; data not shown), were non-crystalline as identified by electron diffraction. Planar structures have also been occasionally detected on grids that sampled bare SBF solution. Interestingly, fanlike structures (Fig. 2c) were observed on peptide films that were incubated for 20 or more days. Nevertheless, these structures have also been occasionally detected on grids that sampled bare interface of SBF. The fibrilar part of these fanlike aggregates show the 3.5 Å spacing that is characteristic of hydroxyapatite (002) spacing (transmission electron microscopy (TEM) images not shown). The fact that these fanlike forms were also observed on grids that sampled bare SBF proves that these features can crystallize autonomously without the peptide template. Noteworthy, since all TEM images showed structural features that are nanometric in scale, it is possible that the mineralized film that was visible to the naked eye did not transfer as a whole to the hydrophobic grid, or alternatively got partially washed off while rinsing the residual SBF solution (see Experimental). We suspect that the fanlike structures crystallize from highly concentrated drops of the solution owing to evaporation. It is well-established that hydroxyapatite crystallizes tend to grow along the c axis. Interfacial interactions and adhesion forces may then govern, or support, the formation of these fibrilar structures, with their long axes parallel to the grid surface. The flat calcium phosphate sheets (Fig. 2d), which were also detected at the interface of bare SBF solution, could be a form of uncharged amorphous calcium phosphate aggregate. Spherical structures, similar to those observed by TEM (Fig. 2a and b) were also formed on peptide fibers suspended in solution, as described below. Therefore, in a solution that is supersaturated with respect to hydroxyapatite, acidic β-sheets induce the heterogeneous nucleation of calcium phosphate spherical particulates that according to TEM diffraction appear to be amorphous. These particulates may have evolved in solution and adhere to the peptide or alternatively grown from inception on the peptide film. Not knowing the nanometer-detailed structure of these spherical particulates we may only speculate at this stage that it is the...
hydroxyapatite. This group also reported that absorption bands at 1045 cm\(^{-1}\) and at 1060 and 1075 cm\(^{-1}\) may be attributed to carbonate-substituted apatite and to apatite respectively. It has also been noted that the percentage area attributed to carbonate-substituted apatite and to apatite mineral forms, appears weaker in the 20 days film, with weakening of the absorption at wavenumbers higher than ca. 1100 cm\(^{-1}\). The peaks at 1630 and 1552 cm\(^{-1}\) could be attributed to amide I and II of antiparallel \(\beta\)-sheets. Apatite and OCP may also show an absorption band at ca. 1630 cm\(^{-1}\) (cf. Fig. 4e).

In a combined X-ray and Fourier transform (FT) IR study of the amorphous calcium-phosphate transition to hydroxyapatite, Mendelsohn, Boesky et al. have shown that the frequency of \(v_3\) absorption band shifted from 1015 to 1030 cm\(^{-1}\) with increase in the crystal c axis length of poorly crystalline hydroxyapatite. This group also reported that absorption bands at 1045 cm\(^{-1}\) and at 1060 and 1075 cm\(^{-1}\) may be attributed to carbonate-substituted apatite and to apatite respectively. It has also been noted that the percentage area attributed to carbonate-substituted apatite and to apatite mineral forms, appears weaker in the 20 days film, with weakening of the absorption at wavenumbers higher than ca. 1100 cm\(^{-1}\). The peaks at 1630 and 1552 cm\(^{-1}\) could be attributed to amide I and II of antiparallel \(\beta\)-sheets. Apatite and OCP may also show an absorption band at ca. 1630 cm\(^{-1}\) (cf. Fig. 4e).

