Scanning Microscopy

Volume 10 | Number 2 Article 17

2-15-1996

The Nucleation and Growth of Calcium Phosphate Crystals at Protein and Phosphatidylserine Liposome Surfaces

G. H. Nancollas
State University of New York at Buffalo

A. Tsortos
State University of New York at Buffalo

A. Zieba
State University of New York at Buffalo

Scanning Microscopy, Vol. 10, No. 2, 1996 (Pages 499-508) 0891-7035/96$5.00+.25
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

THE NUCLEATION AND GROWTH OF CALCIUM PHOSPHATE CRYSTALS AT PROTEIN AND PHOSPHATIDYLSERINE LIPOSOME SURFACES

G.H. Nancollas[1,*], A. Tsortos[1,2] and A. Zieba[1]

Departments of [1]Chemistry and [2]Biophysics, State University of New York at Buffalo, Amherst, NY

(Received for publication June 9, 1995 and in revised form February 15, 1996)

Abstract

The kinetics of calcium phosphate crystal growth at the surfaces of proteins and phospholipids has been investigated using free drift and constant composition methods in supersaturated calcium phosphate solutions (relative supersaturations: with respect to hydroxyapatite, HAP, $\sigma_{HAP} = 15.0$, and with respect to octacalcium phosphate, OCP, $\sigma_{OCP} = 1.9$). Fibrinogen and collagen molecules adsorbed at hydrophobic surfaces as well as uncross-linked collagen fibrils induce ion binding and subsequent nucleation of calcium phosphate. The formation of OCP on phosphatidylserine vesicles introduced to highly supersaturated calcium phosphate solutions probably involves the interaction of the calcium ions with the ionized carboxylic groups of the phospholipid.

Key Words: Biomineralization, calcium phosphate, crystallization, protein matrix, collagen, fibrinogen, matrix vesicles.

*Address for correspondence:
George H. Nancollas
Department of Chemistry,
State University of New York at Buffalo,
Amherst, NY 14260-3000
Telephone number: (716) 645-6800 ext. 2210
FAX number: (716) 645-6947

Introduction

The crystal growth of biominerals such as the calcium phosphates and carbonates is frequently used as a model to explain biomineralization events at both inorganic and organic interfaces. As the concentrations of lattice ions in the contacting aqueous media are increased above the saturation levels, a considerable degree of supersaturation may be required before the nucleation of the new solid phase is initiated. The reason for the appreciable nucleation threshold is associated with the magnitude of the free energy of formation of the new solid surface. This unfavorable, or positive, free energy is balanced by the negative free energy reflecting the release of the supersaturation. It is therefore possible to calculate a free energy corresponding to the formation of the critical nucleus as well as the rate of nucleation. The latter is very difficult to quantify, since it is markedly dependent on concentration, varying with $(\ln S)^{-2}$, where S is the supersaturation ratio. The free energy of nucleation is lowered when suitable surfaces are present upon which nucleation of the biomineral can take place. However, it is sometimes very difficult to distinguish between heterogeneous, or chance, nucleation at the surface and the specific nucleation directed by the molecular structure at the substrata.

In the case of calcium phosphate nucleation and growth, although the final phase formed is usually referred to as "hydroxyapatite" (HAP, $Ca_5(PO_4)_3OH$), at least three other, more soluble crystalline phases have been postulated to be involved in biomineralization: dicalcium phosphate dihydrate (DCPD, $CaHPO_4 \cdot 2H_2O$), tricalcium phosphates (all biological tricalcium phosphates are magnesium substituted and are usually referred to as β-TCMP [34]), and octacalcium phosphate (OCP, $Ca_4H(PO_4)_3 \cdot 2.5H_2O$) [34]. When the calcium phosphate solutions are sufficiently supersaturated, the formation of an apatitic phase is usually preceded by the precipitation of additional calcium phosphate phases, such as amorphous calcium phosphate (ACP), in which no long range order can be detected by X-ray diffraction [3, 13].

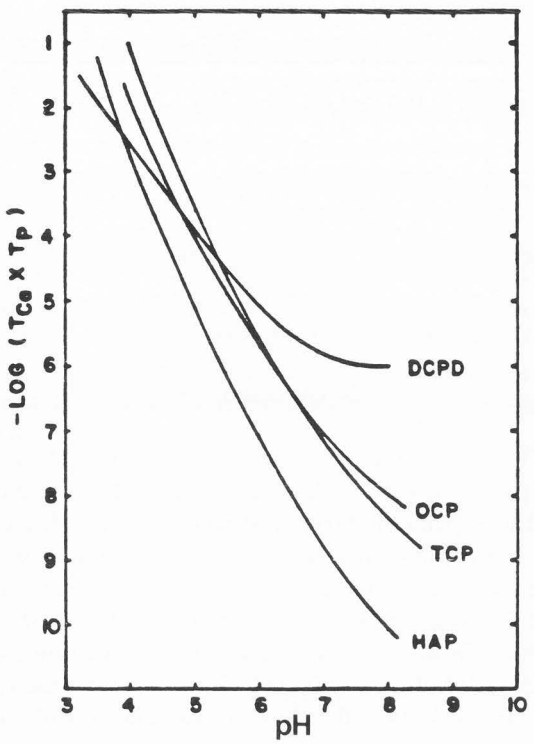

Figure 1. Calcium phosphate solubility isotherms (temperature: 37°C; ionic strength: 0.1 M; T_{Ca}: total calcium concentration; T_P: total phosphate concentration).

The importance of considering all possible calcium phosphates phases when elucidating the kinetic events during biomineralization reactions is readily apparent by examining a typical solubility isotherm diagram in Figure 1. It can be seen that as the acidity of the solution is increased, OCP (pH ≤ 6) or DCPD (pH ≤ 4.5) may be precursor phases to the formation of the thermodynamically more stable TCP or HAP phases, respectively. Moreover, since by the Ostwald Rule of Stages the least stable salt with the highest solubility is most likely to form first in a sequential precipitation, these more acidic phases with different growth mechanisms may be intermediately involved in the overall crystal growth reactions. Elucidation of the reaction mechanisms is especially difficult during free drift precipitation processes in which the concentrations of lattice ions, and, consequently, thermodynamic driving forces, are allowed to decrease. In order to provide a method for investigating nucleation and growth processes at sustained thermodynamic driving forces in which the concentrations of lattice ions remain unchanged, the constant composition (CC) procedure was developed [47]. This procedure enables the growth or dissolution rates to be investigated at known points on the solubility isotherms in Figure 1 with a precision impossible to achieve by conventional free drift techniques. Thus, it has been possible to grow or dissolve not only specific calcium phosphate phases such as DCPD, OCP and HAP, but also a series of defect apatites with the general structure $Ca_{5-u}H_u(PO_4)_3(OH)_{1-u}$ in which u ranges from 0 (for HAP) to 1 (for OCP) [23].

