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In vitro models of collagen biomineralization $\stackrel{\star}{\sim}$

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ABSTRACT

Over the last several years, significant progress has been made toward understanding the mechanisms involved in the mineralization of hard collagenous tissues, such as bone and dentin. Particularly notable are the identification of transient mineral phases that are precursors to carbonated hydroxyapatite, the identification and characterization of non-collagenous proteins that are involved in controlling mineralization, and significant improvements in our understanding of the structure of collagen. These advances not only represent a paradigm shift in the way collagen mineralization is viewed and understood, but have also brought new challenges to light. In this review, we discuss how recent in vitro models have addressed critical questions regarding the role of the non-collagenous proteins in controlling mineralization, the nature of the interactions between amorphous calcium phosphate and collagen during the early stages of mineralization, and the role of collagen in the mineralization process. We discuss the significance of these findings in expanding our understanding of collagen biomineralization, while addressing some of the limitations that are inherent to *in vitro* systems.

1.1. Structure of mineralized collagen

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Early X-ray diffraction studies of bone and teeth showed that

mineralized tissues contain both organic and inorganic material,

consisting of collagen and crystalline apatite, respectively (Clark,

1931; de Jong, 1926). Soon after, Robinson and Watson (1952)

showed that carbonated hydroxyapatite (apatite, in short) crystals

1. Introduction

In vertebrates, the formation of hard connective tissues, such as bone, dentin and cementum, involves the deposition of calcium phosphate within a collagenous matrix, which forms a common building block for these tissues. Understanding the process of mineral deposition in collagen is imperative to the development of treatments for mineralization related diseases, and may also have important implications for the design of bioinspired materials. Several aspects of collagen biomineralization have seen dramatic shifts in viewpoint, and critical gaps in understanding remain. While the structure of mineralized collagen is now well understood, how mineral precipitates with the spatial and hierarchical order found in native tissues is still largely unknown. While in vivo exploration of biomineralization is complex, simplified in vitro models can be used to systematically examine specific aspects of collagen mineralization. Here we review current questions in the field of collagen biomineralization and recent progress using in vitro models in pursuit of answers.

exist within collagen with their *c*-axis nearly parallel with the long axis of the collagen fibril. The crystals are known to be around 2-6 nm thick, 30-50 nm wide, and 60-100 nm long (Beniash, 2011). The first proposal for the 3-dimensional structure of collagen came from the famous electron microscopic study by Hodge and Petruska (1963). Their observations led to the quarter stagger model in which neighboring collagen molecules are offset by a distance of 67 nm (Fig. 1A). Over the years, this model was further refined and the detailed crystal structure of microfibrils was described in 2006 (Fig. 1B) by Orgel et al. (2006). Several studies have shown that mineral nucleates preferentially within the gap regions of collagen fibrils (Jackson, 1957; Robinson and Watson, 1952; Traub et al., 1989) (Fig. 1C), where most crystals are well organized into parallel arrays, in almost continuous dark bands (Traub et al., 1989) (Fig. 1D). It is important to note that while the mineral precipitates preferentially in the gap zones of collagen fibrils, the crystals have been shown to grow in dimension and eventually exceed the size of the gap zone (Landis et al., 1993; Traub et al., 1989). Further, mineral exists both within, and exterior to the collagen fibrils. It is generally accepted, however, that the majority of the mineral exists within the fibrils.

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Fig.1. (A) Cryo-transmission electron microscopy image of a collagen fibril. Black circle: fiducial gold marker for electron tomography. Scale bar: 50 nm. Inset: schematic representation of a collagen fibril, adapted from (Orgel et al., 2001), with permission from Elsevier. Copyright (2001). (B) Unit cell of the crystal structure of collagen, comprising one 67 nm repeat. Adapted from (Orgel et al., 2006), with permission. Copyright (2006) National Academy of Sciences, U. S. A. (C) Schematic representation of hydroxyapatite crystals forming in the gap regions of collagen. Adapted from (Landis et al., 1993), with permission. from Elseiver. Copyright (1993). (D) Transmission electron microscopy image of a section of mineralizing turkey tendon. The dark bands in the collagen constitute hydroxyapatite platelets in the gap region, viewed face-on. Scale bar: 200 nm. Adapted from (Traub et al., 1989), with permission. Copyright (1989) National Academy of Sciences, U. S. A.

1.2. The mineral phase

Collagen mineralization was initially viewed as a classical precipitation and growth of apatite crystals directly from interstitial fluid, which is supersaturated with respect to several calcium phosphate phases (Clark, 1931; Hodge and Petruska, 1963; Robinson and Watson, 1952). The nature of the mineral phase in bone was reconsidered in the 1960s, due to poor X-ray diffraction patterns and variations in the chemical compositions obtained from biological calcium phosphates, as reviewed by Boskey (1998) and Rev et al. (2009). It was suggested then that there was an amorphous phase, which exists in addition to the crystalline apatite phase as a transient precursor (Termine and Posner, 1966). This was supported by the solution precipitation experiments of Eanes et al. (1965), who showed that under near physiological conditions, early precipitates are amorphous and later transform first into octacalcium phosphate (OCP) and only then hydroxyapatite (Blumenthal et al., 1972; Eanes et al., 1965; Eanes and Posner, 1970; Meyer and Eanes, 1978; Termine and Posner, 1970). Taken together, these observations show that calcium phosphate precipitation *in vitro* is kinetically driven, meaning that the mineral does not crystallize directly into the most thermodynamically stable product. Instead, it precipitates first as the kinetically most accessible form, in this case ACP, which subsequently transforms into the most thermodynamically stable phase. The mechanistic details of calcium phosphate precipitation and nucleation *in vitro* have been extensively investigated recently by Habraken et al. (2013).

