Published in final edited form as:

J Struct Biol. 2010 April ; 170(1): 83–92. doi:10.1016/j.jsb.2009.12.018.

Complementary effects of multi-protein components on biomineralization *in vitro*

Xiaolan Ba^a, Miriam Rafailovich^{a,*}, Yizhi Meng^a, Nadine Pernodet^{a,1}, Sue Wirick^b, Helga Füredi-Milhofer^c, Yi-Xian Qin^d, and Elaine DiMasi^e

^aDepartment of Materials Sciences and Engineering, Stony Brook University, Stony Brook, NY 11794-2275, USA

^bDepartment of Physics and Astronomy, Stony Brook University, Stony Brook, NY 11794, USA

^cDepartment of Chemistry, The Hebrew University, Jerusalem, Israel

^dDepartment of Biomedical Engineering, Stony Brook University, Stony Brook, NY 11794, USA

^eNational Synchrotron Light Source, Brookhaven National Laboratory, Upton, NY 11973, USA

Abstract

The extracellular matrix (ECM) is composed of mixed protein fibers whose precise composition affects biomineralization. New methods are needed to probe the interactions of these proteins with calcium phosphate mineral and with each other. Here we follow calcium phosphate mineralization on protein fibers self-assembled *in vitro* from solutions of fibronectin, elastin and their mixture. We probe the surface morphology and mechanical properties of the protein fibers during the early stages. The development of mineral crystals on the protein matrices is also investigated. In physiological mineralization solution, the elastic modulus of the fibers in the fibronectin-elastin mixture increases to a greater extent than that of the fibers from either pure protein. In the presence of fibronectin, longer exposure in the mineral solution leads to the formation of amorphous calcium phosphate particles templated along the self-assembled fibers, while elastin fibers only collect calcium without any mineral observed during early stage. TEM images confirm that small needle-shape crystals are confined inside elastin fibers which suppress the release of mineral outside the fibers during late stage, while hydroxyapatite crystals form when fibronectin is present. These results demonstrate complementary actions of the two ECM proteins fibronectin and elastin to collect cations and template mineral, respectively.

Keywords

Extracellular matrix; Fibronectin; Elastin; Biomineralization; Hydroxyapatite

*Corresponding author. Fax: +1 631 632 5762. mrafailovich@notes.cc.sunysb.edu (M. Rafailovich).

¹Present address: Estée Lauder Inc., 125 Pinelawn Road, Melville, NY 11747, USA.

1. Introduction

The extracellular matrix (ECM) is a complex mixture of structural and functional proteins which regulate both physiological and pathological biomineralization processes. During physiological biomineralization, such as bone or dentin formation, specific ECM components are believed to provide the nucleation sites for hydroxyapatite (HAP) and thereby regulate the morphology, size and composition of the mineral deposits (Schinke et al., 1999). Similarly, in pathological processes, changes in the ECM composition are known to induce such developments as atherosclerotic lesion and vascular calcification (Luo et al., 1997; Simionescu et al., 2007). This interplay between bone maintenance and vascular biology has yet to be clearly defined. However, the phenomenon common to both physiological and pathological biomineralization is the initiation of the calcification processes, mediated by ECM proteins in early stages (Schinke et al., 1999). To understand the fundamental process leading to bone formation or vascular calcification, we have to focus on the development of the ECM and the early stage of biomineral nucleation under the guide of ECM proteins. In this paper, we present an approach to mimic the ECM *in vitro*. We will demonstrate how two non-collagenous proteins can play complementary roles to enhance mineralization when they self-assemble together within one fiber.

Fibronectin (FN) and elastin (EL) are abundant in the ECM of connective tissues and calcified tissues. FN is a hydrophilic glycoprotein with a high molecular weight, ~550–580 kDa. It is central to the ECM architecture and is involved in many fundamental cell functions such as adhesion, growth, differentiation and migration (Pankov and Yamada, 2002). FN has been observed to form a prominent fiber network in early stages of matrix-induced endochondral bone formation, during the proliferation of mesenchymal precursor cells (Weiss and Reddi, 1981). It is ubiquitous during subsequent bone development, highlighting its importance to cellular function during biomineralization. The involvement of FN in early stages of osteogenesis has motivated studies of interactions between FN molecules and apatite particles. Fibrillar FN assembly on sintered hydroxyapatite disks has been observed, dependent upon two bulk solution parameters: protein concentration and ionic strength (Pellenc et al., 2006). The effect of FN on calcium phosphate solution in metastable solution and in agarose gel was probed, but in this study the FN was not in the fibrillar form it takes within the ECM (Couchourel et al., 1999). FN can modify the interfacial energy between surface and mineralization solution and contribute to the early precipitation of biological HAP, a process which occurs during osteointegration of ceramic hydroxyapatite bone implants (Daculsi et al., 1999). FN was further suggested to be the key regulator of arterial calcification via an integrin-based signal pathway (Watson et al., 1998). FN has been linked to transplant arteriosclerosis and has been found in the adventitia of rat aortic grafts (Religa et al., 2003) as well as around calcified lesions in mouse myocardium (Merx et al., 2005). After arterial injury, FN in the extracellular matrix may cause exposed vascular smooth muscle cells (VSMC) to re-differentiate into osteoblast-like cells (Ding et al., 2006; Morla and Mogford, 2000).