Not only is it important to consider the nucleation and growth of biominerals such as the calcium phosphates on other mineral surfaces, but the interaction of biological macromolecules with these calcium phosphate phases may also be an essential aspect of biological mineralization. Thus, the dual role of proteins as crystal growth inhibitors in solutions and as crystal nucleators when immobilized on surfaces is now well established [10, 11, 24, 25, 35, 54]. This has led to an increased interest in the determination of these protein structures in solution. Such considerations must influence their abilities to bind to calcium phosphate surfaces and effectively block growth sites or to present functional groups to the supersaturated solution that are able to initiate nucleation of the mineral phases.

Crystal growth: methods and mechanisms

The nature of the solid phase that forms during mineralization depends upon the relative supersaturation, σ, with respect to that particular phase:

$$\sigma = \{(IP^{1/\nu} - K_{SO}^{1/\nu}) / K_{SO}^{1/\nu}\} = (S - 1) \qquad (1)$$

where ν is the number of ions per formula unit of a precipitating phase having a solubility product, K_{SO}. The ionic product, IP, may be calculated from knowledge on the stoichiometry of the growing phase. A typical example for OCP is shown in eq. (2):

$$IP_{OCP} = [Ca^{2+}]^4[H^+][PO_4^{3-}]^3 y_1\, y_2^4\, y_3^3 \qquad (2)$$

in which brackets enclose molar ionic concentrations and y_x are the activity coefficients of x-charged species. K_{SO} is the equilibrium value of IP. The latter can be calculated using a suitable extended form of the Debye-Hückel equation such as that proposed by Davies [8]. In eq. (1), S, the supersaturation ratio, can also be used to calculate the Gibbs free energy of transfer, ΔG, from the supersaturated solution to an assumed saturated layer at the crystal surfaces using the equation:

$$\Delta G = -RT \ln S \qquad (3)$$

In the CC method for calcium phosphate crystal growth studies, the concentrations of lattice ions are maintained constant by the addition of titrant solutions containing calcium, phosphate and hydroxide ions as well as background electrolyte in order to maintain constant ionic strength and therefore constant activity coefficients. Adjustment of titrant solution stoichiometry to match that of the precipitating phase enables the activities of all ionic species to remain unchanged, even for extended reactions periods.

Sparingly soluble electrolyte crystal growth mechanisms can be divided broadly into two categories: dislocation and surface nucleation. Both may include volume diffusion as a limiting case. For the former, two comprehensive models have been proposed depending on the role of surface diffusion [7, 20, 51]. There is now evidence that many sparingly soluble biominerals undergo crystal growth by surface controlled processes with a rate related to the number of kink sites at developing steps. Such sites provide the most favorable locations for final integration of lattice ions into the crystal lattice. In the direct attachment model, the lattice ions enter active kink sites at crystal surfaces directly from the solution phase. In the indirect attachment model, however, lattice ions are first adsorbed on crystal terraces and an additional surface diffusion process is involved in transporting them to active kink sites. For a number of calcium phosphate phases, the latter, indirect model appears to be an important mechanism for crystal growth [59].

The rate of growth, J, can be expressed by:

$$J = k\, F(m_t/m_o)\, F'(\sigma) \qquad (4)$$

in terms of an effective rate constant, k, and as functions, F and F', of surface properties and solution relative supersaturation, respectively. In eq. (4), m_o and m_t are the crystal masses initially and at time t, respectively. One of the principle features of the CC procedure is that it maintains $F'(\sigma)$ constant and enables studies to be made of changes in the surface activity. The characterization of virtually all biological mineralization reactions as surface controlled processes usually enables the rates to be expressed in terms of a simple empirical rate using eq. (5) with rate constant, k, and effective reaction order, n:

$$J = k\, \sigma^n \qquad (5)$$

A parabolic rate expression with $n = 2$ has been observed for a number of sparingly soluble salts over appreciable ranges of supersaturations. This suggests that the concentration of active kink positions at the surfaces is a function of supersaturation and crystal growth occurs through the addition of ions, mainly at kink sites. Using a non-equilibrium model to express the kink density and setting up equations to describe both kink formation and annihilation, the empirical parabolic rate law {$n = 2$ in eq. (5)} is consistent with this model [57].

Another important feature related to the mechanisms of biomineralization is the sensitivity of the rates to lattice ionic ratios in the supersaturated solutions while maintaining overall thermodynamic driving forces. This dependence can be quantified in terms of a kinetic ionic ratio [58] which, for a simple 2-2 calcium phosphate phase such as DCPD, can be defined by:

$$r = ([Ca^{2+}]\, \xi_{Ca}2+) / ([HPO_4^{2-}]\, \xi_{HPO4}2-) \qquad (6)$$

This introduces not only the ratio of lattice ion concentrations but also the integration frequencies, $\xi_{Ca}2+ / \xi_{HPO4}2-$, of the lattice ions at the kink sites.

Referring to the development of the kink sites, the kinetic ionic ratio concept implies that a "calcium kink" can only advance by the addition of a HPO_4^{2-} ion while a "HPO_4^{2-} kink site" must receive a calcium ion in the growth process. Although the concentrations of Ca^{2+} and HPO_4^{2-} in the supersaturated solution will contribute to the rates at which the ions can arrive at these kink sites, the rate of reaction must also be governed by the ion having the smaller integration frequency. For a number of sparingly soluble salts, CC experiments at sustained thermodynamic driving forces but with different lattice ion ratios in the solutions revealed a striking dependence of the crystal growth rates upon lattice ion molar ratios. Although the interpretation of such effects for more complex biominerals such as OCP and HAP awaits satisfactory theoretical elucidation, there is little doubt that the deposition of biominerals at surfaces must be dependent upon these factors, closely related to zeta potential, as well as conventional thermodynamic driving forces. Unfortunately, most studies to date have been concerned only with these conventional driving forces.