In a combined X-ray and Fourier transform (FT) IR study of the amorphous calcium-phosphate transition to hydroxyapatite, Mendelsohn, Boesky et al. have shown that the frequency of \(v_3\) absorption band shifted from 1015 to 1030 cm\(^{-1}\) with increase in the crystal c axis length of poorly crystalline hydroxyapatite. This group also reported that absorption bands at 1045 cm\(^{-1}\) and at 1060 and 1075 cm\(^{-1}\) may be attributed to carbonate-substituted apatite and to apatite respectively. It has also been noted that the percentage area attributed to carbonate-substituted apatite and to apatite mineral forms, appears weaker in the 20 days film, with weakening of the absorption at wavenumbers higher than ca. 1100 cm\(^{-1}\). The peaks at 1630 and 1552 cm\(^{-1}\) could be attributed to amide I and II of antiparallel \(\beta\)-sheets. Apatite and OCP may also show an absorption band at ca. 1630 cm\(^{-1}\) (cf. Fig. 4e).
peptide and the ions in solution, the thickened mineral layer that developed on the peptide film transformed autonomously into the apatite structure.

Peptide PFD-5 was also found to generate three-dimensional self-supporting hydrogel matrices. TEM images of hydrogel samples (Fig. 4a–c) revealed fibrilar structures that, according to IR absorption spectra (Fig. 4e), are predominantly in β-sheet conformation. PFD-5 hydrogel may form following the dissolution of the acidic peptide in basic solution whereby two processes occur. The initial and fast step is the deprotonation of carboxyl groups to the extent that electrostatic repulsion forces dissolve the peptide. In the second and slower step, peptides assemble via β-sheet hydrogen bonds and hydrophobic interactions between phenylalanine faces of the sheets to form entangled fibers that get locked into a viscoelastic hydrogel structure (rheology measurements will be the scope of a forthcoming manuscript). The hydrogel was prepared by dissolving 0.023 M of the peptide in 0.05 M NaOH (pH 12.7). This peptidic viscous solution at pH ~7 then gels over minutes. Based on simple stoichiometric arguments it is found that the PFD-5 hydrogel that forms at neutral pHs is overall negatively charged. Since each PFD-5 peptide has five carboxyl groups, the actual acid concentration contributed by the peptide is 0.023 \times 5 = 0.115 \text{ M}, which is almost two times more concentrated compared to the hydroxyls concentration (0.05 M) in the system. Consequently, about half of the carboxyl groups could potentially be charged in the hydrogel. However, it is possible that part of these carboxyl groups could be in the protonated state due to stabilizing hydrogen bonds between the aspartic acid side chains. The PFD-5 hydrogel that express acidic β-sheet templates in three dimensions was used to monitor the early stages of calcium phosphate mineralization in bulk solution. PFD-5 matrices which were incubated in a closed vessel with SBF_{1.5} for 20 days (see Experimental) transformed into a biphasic liquid-hydrogel system. Samples of the incubated system which were visualized by TEM revealed spherical particulates decorating elongated fibrilar structures (Fig. 4a–c).

The peptide fibrilar forms could be found at various sizes, with lengths that span a large range, from hundreds of micrometers to several millimeters. The biphasic system was also visualized in-situ, by light microscopy (Fig. 4d), in a manner that is deprived of any sample preparations, revealing in the suspension preferred adsorption between macroscopic peptide fibers and spherical particulates that resemble, by and large the calcium phosphate aggregates that were detected by TEM on the two-dimensional peptide monolayer (cf. Fig. 2a and b). Figure 4b and c shows fibers decorated by spherical particulates that have underwent sample preparation that includes negative staining, which may have affected the observed morphologies. According to electron diffraction measurements all these

---

**Figure 4.** TEM images of amorphous calcium phosphate particulates adhered to PFD-5 hydrogel fibers. a) Unstained sample showing dark dots that appear more abundant on the peptide fibers than in the background. b,c) Negatively stained hydrogel fibers that are ca. 30 nm wide decorated by calcium phosphate spherical particulates. d) Optical microscopy image of peptide fibers decorated by spherical calcium phosphate particulates. The fiber marked by the white arrow reaches more than 1 mm in length and 30 μm in width. e) FTIR spectra of PFD-5 hydrogel (bottom) showing the main absorption bands of amide I and II at ca. 1625 and 1550 cm⁻¹, respectively. Spectrum of SBF_{1.5} stored at 37 °C for 20 days showing mainly two peaks at 1606 and 1637 cm⁻¹ (middle). The biphasic PFD-5 hydrogel sample that was incubated in SBF_{1.5} for 20 days (top). The latter spectrum was scaled in order to have the absorption band at 1637 cm⁻¹ equal to that of the pure SBF_{1.5} sample (marked by arrows equal in length in the middle and top spectra).
spherical particulates appear amorphous. IR spectra of PFD-5 hydrogel (see Experimental) incubated with SBF_{1.5} (Fig. 4e) exhibited a peak centered at around 1060 cm\(^{-1}\). This peak appears more pronounced in presence of the peptide hydrogel, as compared to SBF_{1.5} spectrum. As mentioned above, this peak which may be attributed to \(v_3\) PO\(_4\)^{3-} of carbonated apatite as well as to other nonapatitic calcium phosphate forms, may suggest that the peptide environment enhances the transformation from amorphous towards apatitic and other more ordered calcium phosphate structures.\[^{33}\]