Nevertheless, in the absence of convincing *in vivo* evidence of any precursor phases including amorphous calcium phosphate (ACP) (Roberts et al., 1992), it became generally accepted that although ACP and OCP form in *in vitro* models, they were not likely to form in normal *in vivo* mineralization. Further, the disordered nature of both the amorphous phase and biological mineral made it extremely difficult to separate the two. This has been reviewed thoroughly by Johnsson and Nancollas (1992).

More recently, however, increasing in vivo evidence of transient precursor phases has become available through the application of sensitive techniques with minimal sample preparation artifacts. Crane et al. (2006) showed, using Raman spectroscopy, that OCPlike intermediary phases exist in living mouse calvarial tissue. Later, the presence of transient ACP was reported in mouse enamel (Beniash et al., 2009), growing zebrafish fin bones (Mahamid et al., 2008, 2010), and in rat calvaria and long bones (Mahamid et al., 2011). Mahamid et al. (2010, 2011) further showed that mineral is released in globules (presumably ACP) from cells at the growth zone. The globules, interspersed within the collagen fibrils, then transform into apatite. These results are consistent with previous NMR studies that identified the presence of protonated phosphate groups that are part of non-apatitic components in bone (Wu et al., 1994). Taken together, these observations have precipitated a paradigm shift in the view of the mineral formation pathway in connective tissues. The origin of the mineral, and how it infiltrates the collagen fibrils are still unknown. Several in vitro models have attempted to elucidate the mechanisms involved, as will be discussed in this review.

1.3. Non-collagenous proteins

It is clear that not all collagenous tissues mineralize. In some instances (e.g. in the attachment of the tooth root to the jaw bone via the periodontal ligament) mineralized (bone and cementum) and non-mineralized (periodontal ligament) tissues are sharply juxtaposed. Extracellular matrix non-collagenous proteins (NCPs) are believed to control several aspects of the mineralization, specifically through mechanisms of inhibition and promotion by phosphoproteins and proteoglycans (Beniash et al., 2000; Chen et al., 1984; George and Veis, 2008). The discovery of dentin phosphophoryn (DPP), by Veis and Perry (1967), was the first evidence of phosphoproteins involved in mineralization. DPP was found to be particularly rich in serine, aspartic acid and glutamic acid residues. They hypothesized that this heavily phosphorylated, acidic protein was capable of nucleating hydroxyapatite, when bound to the collagen superstructure in bone and dentin (George et al., 1993; Veis and Perry, 1967). This paradigm supported epitaxial growth of hydroxyapatite, associated to collagen bound NCPs.

Currently there are several known non-collagenous proteins, which are believed to control mineral deposition, orientation, and phase (reviewed by George and Veis (2008)). As such, *in vitro* investigation into the role of proteins in mineralization has been extensive. Some of the first models used gel precipitation to analyze the effect of specific proteins such as bone sialoprotein (BSP) (Boskey, 1989; Hunter and Goldberg, 1993; Silverman and Boskey, 2004). However, while these systems provided valuable information on the role of the protein in the precipitation of hydroxyapatite, they

do not address the full complexity of the interplay between mineral, proteins, and collagen. It has been shown that protein concentration and whether the protein is free or immobilized can profoundly affect function in precipitation models (Boskey at al. 1990; Stetler-Stevenson and Veis, 1983; Termine, J. D., Conn, K. M., 1980).

Much of the *in vivo* evidence regarding the role of specific proteins comes from their distribution and genetic knockout studies. However, many proteins involved in mineralization, (such as osteopontin (Sodek et al., 2000)) have multiple roles, complicating interpretation. Tissue localization can give clues as to the role of a protein, but does not provide any true functional information, especially with regards to proteins with diverse functions (McKee and Nanci, 1996). Further, biological redundancies limit the effectiveness of knockout models. While some knockout studies provide clear evidence for mineral inhibition or promotion, such as the case of matrix gla protein (MGP) knockout (Luo et al., 1997) others present with weak phenotypes, limiting the information which can be attained (Boskey et al., 2002).

In vitro model systems can alleviate many of the issues posed by in vivo models. More specifically, there has been particular success using in vitro models which mimic the mineralized collagen fibril. These models of collagen mineralization employ various collagen substrates, ranging from reconstituted collagen to natural tissues. It is well known that collagen alone will not mineralize in vitro with the same collagen-mineral relationships found in native mineralized collagen (Bachra, 1972). Even the more successful attempts could not achieve intrafibrillar mineralization (Iijima et al., 1994; Saito et al., 1997). Recently, however, Gower and coworkers succeeded in recreating intrafibrillar mineralization using a variety of collagen subtrates (Olszta et al., 2007). Their model uses polyaspartic acid to mimic soluble non-collagenous proteins (as described later). This work and several others that followed have had a profound effect on the envisioned role for non-collagenous proteins in collagen mineralization.

1.4. Scope

In the last years, several *in vitro* systems have been developed, based in large part on the work of Gower and co-workers (Olszta et al., 2007), with the aim of addressing outstanding questions on the mechanism collagen biomineralization. The aim of this review is to discuss how these recent *in vitro* studies have provided details, at the molecular level, on the interplay between collagen, the mineral and the NCPs. While other recent reviews have addressed *in vitro* studies, along with structure, properties and formation of bone (Beniash, 2011; Olszta et al., 2007; Rey et al., 2009), here we focus exclusively on *in vitro*, cell-free models of collagen mineralization in order to highlight the mechanistic details of the mineralization event. We divide the review into four different sections, each focusing on outstanding questions relating to a specific issue, as outlined below:

- (1) The role of the non-collagenous proteins: these proteins are essential for proper osteogenesis and dentinogenesis *in vivo*. How do they modulate collagen mineralization?
- (2) The role of precursor phases: how does the amorphous calcium phosphate infiltrate into the collagen? What controls its transformation into apatite?
- (3) Mineralization in the gap region: how is the spatially-controlled deposition of mineral in the gap region regulated?
- (4) The role of collagen during mineralization: there is increasing evidence suggesting that collagen actively controls apatite formation during mineralization. What is the role of collagen in this process? How does it interact with the mineral?