Knowledge regarding the role of EL, a primary protein with a low molecular weight (~68 kDa) found in connective tissue such as aorta and skin, also hints at a relationship to pathological mineralization. Age-related degradation of EL in the human aorta is directly

involved in elastocalcinosis, or the deposition of HAP in the elastic lamellae of arteries (Bouvet et al., 2008). Recent findings suggest that EL may act as an inhibitor of calcification depending on its protein chemistry and/or structure. The addition of insoluble EL fibers to a collagen scaffold causes calcification in rats one week after implantation, but no calcification occurs when the scaffold contains EL fragments (Daamen et al., 2008). These discussions indicate that both FN and EL have the potential to control the deposition of biominerals. However, the interactions between the protein and inorganic ions and the cooperative effects, if any, of FN and EL on the deposition of calcium phosphate minerals during biomineralization have hardly been explored.

In the present paper, we demonstrate complementary surface sensitive techniques to study the process of biomimetic mineralization from the earliest stages. Using a charged polymer substrate we show that the two ECM proteins, FN and EL, co-adsorb and self-assemble into a common fiber network. Then, by exposing the fibers to calcium phosphate solution at physiological pH and ionic concentration, we are able to track the mineralization process, starting from incorporation of the ions into the proteins, through nucleation of HAP crystals. In previous work we demonstrated that mineralization was templated on FN fibers from supersaturated solution of calcium carbonate (Subburaman et al., 2006). Here we show that templating also occurs from physiological concentration of calcium phosphate solutions. In this case, the concentration is such that nucleation on inert surfaces does not occur. Hence the biomineralization process in this case is assisted by the conformation of the proteins. Since these two proteins are different we also expect their ability to biomineralize to differ. To our aim, Synchrotron-based Scanning Transmission X-ray Microscopy (STXM) can be used to image the calcium in the fibers, as early as 7 days' incubation. Grazing Incidence Synchrotron X-ray Diffraction (GIXD), as well as Transmission Electron Microscopy (TEM) is used to determine the crystallinity of the calcium deposits, while Scanning Electron Microscopy (SEM) with Energy-Dispersive X-ray Spectroscopy (EDXS) is used to probe their elemental composition. The earliest evidence of ionic absorption into the protein fibers, however, is tracked indirectly through measurements of the fiber heights and moduli, where significant changes occur within the first few hours of incubation. In previous work we demonstrated that FN and EL nucleated calcium carbonate crystals exclusively in their fibrillar form (Subburaman et al., 2006), but their efficiency for biomineralization differed. In the present work, we show that these two proteins can have complementary roles in the nucleation and growth of calcium phosphate mineral. Such differences have important implications for the templating of biominerals by proteins.

2. Materials and methods

2.1. Sample preparation

Polished 200 μm thick $\langle 1\ 0\ 0 \rangle$ Si wafers were obtained from Wafer World Corporation, West Palm Beach, FL. Si TEM grids having 100 nm-thick Silicon-Nitride membrane windows were obtained from Silson Ltd., Northampton, England. Both types of substrates were spin-coated with Sulfonated Polystyrene: SPS, $M_w \sim 175$ kDa (Polymer Source Inc., Dorval, Canada) which was dissolved in *N,N*-Dimethylformamide (Sigma-Aldrich, Inc., St. Louis, MO), spun-cast, and vacuum dried as described in our previous work (Subburaman et

al., 2006). FN from bovine plasma and EL from bovine neck ligament (Sigma–Aldrich, Inc., St. Louis, MO) were dissolved in Phosphate-Buffered Saline (PBS) without calcium or magnesium (Invitrogen, Carlsbad, CA). Prepared SPS-coated wafers were incubated in the solutions of FN, EL, and FN–EL in PBS buffer (FN: 100 mg/ml, EL: 5 mg/ml, FN–EL: FN 100 mg/ml and EL 5 mg/ml) for 4 days at 37 °C and 100% RH in 24-well dishes (BD, Franklin Lakes, NJ). After the self-assembled protein network formed, samples were rinsed three times with deionized (DI) water to remove the unabsorbed protein and salts in the PBS.

Samples were immersed in a metastable calcium phosphate solution (Dutour Sikiric et al., 2009) which was prepared as follows: Salts were purchased from Mallinckrodt Baker Inc., Phillipsburg, NJ. Solutions were made in HEPES buffer, 1 M, (Mediatech Inc., Manassas, VA) and diluted to 25 mM with DI water. NaCl solution (137 mM) in 25 mM HEPES buffer was prepared and the pH was adjusted to 7.4. CaCl₂ and Na₂HPO₄ stock solutions with the concentrations of 5.6 and 4 mM, respectively, were prepared separately in the above NaCl solution and the pH was readjusted to 7.4. Each of the stock solutions contained in addition 0.05% (w/v) sodium azide (Sigma–Aldrich, Inc., St. Louis, MO) to avoid bacterial contamination. The metastable calcium phosphate solution was freshly prepared before use by rapidly mixing equal volumes of CaCl₂ and Na₂HPO₄ stock solutions. All the mineralization experiments were carried out at room temperature, at which the solution is known to be metastable and does not precipitate mineral observed by SEM onto bare Silicon wafers or polymer films up to 28 days. After the different mineralization time points (as given in Section 3) samples were rinsed by DI water twice, air-dried overnight and stored in a desiccator for further characterization by different methods. An exception is samples examined by scanning probe microscopy, shear modulation force microscopy and confocal laser scanning microscopy, which were examined under wet conditions as described in the following sections.