Mineralization on macromolecular substrata

One of the dominant theories for the location of the first calcium phosphate mineral deposits involves the collagen fibers for which it was proposed [15, 18] mainly from transmission electron microscopic studies, that the very first crystals are nucleated in the "hole" zones of collagen, nucleating independently at many such sites. It was assumed that collagen in the "hole" zones offered the necessary specific binding sites for calcium and phosphate ions and that this led to the heterogeneous nucleation of calcium phosphate crystals. The possibility of the other proteins being chemically bound or adsorbed in the "hole" zones and thus facilitating the binding of calcium ions has also been strongly implied. Such an example is that of phosphoproteins, which contain phosphoserine and phosphothreonine residues as well as approximately 40% aspartic and glutamic acids. Phosphoproteins are tissue specific and some are known to bind calcium ions *in vitro* [18]. Both collagen types I and II have been found to bind inorganic phosphate in *in vitro* experiments [19, 31]. Collagen type I also strongly binds phospholipid molecules [32] and proteins [17]. Modeling of kinetic results have suggested that for calcium phosphate nucleation by native collagen, the crystal critical nucleus size was approximately 11-13 ions [30]. Comparison with the results of calcium phosphate homogeneous nucleation suggested that the presence of the collagen matrix reduced by a factor of 2 the size of the nucleus needed for mineralization [30]. Although other

studies have also suggested the potential role of collagen in bone mineralization [21], there has been strong disagreement with respect to the exact locus of the mineral deposits [1, 16, 33]. It was suggested that the mineral is not only deposited in the volume of the "hole" zone but also in the "overlap" zone and between collagen fibrils [33, 53]. The shape of the crystals was plate-like rather than needle-like, suggesting the possible participation of an OCP-like phase.

In addition to collagen, there is considerable interest in the ability of other macromolecules, such as fibrinogen and phosphatidylserine (PS) vesicles, to nucleate calcium phosphate crystals in supersaturated solutions. Fibrinogen is an asymmetric molecule composed of three pairs of chains: Aα, Bβ, and γ, containing 610, 461 and 411 amino acids, respectively [22, 45]. The overall structure, held by 29 disulfide bonds [61], can be described as (Aα, Bβ, γ)$_2$. During blood clotting, fibrinopeptides A and B are released to form fibrin monomer with the structure (α, β, γ)$_2$. Fibrinogen readily adsorbs to synthetic biomaterials implanted into the cardiovascular system. This adsorption process often results in blood coagulation and thrombus formation that may undergo pathological calcification involving the calcium and phosphate ions present in the blood at relatively high levels. Thus, both the adsorption of fibrinogen on surfaces as well as its ability to nucleate calcium phosphates are of particular interest. The former has been widely reported in literature [12, 36, 42, 43, 44, 52, 55]. In the present study, the ability of fibrinogen molecules, adsorbed to hydrophobic and hydrophilic surfaces, to nucleate calcium phosphates has been examined.

The role of membrane lipids such as PS vesicles in promoting cartilage and bone calcification has been recently reviewed [4, 5, 6]. These vesicles are enriched in calcium binding proteins and phosphoserine, as well as calcium and inorganic phosphate ions. It appears that these calcium-phosphate-phosphatidylserine complexes can act as calcium phosphate nucleators [5] and some CC kinetic studies are presented in this paper.

Materials and Methods

The ability of PS and immobilized proteins (fibrinogen or collagen) to induce the nucleation of calcium phosphates was investigated using constant composition and free drift methods, respectively. Both types of mineralization experiments were performed in highly supersaturated solutions and prepared in a nitrogen atmosphere by simultaneously mixing equal volumes of two solutions: one containing sodium and calcium chlorides and the other, potassium hydroxide and phosphate. This procedure is very convenient for the preparation of highly supersaturated solutions that are stable for extended periods. NaCl was added to attain an ionic strength of either 0.05 M or 0.15 M, while potassium hydroxide brought the pH of the solution to 7.40 $\pm$ 0.005. The total molar concentrations of calcium, T_{Ca}, and phosphate, T_P, were, respectively, 1.60 x 10^{-3} M and 1.20 x 10^{-3} M (OCP T_{Ca}/T_P molar ratio) or 1.71 x 10^{-3} M and 1.02 x 10^{-3} M (HAP T_{Ca}/T_P molar ratio). These solutions were supersaturated with respect to both HAP (σ_{HAP} = 15.0) and OCP (σ_{OCP} = 1.9).

Calcium phosphate nucleation and subsequent growth on PS vesicles (average size 120 $\pm$ 10 nm), prepared according to published procedures [41, 46], were studied using the CC method. The reaction was initiated by the introduction of 25 mg of the vesicles to the 200 ml nitrogen-purged supersaturated solution. The depletion of lattice ions due to precipitation processes triggered titrant addition which was controlled by a pH electrode and was proportional to the rate of crystal nucleation or growth. The compositions of the two titrant solutions (one containing calcium and sodium chlorides and the other, potassium hydrogen phosphate with potassium hydroxide) with concentrations given by eqs. (7), (8), (9) and (10) were calculated so as to compensate for both dilution effects and the consumption of lattice ions due to the precipitation of an anticipated OCP phase:

$$[CaCl_2]_t = 2\,[CaCl_2]_{rs} + 4C_{eff} \quad (7)$$

$$[NaCl]_t = 2[NaCl]_{rs} - 8C_{eff} \quad (8)$$

$$[KOH]_t = 2[KOH]_{rs} + 5C_{eff} \quad (9)$$

$$[KH_2PO_4]_t = 2[KH_2PO_4]_{rs} + 3C_{eff} \quad (10)$$

where C_{eff} is the effective concentration of the titrants with respect to the precipitating phase and rs and t represent reaction and titrant solutions, respectively.