In summary, the acidic rich peptides that assume predominantly the \(\beta\)-sheet structure induced the adsorption or the in situ nucleation of amorphous calcium phosphate aggregates. The IR spectra provided evidence both for amorphous calcium phosphate accumulation and for the transformation of the mineralized film to apatitic form over time. Nevertheless, since that mineralized-peptide film had been dehydrated before the spectra were measured it is possible that the spectrum also reflects mineralization that was driven by film evaporation. In accordance with these observations the IR spectra of the freeze-dried biphasic liquid-hydrogel (Fig. 4e) may represent better the actual state of the calcium phosphate mineralization on the peptide template, in a system that is abstained of fast mineralization due to dehydration. Electron microscopy images of monolayer films transferred onto carbon coated grids revealed mainly two distinct forms of amorphous calcium phosphate aggregates: flat sheets and spherical particulates. The other form, namely, fan-shaped aggregates that even exhibit electron diffraction typical of crystalline material, appear to have developed from aged solution, in an autonomous manner, triggered by fast evaporation of residual SBF_{1.5} solution. The spherical particulates that formed on the acidic \(\beta\)-sheet structure either in the monolayer system or in the hydrogel biphasic system appeared in various sizes ranging from the nanometer to the micrometer scale. The current study demonstrated that in a solution supersaturated with respect to hydroxyapatite the acidic \(\beta\)-sheet peptides serve predominantly as a charge surface that enhances the adsorption or the in situ nucleation of amorphous calcium phosphate particulates. It is possible that the mineralization state of the adsorbed calcium phosphate aggregates is dominated by the stronger interaction energies between the calcium and the phosphate ions in a manner that overrides the potential templating effect of the peptide carboxyl groups. It is also possible that extensive interactions between the peptide fibers and the nucleated calcium phosphate particulates slow down the transformation towards the hydroxyapatite phase. Nevertheless, it was also apparent that under appropriate conditions, that is, dehydroxyapatite or over longer periods of time, these amorphous clusters may transform into apatitic structures at the peptide interface. Additional control experiments were performed with palmitic acid monolayers on SBF_{1.5}. Fatty acids resemble the acidic and amphiphilic \(\beta\)-sheet structure, in the sense that they may form monolayers, at air/solution interfaces presenting an array of carboxyl groups to the mineralizing solution. A palmitic acid monolayer induced the formation of primarily flat-sheet amorphous calcium phosphate aggregates, similar to those shown in Figure 2d. This result may support the hypothesis (vide supra) that the flexibility of the \(\beta\)-sheet film\[^{25}\] allows the stabilization of the round-shaped calcium-phosphate particulates whereas the rigid monolayer of the fatty acid may stabilize only the flat-sheet amorphous aggregates.

We are currently studying early forms of calcium phosphate mineralization on various other peptide matrices such as those of PFE-5 and with other, more diluted ionic solutions in an attempt to boost the potential templating effect of the \(\beta\)-sheet ordered peptides.