2.2. Scanning Probe Microscopy (SPM) and Shear Modulation Force Microscopy (SMFM)

The early stage imaging and height measurements were made using a Dimension 3100 SPM (Veeco, Santa Barbara, CA) in contact mode with a silicon nitride tip (Veeco, Santa Barbara, CA). After the specific mineralization time point, the sample was rinsed twice by PBS and then immediately measured, in PBS, with scanning force microscopy in a 35 mm petri-dish (BD, Franklin Lakes, NJ). The fiber height was evaluated using SPM cross-sectional images by measuring fiber heights referenced to flat base regions as shown previously (Subburaman et al., 2006). For each protein, two different samples were imaged for each time point and 30 fibers on each sample were analyzed. The error bars shown for fiber height data represent the standard deviation obtained from approximately 60 measurements. Use of the SMFM to measure shear modulus response relies on lateral modulation of the cantilever buried ~3 nm deep into the sample, and measurement of the amplitude response, as detailed previously (Subburaman et al., 2006; Zhang et al., 2003). Significance of observations is determined by *t*-tests.

2.3. Confocal Laser Scanning Microscopy (CLSM)

FN and EL were individually labeled with Oregon Green 488 protein labeling kit (Invitrogen, Carlsbad, CA) before solution preparation. For the mixture, the labeled FN or

EL solution was mixed with unlabeled EL or FN solution to the final concentration of 100 µg/ml FN and 5 µg/ml EL. The SPS-coated Si wafers incubated with the above protein solutions for 4 days were rinsed by PBS twice and kept in a 35 mm petri-dish (BD, Franklin Lakes, NJ) with PBS for imaging by CLSM (Leica, Bannockburn, IL) with a 63× water objective lens.

2.4. Grazing Incidence X-ray Diffraction (GIXD)

Grazing Incidence X-ray Diffraction (GIXD) experiments were carried out on NSLS Beamline X6B at BNL. The X-ray wavelength $\lambda = 0.6525 \text{ \AA}$ and spot size 0.25 mm vertical $\times$ 0.4 mm horizontal were used. The sample was mounted on a goniometer head at a distance of 150 mm from the detector screen. Grazing incidence diffraction patterns with an incident angle of 1.0° were recorded using an X-ray CCD detector (Princeton Instruments, Trenton, NJ). The detector geometry (distance from sample, tilt of detector, center of diffraction pattern) was calibrated using a transmission diffraction pattern of standard Al_2O_3 plate. In addition, synthetic Hydroxyapatite and Amorphous Calcium Phosphate powders (Sigma–Aldrich, Inc., St. Louis, MO) as controls were investigated for reference in both glass capillary tubes (1.0 mm diam.) and as compressed powders on a blank SPS substrate (Meng et al., 2009).

2.5. Scanning Transmission X-ray Microscopy (STXM)

X-ray imaging and spectroscopy were carried out on beamline X1A1 at the National Synchrotron Light Source (NSLS) at Brookhaven National Laboratory (BNL). A monochromatic soft X-ray beam is focused to 50 nm by a Fresnel zone plate, and the sample is scanned through the focal point in two dimensions while recording the intensity of transmitted X-rays. The soft X-ray beam with the energy range from 270 to 800 eV does not cause mineral sublimation through specimen heating. Samples were assembled onto 100 nm-thick Silicon-Nitride membrane windows for X-ray transmission. The carbon K-edge energy calibration was determined using CO_2 and calibrated to peak positions reported by Ma et al. (1991) using a Gaussian fit. Energy near the calcium L-edge peaks was calibrated by assigning measured calcite $\text{L}_{3,2}$ peak positions to 349.3 and 352.6 eV (Benzerara et al., 2004, 2006).

2.6. Transmission Electron Microscopy (TEM)

Samples on Silicon-Nitride membrane windows were observed after 7-day and 21-day mineralization intervals by using a JEOL JEM2100F high-resolution analytical TEM (Center for Functional Nanomaterials, BNL) operating at 200 kV. The electron diffraction patterns of the particles were recorded by using a selected-area aperture allowing observation of a circular area of 1.36-µm diameter. Caution was taken regarding the size and thickness of the examined aggregates, because these parameters may influence the quality of the diffraction patterns.

2.7. Scanning Electron Microscopy (SEM)

SEM measurements (LEO1550, LEO, Germany) were conducted at the Center for Functional Nanomaterials, BNL. The morphology of crystals on the protein matrices after

28 days in calcium phosphate mineralization solution was investigated at 15 kV acceleration voltage and 4 mm working distance.

3. Results

A 20 nm thick coating of Sulfonated Polystyrene (SPS) polymer on either a clean Si wafer (500 μm thick) or a Silicon-Nitride membrane window (100 nm-thick) provides a surface conducive to protein fiber formation (Subburaman et al., 2006; Meng et al., 2009; Pernodet et al., 2003). After 4 days of incubation at 37 $^{\circ}\text{C}$, proteins from solution are adsorbed on the SPS-coated silicon surface. As seen by SPM height imaging, both FN (Fig. 1A) and EL (Fig. 1C) self-assemble into fiber networks, of slightly different dimension. The SPS coating is necessary: when bare silicon substrates are used, no fibrillar ECM network forms; only small amounts of protein globules, <300 nm in height, can be imaged (Meng et al., 2009). In Fig. 1B we show the network formed when both proteins are adsorbed. From the SPM image only a single network is apparent. By labeling each protein in turn with Oregon Green 488 and imaging with confocal microscopy, we demonstrate that in the FN-EL mixture the two proteins have combined into one fiber network, with no evidence of phase segregation. A green fluorescent network is visible when either FN (Fig. 2A) or EL (Fig. 2B) is labeled. At higher magnification (Fig. 2A, inset) it appears that FN forms the backbone of the fiber. This is supported by the SPM friction images of each network in Fig. 2C-E: pure FN, mixed FN-EL, and pure EL have each been examined. The pure networks show contrast from the softer fiber regions (white) compared to the flat (dark), hard substrate areas between them. In Fig. 2D we see that the combined FN-EL fibers are not uniform. Instead they have a backbone which is harder (darker) than the sides of the fibers. Based on the confocal images, we postulate that the harder backbone has more FN than the softer sides, which are EL-rich areas. Based on SPM topography shown in Fig. 1, the combined fibers are thinner (Fig. 3A) and have a smaller mesh size than either pure protein. This is not surprising since the self recognition sites on the proteins can be in different regions than those for the complementary protein, which can lead to different secondary structures. Further study on this aspect using FN fragments is underway, and will be published elsewhere.