2. The role of non-collagenous proteins

In this part of the review, we discuss how *in vitro* models have been exploited to help better understand how NCPs control apatite formation in collagen. Three major approaches will be described: systems which employ synthetic polymers capable of mimicking the activity of the NCPs; models where recombinant proteins are used directly to probe the function of specific NCPs in inducing or inhibiting collagen mineralization on reconstituted collagen; and experiments using demineralized tissue containing matrixbound NCPs.

2.1. Polyaspartic acid as a substitute for non-collagenous proteins

The first study to successfully obtain intrafibrillar mineralization of collagen in vitro was reported by Olszta et al.(2007), followed by Deshpande and Beniash (2008). These studies showed that the formation of apatite crystals inside the collagen, with the same morphology and crystallographic orientation as found in bone, could be obtained by substituting polyaspartic acid (pAsp) for NCPs in the mineralizing solution (Fig. 2). Polyaspartic acid mimics the NCPs as they are also highly negatively charged. Two different mechanisms were proposed for their activity in promoting intrafibrillar mineralization. In their "polymer-induced liquid precursor" (PILP) hypothesis, Olszta et al. (2007) suggest that pAsp acts by inhibiting apatite nucleation in solution and stabilizing the formation of a liquid-like, highly hydrated ACP phase. The polymer-stabilized ACP is thought to infiltrate into the collagen through capillary action, and transforms into oriented apatite crystals. In contrast, Deshpande and Beniash (2008) proposed that pAsp binds to collagen and creates a local supersaturation of calcium ions, and mineralization would occur directly in the fibril. Regardless of mechanism, which is discussed further below, these results were significant in introducing a new concept and understanding about how collagen mineralization may be induced. They results suggest that it is the polyelectrolytic nature of NCPs that is critical in promoting intrafibriallar mineralization through the stabilization of ACP particles. One interesting point is that just as pAsp does not have a secondary structure, the highly acidic domains of proteins are also generally considered intrinsically disordered (Delak et al., 2009; Evans, 2003). It has been proposed that the disorder and lack of folding in these domains allows maximum conformational freedom to interact with the crystal surfaces (reviewed in (Hunter et al., 2010)). It is notable, however pAsp additives did not lead to preferential mineral formation to the gap region, as occurs in vivo (Fig. 2C), but induced mineralization throughout the entire fibril. The issue of mineral formation in the gap region will be discussed in more detail in Section 4.

Mechanistic information on how pAsp induces mineralization has been obtained in more recent studies. It has been demonstrated that the interaction of the polymer with the incipient mineral results in a negatively charged complex that interacts with a positively charged region at the C-terminus end of the gap zone, thus mediating the infiltration of the ACP into the fibril (Nudelman et al., 2010). These mineral-pAsp complexes may correspond to the liquid-like droplets described by (Olszta et al., 2007) and to calcium phosphate aggregates formed on the surface of collagen, as suggested by (Deshpande and Beniash, 2008). Besides forming complexes with ACP, pAsp was also shown to interact with the collagen, through binding of its carboxylate groups to the surface of the fibril (Zeiger et al., 2011). This interaction between collagen and the polymer is essential for the mineralization process and controls the rate of mineral formation, as has been proposed by both Deshpande and Beniash (2008) and Olszta et al. (2007).



Fig.2. (A). Schematic representation of the proposed mechanisms of collagen mineralization directed by polyaspartic acid (pAsp). (a) The polymer stabilizes the formation of liquid-like "droplets" of amorphous calcium phosphate (ACP), which infiltrate into the collagen. (b) Collagen becomes completely impregnated with ACP. (c) The ACP transforms into oriented apatite crystals inside the collagen. (B) Transmission electron microscopy image (TEM) of a collagen fibril containing ACP during mineralization in presence of pAsp. (C) TEM image of a collagen fibril mineralization in presence of pAsp, where all the ACP has already converted into oriented apatite crystals. Inset: electron diffraction of the fibril demonstrates that the mineral phase is apatite and the crystals are oriented with their *c*-axis parallel to the direction of the fibril. Adapted from (Olszta et al., 2007), with permission from Elsevier, Copyright (2007).

2.2. Investigating roles of specific NCPs

The use of a simple synthetic polymer as a proxy for NCPs was critical for understanding basic principles such as the effect of charge interactions, inhibition of nucleation in solution, stabilization of the amorphous phase and mineral-collagen interactions. The next step is to replace the synthetic polymers by recombinant NCPs, in order to probe the roles of specific proteins.

Dentin phosphophoryn (DPP) is a protein that is involved both in dentin (Dimuzio and Veis, 1978) and bone mineralization (George and Veis, 2008). It is rich in aspartic acid and serine with a repeat motif of Asp-Ser-Ser, where 90% of all serines are phosphorylated (Stetler-Stevenson and Veis, 1983). This protein binds selectively to the e-band in the gap region of collagen (Traub et al., 1992). Its ability to induce apatite nucleation is dependent on its degree of phosphorylation (He et al., 2005b). Collagen mineralization experiments in the presence of DPP have shown that this protein, in its non-phosphorylated form, induces only the formation of ACP on the surface of fibrils (Fig. 3A). However, when phosphorylated, it promotes intrafibrillar mineralization of collagen with apatite (Deshpande et al., 2011) (Fig. 3B). Even though this protein binds to collagen specifically at the region between the gap and overlap zones, mineralization still occurred homogeneously along the collagen. Thus, it has been proposed that the mechanism of action of phosphorylated DPP (p-DPP) is similar to that of polyaspartic acid, in that it inhibits calcium phosphate precipitation in solution, allowing the mineral to infiltrate into the collagen. Here, the phosphorylation is thought to play an important role in increasing the charge density of the protein and thus enhancing the inhibitory effect over calcium phosphate precipitation.