Since the nucleation of calcium phosphates at the other investigated surfaces was not as efficient as that at PS and consequently did not induce significant changes in the composition of the reaction supersaturated solution, free drift experiments were applied in these studies. Fibrinogen (from human plasma, over 90% of clottable protein) was adsorbed to clean germanium surfaces or to octadecyltrichlorosilane (OTS)-coated germanium surfaces by immersion in 25 ml aliquots of 10 g/l protein solution for 20 hours at room temperature. Surfaces removed from the fibrinogen solution were washed for up to 3 hours with triple distilled water (TDW). The adsorption of fibrinogen molecules to the germanium surfaces was confirmed by attenuated total internal reflection infrared spectroscopy {(ATIR IR) model 1310, Perkin Elmer, Norwalk, CT} and ellipsometry (model 53702-200E, Rundolph Instrum., Fairfield, NJ) measurements. The same procedure was repeated for the adsorption of collagen (TEC-I® collagen solution composed of Type I collagen derived from bovine extensor tendon using a proprietary acid extraction process that maintains the physical and biological properties of native collagen;

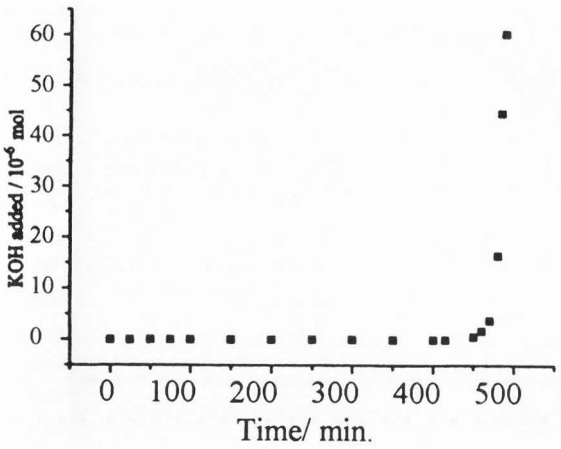

Figure 2. Constant composition nucleation and growth of calcium phosphate on phosphatidylserine vesicles. Plot of titrant added as a function of time.

---

donated by Dr. Paul Kemp, Organogenesis, Cambridge, MA) to OTS-coated germanium prisms. Collagen fibers were obtained in two ways: using TEC-I® acidic type I collagen solution and following the procedure described previously [56], or processing purified bovine reticular collagen type I according to references [28, 29].

The possible onset of calcium phosphate nucleation was investigated in a series of free drift experiments at germanium surfaces: OTS-coated germanium surfaces, fibrinogen adsorbed to untreated germanium surfaces, fibrinogen adsorbed to OTS-coated germanium surfaces, collagen adsorbed to OTS-coated germanium surfaces and at collagen fibers prepared from cross-linked or uncross-linked collagen. Cross-linking was accomplished by severe dehydration (110°C, 3 days, in vacuum) of the fibers. Lysine, arginine, aspartic acid, glutamic acid, threonine and serine amino acid residues are thought to participate in inter-chain cross-linking through esterification or amide formation since they contain accessible side hydroxyl, amino and carboxyl groups.

Following immersion of the germanium surfaces with the adsorbed fibrinogen or collagen molecules or collagen fibers into the nitrogen-purged calcium phosphate supersaturated solutions, the pH was continuously monitored using an Orion (Orion Research, Boston, MA) pH electrode. During the mineralization experiments (either constant composition or free drift), samples of the solution were withdrawn periodically, analyzed for calcium and phosphate ions and examined by an Olympus BH-2 (Olympus America, Inc., Melville, NY) optical microscope in order to test for possible spontaneous precipitation. At the end of each experiment (25-72 hours for free drift experiments, about 9 hours for constant composition experiments), the substrata (PS vesicles, germanium prisms or collagen fibers)

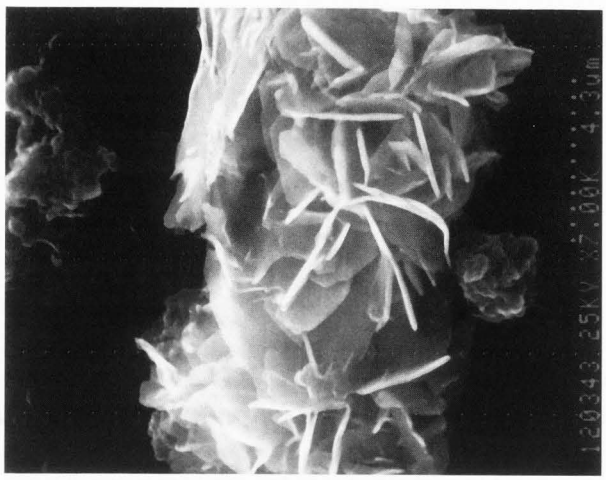

Figure 3. Scanning electron micrograph of the OCP-like crystals nucleated on phosphatidylserine; photo width (P.W.) = 15 μm.

---

were removed from the solution, washed several times with small amounts of TDW and dried at room temperature. Then, they were examined in a S-550 Hitachi (NSA Hitachi Instrum., Mt. View, CA) scanning electron microscope (SEM) and a Hitachi S-900 field-emission SEM fitted for energy dispersive X-ray analysis (EDX) with PGT (Princeton Gamma-Tech., Princeton, NJ) IMIX X-ray micro-analyzer.

Results and Discussion

The results of typical CC experiments using PS vesicles to induce calcium phosphate nucleation and growth are shown in Figure 2 as a plot of added titrant as a function of time. It can be seen that, following an induction period of about 7.5 $\pm$ 0.5 hours, a relatively rapid calcium phosphate precipitation curve was obtained. Such a sharply rising titrant addition was also reported in CC studies of OCP nucleation and growth at foreign surfaces such as DCPD [14].

Scanning electron micrographs of the PS vesicles following the mineralization experiments clearly showed crystal aggregates associated with the organic matrix (Fig. 3). Both the morphology of the crystals and EDX analysis (the Ca/P ionic ratio was 1.30 $\pm$ 0.05, close to that of OCP, 1.33) indicated the formation of an OCP-like phase. However, X-ray diffraction results {Siemens Nicolet/NIC Stoe diffractometer (Stoe Application Laboratory, Darmstadt, Germany), CuK radiation}, showed peaks characteristic of OCP (2θ = 4.71, 31.78, and 25.85) and HAP (2θ = 31.78, 25.85 and 10.59). It is interesting to note that PS vesicles have been shown capable of inducing calcium phosphate precipitation, possibly via interaction with calcium ions, but the nature of the mineral phase remained uncertain [48].