Biom mineralization in the earliest stages is very challenging to observe since the ion concentration is below detection by traditional methods, such as Energy-Dispersive X-ray Spectroscopy (EDXS) or other ion and X-ray scattering. SPM allows us to measure whether any changes in the morphology or the mechanical properties occur once the fibers are immersed in mineralization media. The fiber heights plotted as a function of incubation time in calcium phosphate mineralization solution are shown in Fig. 3A. The fiber height of all proteins increases with exposure time, despite the fact that the ions are as yet undetectable in the first 12 h (Fig. 3A, closed symbols). During the first 4-h mineralization, heights of all of the protein fibers increase quickly ($>0.05 \mu\text{m h}^{-1}$) and then level off near final heights of 1.10, 0.76, and 1.26 μm , which represent increases of 83%, 54%, and 60% for FN, FN-EL, and EL, respectively. When placed in Phosphate-Buffered Saline (PBS) for 12 h, the fiber heights are unchanged (Fig. 3A, open symbols), indicating that neither water nor other ions swell the fibers. Hence the increase of fiber height is specific to the mineralization ion in the solution, which indicates that the ions penetrate and restructure the protein fibers almost immediately. Our previous work, in which both pure EL and FN systems were immersed in

a calcium chloride control solution, demonstrated that either carbonate (Subburaman et al., 2006), or phosphate, as is the case here, are required to produce any time-dependent changes in the SPM experiment.

Structure changes in the fibers can also be sensed by differences in mechanical response. Shear Modulation Force Microscopy (SMFM) measures the relative change in modulus of the fibers. In Fig. 3B we show the moduli of the fibers as a function of time, where the scale is relative to the response of non-biomineralized EL. From the figure we see the modulus of FN is on average 22% higher than that of the EL, consistent with the SPM friction image shown in Fig. 2D. On the other hand, the moduli of the fibers formed from the FN–EL are 12% greater, which is slightly smaller, though not significantly than the value for EL. When exposed to the calcium phosphate mineralization solution, the moduli of the FN and the FN–EL fibers begin to increase rapidly, tracking the changes in fiber height shown previously. These moduli increase at a rate of 0.15 h^{-1} for FN, 0.17 h^{-1} for FN–EL, and only 0.02 h^{-1} for EL for the first 4 h, after which the change becomes more gradual, becoming less than 0.02 h^{-1} for all the fibers. As a result we can see that even though the FN–EL fibers were initially softer than those of EL, they rapidly become harder. During the 12 h period the moduli of the FN and the FN–EL fibers increased by 66% and 68%, respectively, while those of the EL fibers increased only by 8%. The change in fiber moduli in control samples, placed in PBS solutions are shown as open symbols in the figure. Here we can see that no increase and even a slight decrease in modulus occurs with -16% for FN, -7% for FN–EL and EL. The results suggest that mineral ions do not simply adsorb onto the protein fibers non-specifically. Rather, since each of the three networks shows a different behavior on exposure to solution, the interaction may be chemically specific to the protein or protein combination, as well as on the network structure. The next two questions to answer are: (I) when do mineral nuclei appear; and (II) what makes the response of pure EL different? To study spatially resolved Ca deposition and mineral crystallinity, we turn to X-ray scattering and microscopy methods.

Synchrotron Grazing Incidence X-ray diffraction (GIXD) data from crystals templated by FN and FN–EL are shown in Fig. 4A and B. Up to 14 days (data of day 7 not shown), no statistically significant diffraction peaks in either profile could be observed above the background. Thus the presence of crystalline hydroxyapatite within the organic matrix could not be detected. At 21 days, the FN–EL matrix generates a pattern of poorly crystalline HAP with one small broad asymmetric peak corresponding to the $(2\ 1\ 1)/(1\ 1\ 2)$ crystal planes. At 28 days, two broad peaks corresponding to the $(0\ 0\ 2)$ and $(2\ 1\ 1)/(1\ 1\ 2)$ planes of HAP are observed on FN and FN–EL matrices. Using the Scherrer equation, we estimate that the diffraction peaks were obtained from crystallites approximately 10–30 nm in size. These values are comparable to those reported for crystals formed in bone (Kuhn et al., 2000). This is only a lower boundary, however, since further details of grain size and strain are not accessible due to the geometry of the GIXD method, which causes additional peak broadening (see supplemental Fig. 4 of Meng et al. (2009)). The EL sample has no X-ray diffraction pattern even after day 28 (Fig. 4C). These results indicate that only after 28 days of mineralization sufficient bone-like hydroxyapatite is templated on FN and FN–EL to be observed by GIXD. Hence, evidence of biomineralization at earlier stages can only be obtained by other complementary techniques.