Similar studies were performed on dentin matrix protein 1 (DMP1). This protein is also involved in bone (MacDougall et al., 1998) and dentin mineralization (George et al., 1993) and it contains high levels of serine, glutamic acid and aspartic acid in its amino acid sequence, (George et al., 1993). Like DPP, this protein is also highly phosphorylated. It has been shown by in vitro crystallization experiments in a gel-like medium that the N-terminus of DMP1 can stabilize the formation of amorphous calcium phosphate, while the C-terminus end is a very strong nucleator of apatite (Gajjeraman et al., 2007; He et al., 2003). Experiments on collagen mineralization by DMP1 yielded similar results to those obtained with DPP. Intrafibrillar mineralization occurred only when the phosphorylated form (p-DMP1) of the protein was used (Deshpande et al., 2011) (Figs. 3C-D). However, while with p-DPP no calcium phosphate was found outside of the collagen, in the case of p-DMP1 a significant amount of mineral was found also outside the fibrils (Fig. 3D). On the other hand, the intrafibrillar apatite crystals formed in presence of p-DMP1 had a higher degree of crystallographic alignment with respect to the fibril than those formed with p-DPP (Fig. 3B and D). It has been proposed that, in comparison to p-DPP and pAsp, p-DMP1 is a weak inhibitor of apatite nucleation. The activity of p-DMP1 was suggested to be related to the formation of protein–mineral complexes that organize on the surface of the fibrils from where mineralization starts. This hypothesis is consistent with the ability of DMP1 to form supramolecular assemblies in the presence of calcium ions (He et al., 2005a).

Not only highly charged, polyanionic proteins can promote intrafibrillar collagen mineralization. Amelogenin, a hydrophobic protein that is involved both in enamel and dentin formation (Nanci et al., 1998; Papagerakis et al., 2003), was shown to promote apatite formation inside collagen fibrils in vitro (Deshpande et al., 2010). This protein can form filamentous structures on the collagen in the absence of mineral. In the presence of calcium phosphate, it organizes the mineral, in the form of ACP, on the surface of the fibril. Subsequently, the ACP enters the collagen and nucleates into apatite crystals. It is interesting that the ACP particles, when on the surface of the collagen, are assembled in the form of filaments that have their long axis aligned parallel to the long axis of the fibril (Deshpande et al., 2010). The assembly of the ACP particles into such filamentous structures has been shown to be mediated by the charged C-terminus domain of amelogenin (Beniash et al., 2005; Fang et al., 2011). The mechanism of collagen mineralization by amelogenin has been hypothesized to be similar to that of DMP1, namely to act by inducing the formation of ordered mineral complexes on the surface of the fibril, which then enter the collagen. In that respect, in the absence of collagen, both DMP1 and amelogenin were shown to organize mineral particles into bundles of crystals with their c-axes co-aligned (Beniash et al., 2005; Deshpande et al., 2011).

When comparing the proposed mechanism of action of pAsp, and p-DPP with that of DMP1 and amelogenin in promoting collagen mineralization, there are striking differences. As discussed, pAsp and p-DPP act as inhibitors of homogenous apatite nucleation, stabilizing the formation of ACP complexes, which infiltrate into the collagen. This mechanism does not require any specific interaction between these charged macromolecular control agents, the mineral and the collagen, such as the formation of ordered supramolecular assemblies or molecular association to specific sites in the fibril. In contrast, both amelogenin and DMP1 selfassemble into ordered supramolecular complexes in presence of calcium ions and organize the minerals in aligned bundles on the surface of the collagen prior to mineralization. Here, it is likely that



Fig.3. (A) Transmission electron microscopy (TEM) image of collagen mineralized in presence of dentin phosphophoryn. (B) TEM image of collagen mineralized in presence of phosphorylated dentin phosphophoryn 1. Asterisk: apatite crystals formed extrafibrillarly. (C) TEM image of collagen mineralized in presence of dentin matrix protein 1. (D) TEM image of collagen mineralized in presence of phosphorylated dentin matrix protein 1. Adapted from (Deshpande et al., 2011), with permission. Copyright (2011) American Chemical Society.

the mineralization mechanism is not dependent on charge interactions between the collagen and the ACP. Rather, it is conceivable that specific interactions between the mineral, protein and collagen do take place, and are important for mineralization. This would imply that mineral formation in collagen can be mediated through more than one mechanism.

Other proteins have also been shown to promote collagen mineralization. Fetuin, a protein that is abundant in serum and inhibits pathological precipitation of calcium phosphate in the serum, soft tissues and in the extracellular matrix, promotes intrafibrillar mineralization of collagen in vitro (Nudelman et al., 2010; Price et al., 2009). It has been proposed that fetuin inhibits the formation of mineral in solution, allowing the calcium and phosphate ions to enter the collagen. Fetuin itself is too large to penetrate into the collagen and thus remains in solution. Price et al. (2009) argue that inhibitors of apatite nucleation that are small enough to enter the collagen will, in fact, also inhibit the intrafibrillar mineralization. Thus, to achieve mineral formation in the collagen it is essential that the directing agent – fetuin, in this case – be excluded from the internal volume of the fibril. This mechanism has been termed "mineralization by inhibitor exclusion", and stands in contrast to the mechanism proposed for pAsp by Olszta et al. (2007), in which pAsp enters collagen together with the mineral. In fact, the experiments with fetuin showed that it is not required that macromolecules enter the collagen to induce intrafibrillar mineralization.

The above studies raise an important point regarding the classification of NCPs as either promoters or inhibitors of mineralization. This classification derives in many cases from *in vitro* studies showing whether the proteins induce or inhibit the formation apatite when in solution or immobilized on a surface (George and Veis, 2008). However, the studies presented above demonstrated that inhibitors of apatite nucleation in solution in fact promote intra-fibrillar collagen mineralization. Notable examples are pAsp, pAA, fetuin and p-DPP (Deshpande et al., 2011; Liu et al., 2011; Olszta et al., 2007; Price et al., 2009). This highlights the need to specify the context when classifying a protein as a mineral promoter or an inhibitor.