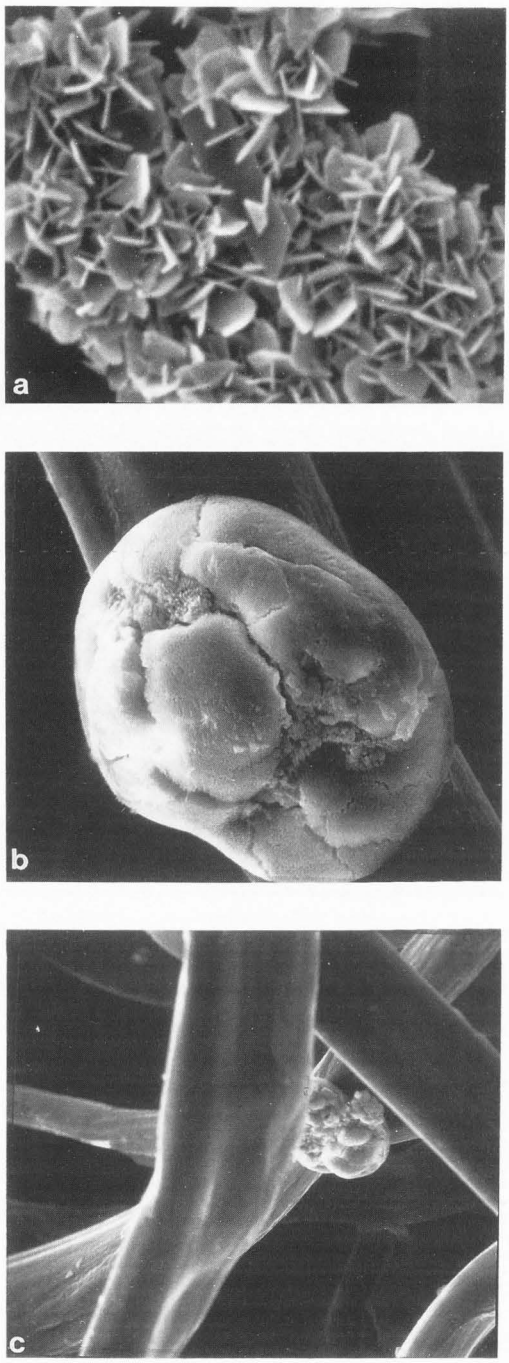

Figure 4. Variable morphologies of the calcium phosphate crystals nucleated on uncross-linked collagen fibers. P.W. = 31 μm (**a**); 67 μm (**b**); and 290 μm (**c**).

The results of the free drift nucleation experiments indicated that only fibrinogen, when adsorbed on the OTS-coated germanium, and collagen, prepared either in the form of fibers or as a film on the OTS-coated germanium, were able to induce calcium phosphate nucleation. None of the other surfaces examined, i.e., OTS-

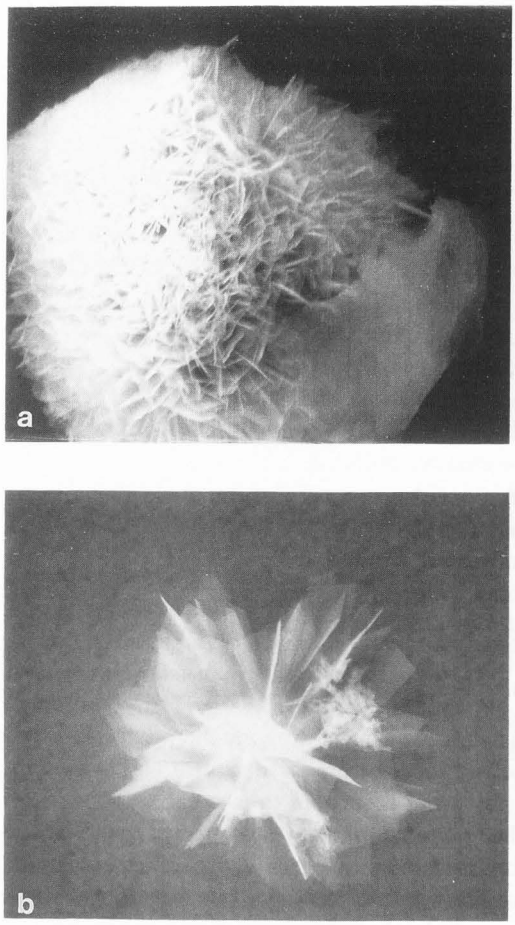

Figure 5. Scanning electron micrographs of OCP-like crystals nucleated on fibrinogen adsorbed to OTS-coated germanium prism. P.W. = 47 μm (**a**); and 17 μm (**b**).

---

coated and uncoated germanium and fibrinogen adsorbed to bare germanium surfaces, appeared to be effective nucleators. Additionally, no evidence for calcium phosphate nucleation in the highly supersaturated reaction solutions was found during any of the extended (25-72 hours) free drift nucleation experiments.

Scanning electron microscopy showed that calcium phosphate crystals nucleated on the uncross-linked collagen fibers while no precipitate was found on the cross-linked fibers. This may be due to the elimination of most of the potentially active sites such as side chains of glutamic and aspartic acid residues during dehydration. The role of glutamic and aspartic acids residues in the mineralization process was demonstrated by Davis and Walker [9]; destruction of these groups diminished the capability of collagen to mineralize *in vitro*. Glycine residues, that constitute 1/3 of the collagen molecule (in terms of the number of the amino acids), have also been proposed as nucleation participants through coordination

of calcium ions [50]. In contrast, it has been documented that the destruction of polar side chains of residues such as lysine, histidine, arginine, serine and tyrosine does not influence nucleation [19].

Crystals, nucleated on uncross-linked collagen, showed variable morphologies and Ca/P ionic ratios by EDX. Both HAP-like crystal aggregates with Ca/P ionic ratios as high as 1.9 and OCP-like phases with lower Ca/P ionic ratios were observed by SEM (Fig. 4). These crystals were randomly distributed on the collagen surfaces, however, their specific location was unresolvable by SEM. Thus, although the process of collagen mineralization has been confirmed, the nature of the induced phase and its possible further transformation as well as its loci with respect to collagen fibers must still be elucidated. It should be added that OCP crystals were grown *in vitro* on a cow tendon collagen disk (0.5 mm) with the c-axis of the OCP crystal parallel to collagen fibril [26]. However, the authors did not exclude that apatite coexisted with OCP crystals in small amounts, undetectable by X-ray spectroscopy. Recently, it has been suggested that collagen cultured with osteoblastic cells undergoes enhanced mineralization [39].