Scanning Transmission X-ray Microscopy (STXM) enables us to obtain protein and calcium distribution maps for self-assembled protein matrices at earlier time points. The synchrotron-based STXM focuses a soft X-ray beam to a 50 nm spot size and photons are transmitted through the fibers. In this measurement, samples have been rinsed by DI water twice and then air-dried, which has the effect of shrinking the fiber height to less than a micron and enabling the soft x-rays to transmit through them. The small beam is an excellent probe of the micron-scale fibers, sensitive to absorption contrast at the C K-edge and Ca L-edge. Images of the same region on the samples after 7-day mineralization at the C K-edge peak (288.2 eV) and at the Ca L-edge (349.3 eV) are shown in Fig. 5 (left and right columns for C and Ca edges, respectively). At 288.2 eV, the amide carbonyl group in protein absorbs x-rays, making fiber areas appear dark. The morphology is similar to that observed by SPM. The same areas of all three 7-day mineralized films are also shown at the Ca L-edge. When FN is imaged at the Ca edge, the fiber contrast is greatly reduced, due to the fact that the energy is far from the carbon K-edge, and this lower absorption contrast also indicates that the fibers do not contain appreciable calcium. Instead small dense spots (arrows) appear on the fibers, evidence for Ca-bearing particles of a few hundred nm in size. The EL network behaves differently. The EL fibers imaged at the Ca edge show a large absorption contrast, indicating that it is along the fibers that the Ca is located. Moreover, no distinct particles are found in EL images we examined. Therefore, Ca in the EL network appears to absorb into the fibers. The FN–EL mixture shows both of these two features. At the Ca edge, fiber contrast is slightly enhanced, and distinct particles also appear. Our interpretation is that EL absorbs Ca in its fibers, while FN nucleates mineral particles. The combination of FN and EL allows these processes to work together.

To investigate the properties of minerals templated on the protein matrices, we turn to Transmission Electron Microscopy (TEM) and Selected-Area Electron Diffraction (SAED) for the same day-7 samples used in STXM. Fig. 6A shows the TEM images of the three different protein matrices, and the small particles of different shapes present on FN and FN–EL. On the FN sample, small platelet-shaped particles consisting of needle-shaped subparticles are uniformly present on and near the protein fibers. On the FN–EL sample, small spherical particles are observed which aggregate beside the protein fibers. Despite their appearance, none of the particles on either substrate produce rings in the electron diffraction patterns shown in Fig. 6B, indicating lack of crystalline order in the particles. Hence the particles formed at the earliest stages of the biomineralization process consist of some type of amorphous calcium containing deposit. The TEM image of EL shows that there are only protein fibers without any particles. In Fig. 6B we also show a representative diffraction pattern similar to those obtained from several EL samples, which only shows the same type of diffuse scattering pattern as observed for FN and FN–EL.

When mineralization develops into late stage (day 21), minerals with different morphology are observed in the STXM images of the FN-bearing surfaces, shown in Fig. 7A. However, the calcium-dense area on EL only follows the morphology of the protein network. The detailed morphology and crystal property of different minerals templated on the protein matrices were characterized by TEM shown in Fig. 7B. Needle-shaped crystals are observed on FN, with clear polycrystalline diffraction rings. On FN–EL, small plate-like crystals surrounding protein fiber produce the weak diffraction rings. On EL, small needle-like

crystals with weak diffraction were confined inside the EL fibers and there is no crystal observed in areas between the fibers. All the samples in Fig. 7C produce different degrees of diffraction patterns exhibiting the (0 0 2) and (2 1 1) hydroxyapatite (HAP) reflections.

SEM images from the back-scattered electron signal for day-28 mineralization are shown in Fig. 8. These images are not sensitive for the protein network which has a lower atomic number value than the substrate (Si). Hence we can clearly see when biomineralized deposits, with higher atomic number, form. In the Fig. 8, we find that the surfaces are uniformly covered with HAP particles on the FN and FN-EL. No particles are seen on the EL. Even in the low resolution images, we can see that the deposits templated on the FN and FN-EL are different. The FN produces large distinct particle aggregates while the FN-EL produces gradual but more uniform particles. In the inset we show a magnified view of the region shown in Fig. 8A and B, where we can clearly see that the particles are composed of discrete crystallites with plate and needle morphologies. Pure EL viewed by SEM shows that the production of large coverage of mineral particles has been suppressed; the nanoscale needles viewed by TEM are not numerous enough to provide the electron contrast needed to image them by SEM (Fig. 8C). HAP went undetected by GIXD as well, suggesting that perhaps only a fraction of the Ca-rich material on EL became crystalline. The raw EDXS response was used to compare Ca/P ratios of these particles. The Ca/P ratios deduced from EDXS measurements calibrated using synthetic HAP as a standard are 1.55 ± 0.12 for FN and 1.46 ± 0.24 (standard deviation of three measurements per sample) for FN-EL. These values are comparable to each other, but less than 1.67 obtained for the HAP standard measured. The proteins nucleate a form of calcium deficient HAP which is consistent with the theory reported by Olszta et al. (2007), where they proposed that non-stoichiometry of the HAP bestows the bone mineral solubility for resorption of the bone by osteoclasts during bone remodeling and repair process.

4. Discussion

The actions of non-collagenous proteins (NCPs) are central to the fundamental mechanism of biomineralization *in vivo*. Based on our data, we propose that NCP-mediated biomineralization occurs in three stages. The first step involves ionic adsorption into the fibrillar protein matrix, leading to the modification of structural and mechanical properties of protein fibers (Fig. 3). The second step is the nucleation of nanometer-size particles Fig. 5, which occurs only on certain proteins. Neither synchrotron GIXD (Fig. 4) nor TEM (Fig. 6) detects crystalline order in these particles. The third step is that the nanometer-sized particles grow into micrometer size by further ionic deposition and/or aggregation and undergo phase transformation into HAP crystals (Figs. 7 and 8). With further incubation, particles seen on FN become crystalline and grow significantly larger, while on EL the formation of distinct particles outside the fibers is suppressed.