2.3. Demineralized collagenous tissues as mineralization substrate

The above experiments were mostly performed on reconstituted collagen, which lacks the collagen-bound macromolecules present in native tissues, as well as collagen cross-linking. In this respect, models that use demineralized tissues as the mineralization substrate have an advantage, since they still contain some of the non-collagenous macromolecules that were present during mineralization of the tissue and are involved in controlling mineral formation. Examples are turkey leg tendon (Freeman and Silver, 2005; Jee et al., 2011), demineralized bone (Chen et al., 2005; Price et al., 2009; Thula et al., 2011) and periodontal ligament (Kirkham et al., 1995). Recently, a model using fixed, demineralized murine periodontal tissues has been developed to study the molecular factors that control collagen mineralization (Lausch et al., 2013). These tissues contain very well defined mineralized and non-mineralized collagenous regions in close proximity, allowing for a direct comparison between hard and soft tissues. In this work, Lausch et al. have shown that in demineralized tissue sections exposed to simulated body fluid, the natively mineralized tissues (cementum and dentin) are selectively remineralized over the periodontal ligament, which is a soft tissue (Fig. 4).

Achieving precise spatial control over mineral formation (mimicking the natural pattern of mineralization), in a solution without any organic additives, shows that the extracellular matrix itself can regulate the rate of mineral deposition in different tissues. While the mineral formed from simulated body fluid was ACP, in other mineralizing solutions containing pAsp, oriented apatitic mineral was formed, which suggests that soluble macromolecules are necessary to mimick the native collagen-mineral relation. It is notable the rate of mineralization differed not only between hard and soft tissues, but also among the mineralized tissues themselves. Dentin and cementum mineralized faster than bone, while peritubular dentin was preferentially mineralized over intertubular dentin. This may result from the different content and distribution of NCPs such as dentin sialoprotein, osteopontin, proteoglycans and other proteins within the tissues, and may also correlate with differences in the collagen itself, such as cross-linking and collagen type. Ultimately this model may be useful in probing the roles of specific molecules in the mineralization process through a top-down approach in which different components are selectively removed by enzymatic digestion prior to remineralization. Although there are limitations to using fixation to retain non-collagenous macromolecules, the multiple-level of control demonstrated by this system suggests that much can be learned from it.

3. The role of precursor phases

In vivo experiments show that an amorphous precursor phase is delivered by osteoblasts to the collagen fibrils at the bone growth front (Mahamid et al., 2010, 2011). An important question that arises is how the ACP penetrates into the collagen. Here, we propose two different scenarios. One possibility is that infiltration of calcium phosphate into the collagen could occur through a dissolution-re-precipitation mechanism, where once deposited in the extracellular medium, the ACP could dissolve and the ions diffuse into the collagen. This hypothesis reconciles the idea that mineralization occurs through the diffusion of ions into the collagen and their subsequent nucleation into apatite (Silver and Landis, 2011). There is no evidence, however, for dissolution of the ACP and re-precipitation inside collagen. A second hypothesis is that the ACP itself enters the collagen. It was initially proposed that ACP exists in a liquid-like state which can diffuse into the collagen through capillary forces (Gower, 2008; Olszta et al., 2007). Later studies showed that the infiltration of the ACP into the collagen is mediated by charge interactions between the polymer-mineral complex and the fibril (Nudelman et al., 2010; Zeiger et al., 2011) at specific sites in the collagen. In this process, it is not immediately clear whether pAsp enters the collagen fibril together with the mineral, and whether the infiltration process is solely dependent on charge interactions and/or capillary forces, or if additional factors are also involved. It is known, however, that it is not necessary that pAsp infiltrates into the collagen for mineralization to be induced (Nudelman et al., 2010; Price et al., 2009).

In vitro studies have also shown that the size of the ACP aggregates is important for the mineralization, in that smaller aggregates result in faster infiltration into the fibril and apatite formation (Nudelman et al., 2012) (Fig. 5). These results are in accordance with the size-exclusion characteristic of collagen, which limits the size of molecules that can penetrate into the fibril. Molecules that are larger than 40 kDa do not to diffuse into the collagen, while the ones smaller than 6 kDa can easily penetrate into the fibril (Toroian et al., 2007). It is interesting that using polyaspartic acid (pAsp) with higher molecular weight caused the mineralization to proceed faster and increased the mineral content inside the fibril (Jee et al., 2010). In this case, it has been proposed that the rate of collagen mineralization is connected with the stability of the ACP in the solution. Higher molecular weight polymers have a higher inhibitory effect on apatite nucleation and may stabilize the ACP in the form of small particles, or "nanodroplets", for longer periods when compared to lower molecular weight polymers. These nanodroplets are small enough to infiltrate into the collagen, yielding a higher mineral content.

In remineralization experiments on demineralized mouse periodontal tissue sections, Lausch et al. evaluated the mineral phase formed from a series of metastable calcium phosphate solutions stabilized by pAsp. They showed that when the overall rate of mineral deposition was slow, only ACP was formed, while with a higher rate of mineral deposition, oriented apatite crystals formed (Lausch et al., 2013). They suggest that the more rapidly formed ACP is less stable, and therefore more rapidly transforms into apatite. This hypothesis is corroborated by mineralization experiments performed in the presence of copper ions (Nudelman et al., 2012), which are known to stabilize ACP and inhibit its transformation into apatite (Okamoto and Hidaka, 1994). ACP formed with simulated body fluid or in the presence of copper ions remains stable for long periods of time (Nudelman et al., 2012). The above results



Fig.4. (A) TEM image of tissue remineralized with simulated body fluid for 13 days, showing the sharp mineralization front at the cementum (C) /periodontal ligament (L) junction (arrow). Contrast is given by the presence of mineral – areas of high electron density (cementum, mantle dentin (MD) and circumpulpal dentin) are mineralized, whereas areas with low electron-density (the periodontal ligament) remains unmineralized. (B) TEM image of demineralized section, where no mineral is present. Adapted from Lausch et al. (2013), with permission. Copyright (2013) WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.