At OTS-coated germanium surfaces supporting adsorbed fibrinogen, SEM showed that the nucleated OCP-like crystals were specifically associated with the fibrinogen matrix (Fig. 5) [49]. EDX analysis of these crystals (Fig. 6) also suggested the exclusive nucleation of OCP crystals with Ca/P ionic ratio 1.33 $\pm$ 0.10. The number of the nucleated OCP-like crystal aggregates increased as the reaction time was increased to 72 hours. Decrease of the ionic strength from 0.15 M to 0.05 M also appeared to enhance the nucleation of the OCP-like calcium phosphate crystallites. As suggested previously [60], this may be explained in terms of the inhibitory influence of sodium ions on OCP nucleation due to their relatively strong adsorption at calcium sites located at the peripheries of the subcritical OCP nuclei.

Conformational differences between the fibrinogen molecules adsorbed on hydrophobic, e.g., the OTS-coated germanium surfaces, and on hydrophilic surfaces, e.g., germanium surfaces, probably account for their ability to induce the nucleation of calcium phosphate [2, 27, 40, 49]. On high energy surfaces such as untreated germanium, fibrinogen molecules lie flat, inducing significant strain in the central domain of the molecule formed by the NH_2 termini of all six polypeptide chains. Subsequent polymer formation involves two terminal "D" regions, each comprising COOH termini of the Bβ and γ chains, and flexible COOH termini of the Aα chains. These COOH termini of the Aα polypeptide chains (from 400 to 610 amino acid residues) constitute the specifically charged regions of the fibrinogen molecules with clusters of positive and negative charges [52].

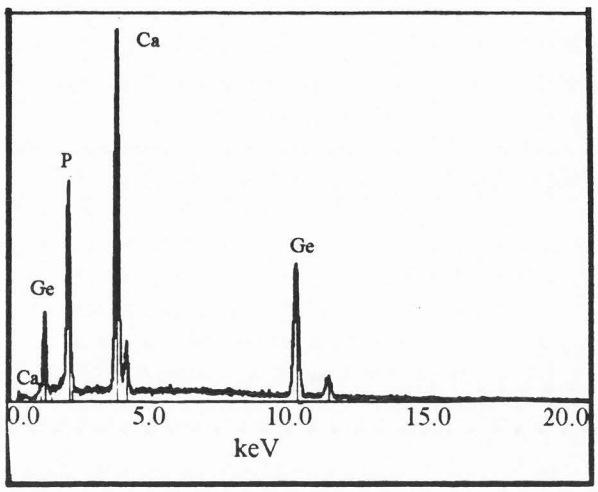

Figure 6. EDX spectrum of OCP-like crystals nucleated on fibrinogen adsorbed to OTS-coated germanium prism. The Ca/P atomic ratio is 1.33 $\pm$ 0.15. The calibration curve for EDX was prepared using synthetic calcium phosphate phases.

They have been suggested as potential calcium binding sites [38]. On low energy surfaces such as hydrophobicized germanium, the less tenaciously adsorbed fibrinogen can be distributed as single molecules and small aggregates [27]. Scanning force microscopy observations have confirmed that the fibrinogen molecule adsorbed to hydrophobicized surfaces retains its native structure [42], probably exposing active COOH termini of Aα chains. C-termini of the Aα chains might be hidden in more flat structures such as those assumed by fibrinogen molecules adsorbed on hydrophilic surfaces. It should also be added that fibrinogen molecules, adsorbed to hydrophobicized surfaces, form non-uniform layers containing localized pockets and grooves. These disruptions, especially if highly charged, can accumulate ions and stabilize the bulk interactions that result in nucleation [37]. Thus, although many questions remain, it appears that specific fibrinogen conformations contribute differently to calcium binding and subsequent nucleation.

Acknowledgment

We thank The National Institute of Dental Research for a grant (DE03223) in support of this work.

References

[1] Arsenault AL (1991) Image analysis of collagen-associated mineral distributions in cryogenically prepared turkey leg tendon. Calcif Tiss Int **48**: 56-62.

[2] Baier RE (1985) Modification of surfaces to meet bioadhesive design goals: A review. In: Proc. Eighth Meeting of the Adhesion Society. Adhesion Society, Inc., Blacksburg, VA. pp. 171-186.

[3] Betts F, Blumenthal NC, Posner AS (1981) Bone mineralization. J Crystal Growth **53**: 63-73.

[4] Boskey AL (1989) Phospholipids and calcification. In: The Connective Tissue Matrix III. Hukins DWL (ed.). MacMillan Press, London. pp. 110-115.

[5] Boskey AL (1992) Mineral-matrix interactions in bone and cartilage. Clin Orthop **281**: 244-274.

[6] Boyan BD, Schwartz Z, Swain LD, Khare A (1989) Role of lipids in calcification of cartilage. Anat Rec **224**: 211-222.

[7] Chernov AA (1961) The spiral growth of crystals. Soviet Phys Uspehi **5**: 116-128.

[8] Davies CW (1962) Ion Association. Butterworths, London. pp. 39-40.

[9] Davis NR, Walker TE (1972) The role of carboxyl groups in collagen calcification. Biochem Biophys Res Commun **48**: 1656-1662.

[10] Doi Y, Horiguchi T, Kim S, Moriwaki Y, Wakamatsu N, Adachi M, Ibaraki K, Sasaki S, Shimokawa H (1992) Effects on non-collagenous proteins on the formation of apatite in calcium-glycerophosphate solutions. Arch Oral Biol **37**: 15-21.

[11] Doi Y, Horiguchi T, Kim S, Moriwaki Y, Wakamatsu N, Adachi M, Shigeta H, Sasaki S, Shimokawa H (1993) Immobilized DPP and other proteins modify OCP formation. Calcif Tiss Int **52**: 139-145.

[12] Drake B, Prate CB, Weisenhorn AL, Gould SAC, Albrecht TR, Quate CF, Canneli DS, Hansma HG, Hansma PK (1989) Imaging crystals, polymers, and processes in water with the atomic force microscopy. Science **243**: 1586-1589.

[13] Eanes ED, Gillessen IH, Posner AS (1965) Intermediate states in the precipitation of hydroxyapatite. Nature **23**: 365-367.

[14] Ebrahimpour A, Zhanng J, Nancollas GH (1991) Dual constant composition method and its application to studies of phase transformation and crystallization of mixed phases. J Crystal Growth **113**: 83-91.

[15] Endo A, Glimcher MJ (1989) The effect of complexing phosphoproteins to decalcified collagen on *in vitro* calcification. Connect Tiss Res **21**: 179-196.