As we can see in Fig. 4, The X-ray data exhibit weak peaks on a large background. TEM is more sensitive since the beam can be focused on individual particles. Well defined diffraction rings are observed. To quantify these TEM diffraction patterns, we performed a powder-like integration of the diffracted intensity plotted against momentum transfer q in Fig. 9A². For comparison, synchrotron powder X-ray data of commercial HAP and ACP

also plotted against q , shown in Fig. 9C. Note that the strong peaks of HAP are in the region $q = 1.83$ and $2.25 \text{ \AA}^{-1}$ which is corresponding to the (0 0 2) and (2 1 1) planes, while the broad peak in the bulk structure factor of ACP is at $q = 2.05 \text{ \AA}^{-1}$. In TEM data at day 7 (dashed line) no sharp diffraction peaks are present. To clarify the details of peaks near $2.05 \text{ \AA}^{-1}$, we use the featureless spectrum of EL-7 day as a baseline, and all spectra shown in Fig. 9B have been subtracted by that spectrum. The spectra of FN-7 day and FN-EL-7 day have a broad peak with its maximum in the same position as the ACP structure factor shown in Fig. 9B, which suggests that the nanometer-sized particles on FN containing samples incorporate ACP. By day 21, peaks in FN containing samples are clear which correspond to the (0 0 2) and (2 1 1) planes of HAP. A number of studies point to the possibility that the initially deposited ACP transforms into a crystalline mineral phase (Olszta et al., 2007; Termine and Posner, 1966; Lowenstam and Weiner, 1985; Glimcher, 1984). Our study demonstrates how ACP formation may be initiated by NCPs in the early stage of biomineralization *in vitro*, following which it is transformed into HAP.

In our study, we also demonstrate that different NCPs play different roles during the biomineralization process. Based on the above results, we propose to take into account the proteins' relative structural heterogeneity. The FN molecule is a dimer with specific dissimilar domains known to interact with each other when the protein unfolds to form fibrils (Ng et al., 2007). Since FN alone can nucleate calcium phosphate crystals from physiological calcium phosphate solution, we surmise that both cations and anions must bind to FN in such a way as to be brought into proximity with each other under our experimental conditions. We propose that FN has specific sites which capture phosphate and calcium, though not in large amounts. When fibrillogenesis occurs, the adjacent FN molecules bring calcium and phosphate in proximity with each other, enhancing nucleation, and eventual crystal growth. However, EL is expected to be very different based on the properties of the molecule. Its hydrophobic subunits are structurally similar to each other, and the extended network is mechanically flexible. This means that even when the conformational change into fibers occurs, the local environment seen by ions near the protein is similar throughout the fiber. We have no evidence that the protein in its globular form interacts with ions. STXM images (Fig. 5) show that in the fiber structure, calcium is bound to the protein in significant amounts. However, the presence of phosphate in solution is required for this to occur: our previous work showed that EL fibers underwent no detectable changes when immersed in CaCl_2 control solutions (Subburaman et al., 2006). Despite the abundance of Ca and the implied proximity of available phosphate, the release of mineral particles outside the protein fibers is evidently suppressed by EL. Although the role of phosphate remains to be determined, an understanding of the cooperative effect in the FN-EL mixture is suggested by our model. The collection of calcium by EL, and the structural control exerted by FN, work together to nucleate a greater amount of calcium phosphate particles, which are amorphous in the early stages but later transform into calcium deficient apatite crystals. Even though many crystallites are obtained, the TEM and STXM data imply that a significant amount of calcium in the protein mixture remains un-associated

²Calibration and resolution of integrated TEM data are not as reliable as the X-ray technique, leading to slight inaccuracy in q values for TEM.

with the crystals – either as bound calcium ions or in amorphous mineral form – based upon the images showing contrast from fibers co-existing with mineral particles.

In summary, our work provides direct evidence that two specific NCPs can combine to provide the molecular design necessary for controlling biomineralization. Our next step is to investigate how FN and EL affect mineralization of collagen, the most abundant component of the ECM. The retarding mechanisms in biomineralization, which are crucial to prevent pathological mineralization in the early stage, are under intense investigation. We emphasize here that an apparent retarded biomineralization, as by EL alone, can become an enhancer of mineralization by sequestering cations, if a complementary protein in the system provides the structure that overcomes barriers to nucleation.

Acknowledgments

This work is supported by NSF-MRSEC Program (DMR0606387), the Brookhaven National Laboratory-Stony Brook University Seed Grand Program and NIH program (R01 AR52379 and R01 AR49286). Research carried out in part at the Center for Functional Nanomaterials and National Synchrotron Light Source, Brookhaven National Laboratory, which is supported under USDOE Contract DE-AC02-98CH10886.