Fig.5. CryoTEM images of collagen mineralized with calcium phosphate for 24 h in presence of different concentrations of pAsp. (A) 1.5 µg/ml of pAsp. White arrows: apatite crystals nucleating and growing within the amorphous calcium phosphate phase. (B) 3 µg/ml of pAsp. (C) 6 µg/ml of pAsp. (D) 10 µg/ml of pAsp. White arrows: amorphous calcium phosphate (ACP) particles infiltrating into the collagen. The higher the concentration of pAsp, the larger the ACP aggregates. (A–C) adapted from (Nudelman et al., 2012), with permission, Copyright (2010), Macmillan Publishers Ltd. (D) Adapted from (Nudelman et al., 2010), with permission of the Royal Society of Chemistry, Copyright (2012).

suggest that ACP particles have to be stable for long enough in order to diffuse into the collagen, but not too stable to the point that their crystallization into apatite inside the collagen is inhibited. Based on these *in vitro* studies, it is tempting to assume that the ACP that deposits on the collagen fibrils *in vivo* will have similar characteristics as reported *in vitro* – negative net charge and composed of loosely packed aggregates of calcium phosphate complexes that could diffuse into the collagen. However, whether this is indeed the case still needs to be verified.

4. Mineralization starting in the gap region

The factors that direct nucleation in the gap region of collagen remain elusive. Several *in vitro* approaches have been used to investigate the mechanisms underlying the spatial specificity of apatite formation in the collagen. Analysis of the amino acid sequence and structure of collagen has shown that the specific site in the gap region where crystals have been reported to nucleate (Traub et al., 1992) contains clusters of both positively and negatively charged amino acids. (Chapman, 1974; Chapman and Hardcastle, 1974; Dahl et al., 1998) It has been suggested that the side-chains of these amino acids could form a 3-dimensional environment capable of coordinating calcium and phosphate ions and thus providing nucleation sites for apatite (Dahl et al., 1998; Landis and Silver, 2002; Silver et al., 2001; Silver and Landis, 2011). Furthermore, it has been proposed that the preferential binding of calcium and phosphate ions to the gap zone could also be a consequence of the flexibility of this region (Landis and Silver, 2002; Silver et al., 2001). However, most of the in vitro studies have reported that the apatite crystals nucleate equally in the gap and overlap regions, suggesting that there must be an additional mechanism that directs apatite formation to specific sites (Deshpande and Beniash, 2008; Nudelman et al., 2010; Olszta et al., 2007). An additional hypothesis is that the gap regions of adjacent fibrils create channels that provide space and allow the mineral to form (Katz and Li, 1973; Landis et al., 1996, 1991; Landis and Song, 1991; Landis et al., 1993). Although space constraints may indeed play a role, the fact that in vitro apatite nucleation in the collagen was observed both in the gap and overlap regions, suggests that availability of space does not limit crystal growth (Deshpande and Beniash, 2008; Nudelman et al., 2010; Olszta et al., 2007). In fact, the crystals are capable of pushing away the collagen molecules as they grow, thus allowing mineral formation in the overlap region as well (Fratzl et al., 1993; Nudelman et al., 2010, 2012).

It has been proposed that non-collagenous proteins could also direct the formation of apatite to the gap region (Dahl et al., 1998; Traub et al., 1992). Several *in vitro* studies on the interactions between these proteins and the collagen have shown that dentin phosphophoryn (DPP), dentin matrix protein 1 (DMP1) and bone sialoprotein (BSP) are all capable of binding to the gap region of collagen (He and George, 2004; Traub et al., 1992; Tye et al., 2005). DPP, in particular, binds predominantly to the collagen e-band, which is the site where apatite crystals are observed to nucleate (Traub et al., 1992). However, in the *in vitro* mineralization experiments

performed by Deshpande et al., DPP did not induce preferential apatite formation in the gap zone (Deshpande et al., 2011). It is notable that in remineralization of demineralized mouse dentin banding was observed in remineralized collagen fibrils (Lausch et al., 2013). This is in contrast to most reconstituted collagen systems, and supports the notion that NCPs could direct preferential gap zone mineralization, as in the demineralized tissue model some NCPs are retained by simultaneous fixation during demineralization (Mckee et al., 1991). Recently, preferential apatite formation in discrete bands along the collagen fibril was obtained using a combination of polyacrylic acid (pAA) and inorganic polyphosphates as additives to induce intrafibrillar mineralization of collagen (Liu et al., 2011) (Fig. 6). The authors hypothesize that the regions where the mineral form correspond to the gap zone of collagen, thus mimicking in vivo collagen mineralization. However, it is not clearly demonstrated whether the mineralized regions do correspond to the gap zones. The pAA, in this system, has a function that is similar to that of pAsp. in that it promotes intrafibrillar mineralization. However, while pAsp induces the formation of discrete crystals homogeneously distributed throughout the fibril, pAA induces the formation of long crystals of apatite that fill the intermolecular spaces in the fibril. The combination of pAA with polyphosphates promotes the formation of smaller, discrete crystals in periodic bands within the collagen. The authors suggest that the polyphosphates replicate the purported function DPP, DMP1 and BSP in directing the mineralization to the gap region (Dai et al., 2011; Liu et al., 2011). They hypothesize that similar to DPP, DMP1 and BSP, polyphosphates bind to the collagen at the gap region, and from there template apatite nucleation. However so far, no direct experimental evidence was provided in this regard.