[16] Erts D, Gathercole LJ, Atkins EDT (1994) Scanning probe microscopy of intrafibrillar crystallites in calcified collagen. J Mater Sci Mater Med **5**: 200-206.

[17] Fujisawa R, Kuboki Y (1992) Affinity of bone sialoprotein and several other bone and dentin acidic proteins to collagen fibrils. Calcif Tiss Int **51**: 438-442.

[18] Glimcher MJ (1989) Mechanism of calcification. Role of collagen fibrils and collagen-phosphoprotein complexes *in vitro* and *in vivo*. Anat Rec **224**: 139-153.

[19] Glimcher MJ, Krane SM (1968) Treatise on Collagen. Vol 2. Gould BS (ed.). Academic Press, New York. pp. 54-60.

[20] Glimmer GH, Ghez R, Carbera N (1971) An analysis of combined surface and volume diffusion process in crystal growth. J Crystal Growth **8**: 79-93.

[21] Grynpas MD, Tenenbaum HC, Holmyard DP (1989) The emergence and maturation of the first apatite crystals in an *in vitro* bone formation system. Connect Tiss Res **21**: 227-237.

[22] Henshen AH (1993) Human fibrinogen: Structural variants and functional sites. Thromb Haemost **70**: 42-47.

[23] Heughebaert JC, Zawacki SJ, Nancollas GH (1990) The growth of nonstoichiometric apatite from aqueous solution at 37°C. I. Methodology and growth at pH 7.4. J Colloid Interf Sci **135**: 20-31.

[24] Hunter GK, Goldberg HA (1994) Modulation of crystal formation by bone phosphoproteins: Role of glutamic acid-rich sequences in the nucleation of hydroxyapatite by bone sialoprotein. Biochem J **302**: 175-179.

[25] Hunter GK, Kyle CL, Goldberg HA (1994) Modulation of crystal formation by bone phosphoproteins: Structural specificity of the osteopontin-mediated inhibition of hydroxyapatite formation. Biochem J **300**: 723-728.

[26] Iijima M, Moriwaki Y, Kuboki Y (1994) *In vitro* crystal growth of octacalcium phosphate on type I collagen fiber. J Crystal Growth **137**: 553-560.

[27] Karlsson C, Carlsson A, Stenberg M, Nygren H (1992) Cooperativity in the adsorption of cellulose ethers and fibrinogen at liquid-solid interfaces. Colloid Polym Sci **270**: 377-383.

[28] Kato YP, Silver FH (1990) Formation of continuous collagen fibres: Evaluation of biocompatibility and mechanical properties. Biomaterials **11**: 169-175.

[29] Kato YP, Christiansen DL, Hahn RA, Shieh SJ, Goldstein JD, Silver FH (1989) Mechanical properties of collagen fibres: A comparison of reconstituted and rat tail tendon fibres. Biomaterials **10**: 38-42.

[30] Katz EP (1969) The kinetics of mineralization *in vitro*. I. The nucleation properties of 640 collagen at 25°C. Biochim Biophys Acta **194**: 121-129.

[31] Koutsoukos PG, Nancollas GH (1986) The adsorption of inorganic phosphate by collagen. Colloids Surf **17**: 81-90.

[32] Le Lous M, Boudin D, Salmon S, Polonovski J (1982) The affinity of type I collagen for lipid *in vitro*. Biochim Biophys Acta **708**: 26-32.

[33] Lees S, Prostal KS, Ingle VK, Kjoller K (1994) The loci of mineral in turkey leg tendon as seen by atomic force microscope and electron microscopy. Calcif

Tissue Int **55**: 180-189.

[34] LeGeros RZ (1991) Calcium Phosphates in Oral Biology and Medicine. In: Monographs in Oral Science. Vol. 15. Myers HM (ed.). Karger, Basel, Switzerland. pp. 52-55.

[35] Linde A, Lussi A, Crenshaw MA (1989) Mineral induction by immobilized polyanionic proteins. Calcif Tiss Int **44**: 286-295.

[36] Lu CF, Nadarajah A, Chittur KK (1994) A comprehensive model of multiprotein adsorption on surfaces. J Coll Interf Sci **75**: 152-161.

[37] Mann S (1989) Crystallochemical strategies in biomineralization. In: Biomineralization: Chemical and Biochemical Perspectives. Mann S, Webb J, Williams RJP (eds.). VCH Verlagsgesellschaft, Weinheim, Germany. pp. 35-62.

[38] Marguerie G (1977) The binding of calcium to fibrinogen: Some structural features. Biochim Biophys Acta **494**: 172-181.

[39] Nagahara K, Mouri K, Kanematsu N, Shrestha P, Meenaghan MA (1995) Stimulation of *in vivo* calcification using collagen membranes cultured with osteoblastic cells *in vitro*: A preliminary report. Int J Oral Maxillofac Implants **10**: 109-113.

[40] Nygren H, Stenberg M, Karlsson C (1992) Kinetics supramolecular structure and equilibrium properties of fibrinogen adsorption at liquid-solid interfaces. J Biomed Mat Res **26**: 77-91.

[41] Ohki S (1984) Effects of divalent cations, temperature, osmotic pressure gradient and vesicle curvature on phosphatidylserine vesicle fusion. J Membrane Biol **77**: 265-277.

[42] Rao SPS, Poojary MD, Elliott BW, Jr, Melanson LA, Oriel B, Cohen C (1991) Fibrinogen structure in projection at 18 resolution. Electron density by coordinated cryo-electron microscopy and X-ray crystallography. Mol Biol **222**: 89-98.

[43] Retzinger GS, Cook BC, DeAngelis AP (1994) Two binding of fibrinogen to surfaces and the identification of two distinct surface-bound species of the protein. J Coll Interf Sci **168**: 514-52.

[44] Soderquist ME, Walton AG (1990) Structural changes in proteins adsorbed on polymer surfaces. J Coll Interf Sci **75**: 386-397.

[45] Stryer L (1989) Biochemistry. Freeman WH and Company, New York. pp. 249-253.

[46] Szoka F Jr, Papahadjopoulos (1978) Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. Proc Natl Acad Sci **75**: 4194-4198.

[47] Tomson MB, Nancollas GH (1978) Mineralization kinetics: A constant composition study. Science **200**: 1059-1061.