Abbreviations

ECM	extracellular matrix
FN	fibronectin
EL	elastin
FN-EL	mixture of fibronectin and elastin
HAP	hydroxyapatite

References

- Benzerara K, Yoon TH, Tyliszczak T, Constantz B, Spormann AM, B GE JR. Scanning transmission X-ray microscopy study of microbial calcification. *Geobiology*. 2004; 2:249–259.
- Benzerara K, Miller VM, Barell G, Kumar V, Miot J, Brown GE, Lieske JC. Search for microbial signatures within human and microbial calcifications using soft X-ray spectromicroscopy. *J Invest Med*. 2006; 54:367–379.
- Bouvet C, Moreau S, Blanchette J, de Blois D, Moreau P. Sequential activation of matrix metalloproteinase 9 and transforming growth factor beta in arterial elastocalcinos. *Arterioscler Throm Vasc Biol*. 2008; 28:856–862.
- Couchourel D, Escoffier C, Rohanizadeh R, Bohic S, Daculsi G, Fortun Y, Padrines M. Effects of fibronectin on Hydroxyapatite formation. *J Inorg Biochem*. 1999; 73:129–136. [PubMed: 10331242]
- Daamen WF, Nillesen STM, Wismans RG, Reinhardt DP, Hafmans T, Veerkamp JH, Van Kuppevelt TH. A biomaterial composed of collagen, solubilized elastin enhances angiogenesis, elastic fiber formation without calcification. *Tissue Eng A*. 2008; 14:349–360.
- Daculsi PPG, Cottrel M, Guicheux G. Role of fibronectin during biological apatite crystal nucleation: ultrastructural characterization. *J Biomed Mater Res*. 1999; 47:228–233. [PubMed: 10449634]
- Ding, WH CG, Zhang, Tian-Lan, Kui Wang. Fibronectin enhances in vitro vascular calcification by promoting osteoblastic differentiation of vascular smooth muscle cells via ERK pathway. *J Cell Biochem*. 2006; 99:1343–1352. [PubMed: 16795048]

- Dutour Sikiric M, Gergely C, Elkaim R, Wachtel E, Cuisinier FJG, Füredi-Milhofer H. Biomimetic organic-inorganic nanocomposite coatings for titanium implants. *J Biomed Mat Res A*. 2009; 89A: 759–771.
- Glimcher MJ. Recent studies of the mineral phase in bone, its possible linkage to the organic matrix by protein-bound phosphate bonds. *Philos Trans R Soc Lond Ser B*. 1984; 304:479–508. [PubMed: 6142489]
- Kuhn LT, Xu YT, Rey C, Gerstenfeld LC, Grynblas MD, Ackerman JL, Kim HM, Glimcher MJ. Structure, composition, and maturation of newly deposited calcium–phosphate crystals in chicken osteoblast cell cultures. *J Bone Miner Res*. 2000; 15:1301–1309. [PubMed: 10893678]
- Lowenstam HA, Weiner S. Transformation of amorphous calcium phosphate to crystalline dahillite in the radular teeth of chitons. *Science*. 1985; 227:51–53. [PubMed: 17810022]
- Luo G, Ducey P, McKee MD, Pinero GJ, Loyer E, Behringer RR, Karsenty G. Spontaneous calcification of arteries and cartilage in mice lacking matrix GLA protein. *Nature*. 1997; 386:78–81. [PubMed: 9052783]
- Ma Y, Chen CT, Meigs G, Randall K, Sette F. High-resolution K-shell photoabsorption measurements of simple molecules. *Phys Rev A*. 1991; 44:1848–1858. [PubMed: 9906154]
- Meng Y, Qin YX, DiMasi E, Ba X, Rafailovich M, Pernolet N. Biomineralization of a self-assembled extracellular matrix for bone tissue engineering. *Tissue Eng A*. 2009; 15:355–366.
- Merx MW, Schafer C, Westenfeld R, Brandenburg V, Hidajat S, Weber C, Ketteler M, Jahnke-Dechent W. Myocardial stiffness, cardiac remodeling and diastolic dysfunction in calcification-prone fetuin-A-deficient mice. *J Am Soc Nephrol*. 2005; 16:3357–3364. [PubMed: 16177000]
- Morla AO, Mogford JE. Control of smooth muscle cell proliferation and phenotype by integrin signaling through focal adhesion kinase. *Biochem Biophys Res Commun*. 2000; 272:298–302. [PubMed: 10872843]
- Ng SP, Billings KS, Ohashi T, Allen MD, Best RB, Randles LG, Erickson HP, Clarke J. Designing an extracellular matrix protein with enhanced mechanical stability. *Proc Natl Acad Sci USA*. 2007; 104:9633–9637. [PubMed: 17535921]
- Olszta MJ, Cheng X, Jee SS, Kumar R, Kim Y, Kaufman MJ, Douglas EP, Gower LB. Bone structure, formation: a new perspective. *Mater Sci Eng R*. 2007; 58:77–116.
- Pankov R, Yamada KM. Fibronectin at a glance. *J Cell Sci*. 2002; 115:3861–3863. [PubMed: 12244123]
- Pellenc D, Berry H, Gallet O. Adsorption-induced fibronectin aggregation, fibrillogenesis. *J Colloid Interface Sci*. 2006; 298:132–144. [PubMed: 16375913]
- Pernolet N, Rafailovich M, Sokolov J, Xu D, Yang NL, McLeod K. Fibronectin fibrillogenesis on sulfonated polystyrene surfaces. *J Biomed Mater Res A*. 2003; 64A:684–692. [PubMed: 12601780]
- Religa P, Bojakowski K, Gaciong Z, Thyberg J, Hedin U. Arteriosclerosis in rat aortic allografts: dynamics of cell growth apoptosis and expression of extracellular matrix proteins. *Mol Cell Biochem*. 2003; 249:75–83. [PubMed: 12956401]
- Schinke T, McKee MD, Karsenty G. Extracellular matrix calcification: where is the action? *Nat Genet*. 1999; 21:150–151. [PubMed: 9988260]
- Simionescu A, Simionescu DT, Vyavahare NR. Osteogenic responses in fibroblasts activated by elastin degradation products and transforming growth factor β 1: role of myofibroblasts in vascular calcification. *Am J Pathol*. 2007; 171:116–123. [PubMed: 17591959]
- Subburaman K, Pernolet N, Kwak SY, DiMasi E, Ge S, Zaitsev V, Ba X, Yang NL, Rafailovich M. Templated biomineralization on self-assembled protein fibers. *Proc Natl Acad Sci USA*. 2006; 103:14672–14677. [PubMed: 17003131]
- Termine JD, Posner AS. Infrared analysis of rat bone: age dependency of amorphous and crystalline mineral fractions. *Science*. 1966; 153:1161–1164.
- Watson KE, Parhami F, Shin V, Demer LL. Fibronectin, collagen I matrixes promote calcification of vascular cells in vitro, whereas collagen IV matrix is inhibitory. *Arterioscler. Thromb Vasc Biol*. 1998; 18:1964–1971.
- Weiss RE, Reddi AH. Appearance of fibronectin during the differentiation of cartilage, bone and bone-marrow. *J Cell Biol*. 1981; 88:630–636. [PubMed: 7217207]