5. The role of collagen in mineralization

One of the main questions regarding collagen mineralization is the nature of the collagen-mineral interactions. On the one hand, it is well known that collagen alone does not induce hydroxyapatite formation, requiring the presence of NCPs (Bradt et al., 1999; Hunter et al., 2001; Saito et al., 1997). On the other hand, the structure of collagen is necessary to guide the organization and growth of the crystal. Mutations in the amino acid sequence of collagen, as in osteogenesis imperfecta, can seriously impair the formation, orientation and organization of the apatite crystals, which significantly increases the brittleness of bone (Fratzl et al., 1996). Therefore, it is clear that there is a relationship between the fibril structure and mineral formation. Several *in vitro* methods have been developed that simplify the physiological process of collagen mineralization to the point that one can investigate specifically the collagen-mineral interactions, from the atomic level to the micrometer level. These *in vitro* methodologies have allowed scientists to investigate the involvement of collagen in controlling the infiltration of calcium phosphate into the collagen; nucleation of apatite; control over crystal size and morphology; and orientation of the crystallographic *c*-axis of the apatite crystals with respect to the fibrils.

5.1. The role of collagen in controlling mineral infiltration and apatite nucleation

The development of an *in vitro* system by Olszta et al. (2007) where intrafibrillar mineralization of single collagen fibrils was achieved by replacing the NCPs with a single synthetic polymer have provided an excellent platform to investigate collagen-apatite interactions during mineralization. Combining this in vitro system with cryogenic transmission electron microscopy and tomography (Frederik and Sommerdijk, 2005; Nudelman et al., 2011), it was shown that collagen controls two very important steps in mineralization - the infiltration of the mineral phase, in the form of an amorphous precursor, into the fibril, and its subsequent transformation into oriented apatite crystals (Nudelman et al., 2010). Polyaspartic acid, which was used as a mineralization-directing agent, forms a negatively charged complex with ACP, which interacts with a positively charged region of collagen at the C-terminus end region of the gap zone (Fig. 7A-B and D-E). This charge interaction between the pAsp-ACP complex and the collagen is what mediates infiltration of the mineral into the fibril. The nucleation of apatite is induced by nucleation sites formed by clusters of charged amino acids that are present throughout the 67 nm repeat of the collagen (Fig. 7C and F). These results provided the first experimental evidence of the active role of collagen in controlling mineralization. They support the notion that the charged groups in the collagen provide nucleation sites that induce nucleation of apatite (Kawska et al., 2008; Landis and Silver, 2009; Silver and Landis, 2011). It is also conceivable that the 3-dimensional arrangement of the charged groups presents a surface that forms an epitaxial template that controls the crystallographic orientation of the incipient crystals. In this case, however, one could also argue that it is due to spatial constrains that the apatite crystals nucleate and grow with their *c*-axis parallel to the long axis of the fibril. Since the collagen molecules are aligned in the axial direction of the collagen fibril, it



Fig.6. (A) Collagen mineralization in presence of polyacrylic acid. Mineralization is homogeneous throughout the fibril. Inset: electron diffraction pattern, showing reflections characteristic of hydroxyapatite. (B) Collagen mineralization in presence of polyacrylic acid and sodium tripolyphosphate. Mineralization occurs specifically in bands along the collagen. Adapted from (Liu et al., 2011), with permission from Elsevier. Copyright (2011).



Fig.7. (A) CryoTEM image of non-mineralized collagen stained with uranyl acetate. Staining bands are labeled according to (Chapman et al., 1990). White circle: 10 nm gold marker for electron tomography. (B) CryoTEM image of collagen mineralized for 24 h and stained with uranyl acetate. Calcium phosphate is associated to the fibril in a regular pattern, following the staining bands (black arrows). (C) CryoTEM image of a fibril mineralized for 48 h and stained with uranyl acetate. Apatite crystals are found within an amorphous calcium phosphate bed, which can still be seen infiltrating into the fibril through the *a*-band (inset 1, black arrow). Insets 2–5 show crystals nucleating on the staining bands. (D) Intensity profile of **A**, non-mineralized collagen. Labels in the peaks are according to the corresponding staining band. (E) Intensity profile of **B**, collagen mineralized for 24 h. (F) Histogram of the distribution of the number of nucleating crystals per staining band. Scale bars: 50 nm. Adapted from (Nudelman et al., 2010), with permission, Copyright (2010), Macmillan Publishers Ltd.

would be energetically favorable for the crystals to grow preferentially aligned parallel to the fibril. Nevertheless, even though collagen actively mineral formation, controls intrafibrillar mineralization only occurs in the presence of inhibitors of apatite nucleation in solution. While these experiments were performed on reconstituted single collagen fibrils, it was later demonstrated the same method could be used to induce intrafibrilar mineralization of dense 3-dimensional collagenous matrices (Nassif et al., 2010). More recently, Wang et al. (2012) succeeded in achieving collagen mineralization in vitro without the use of polymer additives as mineralization directing agents, further demonstrating that it is possible that collagen directs apatite nucleation and growth. In this study, they were able to achieve intrafibrillar mineralization of high density matrices, however it was necessary to impregnate the collagen with high concentrations of calcium, phosphate and carbonate ions during fibrilogenesis. In addition, Wang et al. (2012) did not discard that in vivo, extracellular matrix proteins such as NCPs may have the function of driving the mineral ions into the collagen, similar to the action exerted by polyaspartic acid in vitro. The most important conclusion from these in vitro model studies is that collagen is not a passive scaffold as has been previously thought; rather, it actively controls and templates apatite formation during mineralization by directing ACP infiltration and mediating its nucleation into the crystalline phase.

The role of collagen cross-links on mineralization has also been investigated *in vitro*. This issue is important since the reconstituted collagen normally used in the mineralization experiments lacks the cross-linking between the molecules that is present in the fibrils in living mineralizing tissue. Li et al. (2012) demonstrated that crosslinking, in fact, enhanced the mineralization of the collagen. The authors proposed that when the molecules were cross-linked, the spaces between the collagen molecules in the fibril increased, allowing easier diffusion of the ACP particles. These results need to be taken with care, though, since the cross-links induced *in vitro* were substantially different from the ones that are present *in vivo*. In this work, the reaction of carbodiimide with N-hydroxysuccinimide was used to form amide bonds between the collagen molecules (Kuijpers et al., 2000). In bone, the cross-links are based on aldehyde formation and condensation between peptidyl lysine and hydroxylysine residues, which results in immature ketoimine cross-links that subsequently matures to pyridinoline and pyrrole cross-links (Knott and Bailey, 1998; Veis, 1997).