This study also demonstrates the utilization of the well-characterized peptide matrices for the bottom-to-top design of negatively charged \(\beta\)-sheet scaffolds that may serve as templates for calcium phosphate mineralization. The physical and chemical properties of these novel composite peptide matrices can be fine-tuned both by the design of different amino acid sequences along the peptide and by the mineralizing solution. These acidic \(\beta\)-sheet peptides can be self-assembled into either anionic monolayers at interfaces or into three-dimensional hydrogel matrices composed of entangled \(\beta\)-sheet nanofibers. These hydrogels may become useful as multifunctional biomimetic mineralizing scaffold for bone tissue regeneration.

**Experimental**

SBF_{1.5}, which has ions concentrations 1.5 times those of human plasma, was prepared using the method of Kokubo \[^{34}\]. NaCl (213 mM), NaHCO\(_3\) (6.3 mM), KCl (3.5 mM), K\(_2\)HPO\(_4\) (1.5 mM), MgCl\(_2\) (2.3 mM), CaCl\(_2\) 2H\(_2\)O (3.8 mM) and Na\(_2\)SO\(_4\) (0.75 mM) were dissolved consecutively in double distilled water at 36 \(^{\circ}\)C and buffered at pH~7.35 using 75 mM of tris(hydroxymethyl)aminomethane, along with an appropriate volume of 1M HCl. Prior to experiments the solution was stored for 24h, at 4 \(^{\circ}\)C, in polypropylene flasks to reach equilibrium.

Langmuir isotherms were measured in KSV MiniTrough at ambient temperature. PFD-5 (AnaSpec, CA) monomolecular films were formed at the interface of air/water or air/SBF_{1.5} using peptide/TFA/chloroform (~0.5 mg/0.7 mL/0.3 mL) solution. Monomolecular films were also prepared in a similar manner in home made troughs ~10 cm in diameter, at area per molecule that corresponds to ~10 mN m\(^{-1}\). Samples of the interfacial films were placed over the interface for ca. 1 h and picked on carbon-coated electron microscopy gold grids of 300 mesh. The grids were then rinsed with ~5 mL of water and allowed to dry at room temperature over night. TEM images were acquired by Tecnai 12 (FEI) TEM operated at 120 kV and equipped with a Gatan 794 CCD camera. The interfacial films were also transferred onto ZnSe ATR prisms for ATR-IR measurements in the horizontal mode, simply by bringing the prism into contact with the solution interface. ATR-FTIR measurements were carried out using a Bruker EQUINOX 55 spectrometer.

PFD-5 hydrogel was prepared by dissolving the peptide in 100 \(\mu\)L, 0.05 M NaOH to a final concentration of 0.023 M. Hydrogel appeared to have structured after 30 min. pH values were estimated by transferring an aliquot of the hydrogel onto universal indicator paper showing pH~7. The hydrogels were freeze-dried in liquid nitrogen for ~1 min and lyophilized overnight. Next, hydrogels were reformed in 100\(\mu\)L SBF\(_{1.5}\). A second portion of 100 \(\mu\)L of SBF\(_{1.5}\) was added leaving a thin liquid layer on top of the gel. These biphasic liquid-hydrogel samples were stored in closed vials at 37 \(^{\circ}\)C for 20 days. The incubated
Reference spectra were obtained using a bare CaF$_2$ plate. Hydrogel samples were lyophilized and then laid on CaF$_2$ plates. Measurements were performed with a Bruker EQUINOX 55 equipped with IRscope-II under ambient atmosphere. The FTIR optical microscopy image was obtained by placing the vial with the biphasic liquid hydrogel sample on an inverted light microscope. TEM images were acquired with the same instrument mentioned above. The few of the grids were negatively stained using 3% uranyl acetate. TEM samples were 4 × diluted in water. A few drops of the diluted solution were placed on carbon-coated copper grids of 300 mesh, and allowed to equilibrate at room temperature for 1 h. Finally, the grids were washed a few times with water in order to remove residual SBF$_{1.5}$ solution. A few of the grids were negatively stained using 3% uranyl acetate. TEM measurements were performed with a Bruker EQUINOX 55 which was equipped with IRscope-II under ambient atmosphere. The hydrogel samples were lyophilized and then laid on CaF$_2$ plates. Reference spectra were obtained using a bare CaF$_2$ plate.

Received: August 22, 2007
Revised: October 23, 2007