[48] Tsortos A (1993) The role of macromolecules as inhibitors and nucleators of calcium phosphate phases: A physico-chemical study. PhD thesis, State Univ. New York (SUNY) at Buffalo, Buffalo, NY.

[49] Tsortos A, Ohki S, Zieba A, Baier RE, Nancollas GH (1995) The dual role of fibrinogen as inhibitor and nucleator of calcium phosphate phases. The importance of structure. J Colloid Interf Sci **177**: 257-262.

[50] Urry DW (1971) Neutral sites for calcium ion binding to elastin and collagen: A charge neutralization theory for calcification and its relationship to atherosclerosis. Proc Natl Acad Sci **68**: 810-814.

[51] Van der Eerden JP (1982) The advance velocity of steps under the influence of volume and surface diffusion, by direct and indirect incorporation of growth units. J Crystal Growth **56**: 174-188.

[52] Veklich YI, Gorkun OV, Medved LV, Nieuwenhuizen W (1993) Carboxyl-terminal portions of the chains of fibrinogen and fibrin. Localization by electron microscopy and the effects of isolated C fragments on polymerization. J Biol Chem **269**: 13577-13585.

[53] Weiner S, Traub W (1992) Bone structure: From angstroms to microns. FASEB J **6**: 879-885.

[54] Wheeler AP, Sikes CS (1989) Matrix-crystal interactions in $CaCO_3$ biomineralization in Biomineralization. In: Chemical and Biochemical Perspectives. Mann S, Webb J, Williams RJP (eds.). VCH Verlagsgesellschaft, Weinheim, Germany. pp. 95-132.

[55] Wigren R, Elwing H, Erlandsson R, Welin S, Lundstrom I (1991) Structure of adsorbed fibrinogen obtained by scanning force microscopy. FEBS Lett **290**: 225-228.

[56] Wood GC, Keech MK (1960) The formation of fibrils from collagen solutions. 1. The effect of experimental conditions: Kinetic and electron-microscope studies. Biochem J **75**: 588-598.

[57] Zhang J, Nancollas GH (1990) Kink densities along a crystal surface step at low temperatures and under nonequilibrium conditions. J Crystal Growth **106**: 181-190.

[58] Zhang J, Nancollas GH (1992) Influence of calcium/sulphate molar ratio on the growth rate of calcium sulphate dihydrate at constant supersaturation. J Crystal Growth **118**: 287-294.

[59] Zhang J, Nancollas GH (1992) Interpretation of dissolution kinetics of dicalcium phosphate dihydrate. J Crystal Growth **125**: 251-269.

[60] Zhang J, Ebrahimpour A, Nancollas GH (1992) Dual constant composition studies of phase transformation of dicalcium phosphate dihydrate into octacalcium phosphate. J Coll Interf Sci **152**: 132-140.

[61] Zhang JZ, Kurdyk B, Redman CM (1993) Symmetrical disulfide bonds are not necessary for assembly and secretion of human fibrinogen. J Biol Chem **268**: 11278-11282.

Discussion with Reviewers

C. Rey: *In vivo*, soluble abundant proteins such as albumin are strong inhibitors of crystal growth and would prevent crystal formation on the surface of the materials but not inside due to their poor diffusity in a dense organic matrix. Were the calcium phosphate crystals observed also inside the protein fibers and/or layers? If yes, were they different from those on the surface? Was the mineral formed in the hole zone of collagen at the very beginning of nucleation stage? Were the crystals all over the material's surface or randomly distributed at certain spots?

Authors: As noted in **Results and Discussion**, the specific location of the nucleated calcium phosphate crystals with respect to collagen fibers was unresolvable by SEM. The micrographs presented in Figures 4a, 4b and 4c characterize the morphological features of the crystals formed, probably, on the surface of the collagen fibers. These features were uniformly consistent in all collagen samples examined. Preliminary transmission electron micrographs of calcium phosphate crystals nucleated on the collagen fibers were also unable to provide additional information on the specific location of the mineral crystals.

C. Rey: It has been mentioned that cross-linked collagen was a poor nucleator of calcium phosphate crystals. *In vivo*, however, cross-linked collagen, e.g., pig heart valves cross-linked with glutaraldehyde, have been shown to calcify heavily inside the material not on its surface. How can this discrepancy between the presented *in vitro* studies and the *in vivo* observations be explained?

Authors: We would not regard this as a discrepancy since *in vivo* other factors, such as the presence of proteins and other macromolecules, might be involved in calcium phosphate precipitation.

J.P. Kavanagh: For how long were the solutions used in free drift experiments stable and what were the induction times for the test systems which showed nucleation?

Authors: No evidence for spontaneous calcium phosphate precipitation in the highly supersaturated reaction solutions was found during any of the nucleation experiments. It is possible that traces of protein desorbed from the germanium prisms (partial desorption of proteins from germanium prisms was confirmed by ATIR IR studies) or from the core of the collagen fibers prevented calcium phosphate nucleation in the reaction solution. It should be added that fibrinogen is a relatively good inhibitor of calcium phosphate mineralization when present in the supersaturated solutions.

The very first crystals nucleated on fibrinogen or collagen molecules adsorbed to hydrophobic surfaces as well as on uncross-linked collagen fibrils were observed after a period of 24 hours.

D. Christiansen: The refined capacity of the constant composition method in measuring the kinetics and thermodynamics of mineral nucleation is clearly defined in **Materials and Methods**. Why was this method not used in nucleation experiments employing collagen and fibrinogen immobilized on surfaces?

Authors: Nucleation of calcium phosphates on collagen and fibrinogen immobilized on surfaces appeared to be relatively slow as compared with precipitation on PS. As noted in **Materials and Methods**, following immersion of surfaces carrying the immobilized proteins into the reaction solution, the pH was continuously monitored and the concentrations of calcium and phosphate were periodically measured. The pH of the reaction solution as well as the calcium and phosphate concentrations remained virtually unchanged even during extended reaction times. The CC method that can be used to quantify the kinetics of reactions involving more extensive crystal nucleation and growth could not be applied in the limited deposition at fibrinogen and collagen surfaces. It was therefore necessary to use SEM methods. Although increasing the supersaturation level of the reaction solutions would have increased the extent of nucleation, it also reduced the stability of the solutions.

Reviewer VI: Is it not possible that the loss of water inside the fibrils and the thinning of the fibrils during severe dehydration were sufficient to inactivate the collage template?

Authors: Yes, it is quite possible that the dehydration inactivated the collagen.