Zhang Y, Ge S, Rafailovich MH, Sokolov JC, Colby RH. Surface characterization of cross-linked elastomers by shear modulation force microscopy. *Polymer*. 2003; 44:3327–3332.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 1.
Contact mode SPM topography images ($50 \times 50 \mu\text{m}$) of self-assembled protein network on SPS-coated silicon wafer after 4-day incubation in protein solutions: (A) FN; (B) FN-EL; (C) EL.

Fig. 2.

Confocal images of two FN–EL samples fluorescently labeled with Oregon Green 488 prior to adsorption on SPS-coated Si wafers: (A) The FN is labeled with Oregon Green 488 while the EL is unlabeled. Inset: higher magnification image showing FN-rich (green) region on the backbone of the fibers. (B) The EL is labeled with Oregon Green 488 while the FN is unlabeled. Uniform fluorescence intensity in the fiber patterns in both images indicates that the proteins combine to form uniform common fiber structures, rather than phase separated networks composed of the individual separate two proteins. Scale bar in (A), (B) = 10 μm . Lateral friction images (50 $\times$ 50 μm) corresponding to (C) a pure FN network showing fibers of uniform mechanical response which appear soft (white) fibers against the hard (dark) Si wafer background. (D) A mixed FN–EL network, where the fiber backbone appears harder (darker) than the fiber edges (white). This image corresponds to that shown in the inset of (A) and hence we infer that the backbone is stiffer since it is enriched in the FN, the stiffer of the two proteins. (E) Uniform fibers of the smaller EL network, which also appear softer (white) than the surrounding flat regions (dark). (For interpretation of color mentioned in this figure, the reader is referred to the web version of this article.)

Fig. 3.

(A) Average fiber heights measured by SPM for FN, FN-EL, and EL networks as function of time immersed in calcium phosphate solution (closed symbols) or buffer control solution (opened symbols). See figure key for symbols. Lines are guides for the eye. (B) Relative elastic modulus of protein fibers measured by SMFM technique under each condition (see figure key). All values are normalized to the response of EL in calcium phosphate solution at $t = 0$, prior to mineralization.

Fig. 4. Synchrotron Grazing Incidence X-ray Diffraction (GIXD) patterns of 14-, 21-, 28-day (A) FN; (B) FN-EL; and (C) EL on SPS-coated silicon wafer (Wavelength = 0.65255 Å). Inset: Gaussian fitting of (0 0 2) diffraction peak on 28-day samples. Lines on 2-theta axis indicate the intense lines of the standard HAP diffraction pattern.

Fig. 5.

STXM images of self-assembled protein matrices after 7-day incubation in calcium phosphate mineralization media. Left column: C K-edge map at 288.2 eV, highlighting protein-dense regions. Right column: Ca L-edge map at 349.3 eV. Rows: FN network, FN–EL mixture, and EL network as labeled. Arrows show where calcium-rich deposits appear as submicron-scale particles, in samples containing FN. The EL sample fibers are calcium-rich (darkest in the EL Ca L-edge map) but no discrete Ca-bearing particles separate from fibers are observed. Image intensity scales in each column have been set to identical range and contrast parameters and have been taken under comparable beam currents.

Fig. 6. (A) High-resolution TEM images and (B) SAED pattern of mineral particle morphologies in different protein matrices after 7-day incubation in calcium phosphate mineralization media. Columns: FN network, FN-EL mixture and EL network as labeled. (Scale bar: 250 nm). Corresponding SAED patterns exhibit diffuse background scattering only.

Fig. 7. (A) STXM images correlated with (B) High-resolution TEM and (C) SAED of mineral templated on protein matrices after 21-day incubation in calcium phosphate mineralization media. Columns: FN network, FN-EL mixture, and EL network as labeled. Scale bar in (A): 2.5 μm . TEM images show the detail morphology of crystals and protein fibers. (Scale bar in (B): 0.3 μm in FN and 1 μm in FN-EL and EL).

Fig. 8. SEM micrographs of mineralized samples on day 28. The low-magnification images (1000 $\times$) of mineralized self-assembled protein matrices: (A) FN, (B) FN-EL, and (C) EL. (Scale bar = 20 μ m). Inset magnification (A and B): 10,000 $\times$. (Scale bar = 2 μ m.) No particles were found at low or high magnification for EL.

Fig. 9.

(A) Integrated TEM diffraction spectra of mineralized samples (Figs. 6 and 7) with Si wafer for calibration. (B) Integrated TEM diffraction spectra subtracted by the spectrum of EL-7 day. (C) Synchrotron powder X-ray diffraction patterns of synthetic hydroxyapatite (HAP) and amorphous calcium phosphate (ACP). (A–C) are plotted in equivalent units of momentum transfer q .