5.2. The role of collagen in controlling the crystallographic orientation of the apatite crystals

The regulation of the crystallographic orientation of the apatite crystals in bone and dentin is also an important question. It has been hypothesized that stereochemical features of collagen may be fundamental in directing ion binding, apatite nucleation, crystal size, shape, orientation and alignment (Landis and Silver, 2009; Silver and Landis, 2011). The above *in vitro* mineralization models suggest that this hypothesis may indeed be correct, since the additives used as mineralization-directing agents probably do not provide an epitaxial relationship with apatite (Olszta et al., 2007). Collagen mineralization has been achieved using fetuin as an

additive, a protein that is too large to infiltrate into the fibril. In this case, only the collagen can be responsible for inducing oriented nucleation (Nudelman et al., 2010). Furthermore, oriented nucleation of apatite and the formation of well-organized arrays of crystals were only observed when the collagen fibril was well organized. Poorly organized fibrils contained only randomly oriented crystals, indicating the absence of a structural template for mineral formation (Chen et al., 2005; Nudelman et al., 2012). One must still take into account that all these experiments were performed in vitro, using synthetic components to replace the proteins responsible for directing mineralization. In the biological tissue, where all the NCPs are present, it still cannot be discarded that these proteins will also play a role in inducing oriented nucleation and alignment of the apatite crystals with respect to the collagen. DMP1, for instance, was shown to orient apatite crystals both intra- and extrafibrillarly (Deshpande et al., 2011).

6. Outlook

Using in vitro models to investigate collagen mineralization has provided several important advances in our understanding on collagen mineralization in the contexts of bone and dentin formation. The infiltration of the ACP into the collagen is dependent on a number of factors, such as particle size, stability of the ACP and net charge. Controlling these parameters is part of the function of the NCPs, some of which induce collagen mineralization on their own and are thought to direct the mineral preferentially to the gap region. It has also been shown that collagen is an active scaffold which provides an appropriate environment for mineral infiltration through charge interactions with the ACP and mediates its crystallization into apatite crystals. Here, the fibrillar structure of collagen is essential to produce an organized array of crystals with the same crystallographic orientation. These conclusions represent important mechanistic details on collagen mineralization that could only be obtained through a reductionist approach, which allowed each parameter to be isolated and individually investigated.

It must be noted that all the above conclusions were derived from synthetic systems that contained very few components, as opposed to the complex environment found in vivo. Furthermore, in vitro systems cannot replicate the dynamic biochemical and cellular processes that occur in the living tissue. However, the insights obtained from the in vitro models constitute the first step in unraveling the mechanisms of collagen mineralization in vivo. Applying the knowledge obtained from in vitro models to in vivo investigations is the next step toward answering remaining questions on the molecular mechanisms of mineralized tissue formation, with increasing physiological significance. A very important question that cannot be addressed through in vitro experiments however, is the origin of the calcium phosphate that is deposited in the collagen. This issue has been the subject of discussion for a long time, with two major hypotheses. First the crystals are actively nucleated from the interstitial fluid, which is supersaturated with respect to multiple calcium phosphate phases. In this case, apatite formation is largely controlled by the NCPs that are associated to the collagen (Glimcher and Muir, 1984; Veis and Perry, 1967). Second, matrix vesicles accumulate ions and are transported to the bone growth front (Ali et al., 1970; Anderson et al., 2005).

Regarding the presence of matrix vesicles, it has been recently demonstrated in *in vivo* experiments that osteoblasts concentrate calcium phosphate, presumably as amorphous calcium phosphate, within intracellular compartments. This precursor phase is then delivered to the collagen at the bone growth front, where it infiltrates the fibrils and crystallizes into apatite (Mahamid et al., 2010, 2011). It was further suggested, based on cell culture experiments, that the intracellular ACP deposits originate from the mitochondria (Boonrungsiman et al., 2012). It is possible that phosphate ions are condensed in the form of polyphosphates, which then complex calcium ions, forming storage granules that can be transported to the extracellular medium (Omelon et al., 2009). The polyphosphates then are degraded by alkaline phosphatase, releasing calcium and phosphate ions for mineralization. Polyphosphates, as a medium to store calcium and phosphate within cells, is an effective way to decrease the free ion concentrations, enabling them to contain high concentrations of calcium and phosphate, without precipitating apatite crystals in the intracellular compartments. These issues certainly could not have been investigated using in vitro systems. Much work still needs to be done to completely understand how the mineral is accumulated into the cells, how it is transported, and its chemical composition/phase, etc. Here, we believe that only in vivo and in situ methods, where the biological tissue is directly investigated during collagen mineralization can provide the answers to these issues.

The availability of techniques such as cryoTEM and tomography, cryo-scanning electron microscopy, electron diffraction, chemical analysis (EDX/EELS), gene knock-out, and molecular modeling, allows the investigation of the mechanisms of mineralization with nanometer resolution while ensuring the preservation the molecular structures in a close to native state. Thus, important questions such as how the ACP infiltrates into the collagen: deposition of mineral by the cells; sub-cellular localization of mineral and proteins; intracellular transport mechanisms; and the spatial and temporal relationship between collagen, ACP and the NCPs can be addressed, providing new insights into the function of the key players in collagen mineralization. A very important issue, and perhaps the most challenging, is to understand how the different components (i.e. the collagen, the NCPs, and the mineral) interact with each other and function in synergy to control mineralization. A combination of both in vitro and in vivo studies can lead to very powerful approaches to understand the mechanisms of formation of the hard tissues in the body.

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References

- Ali, S.Y., Sajdera, S.W., Anderson, H.C., 1970. Isolation and characterization of calcifying matrix vesicles from epiphyseal cartilage. Proceedings of the National Academy of Sciences of the United States of America 67, 1513.
- Anderson, H.C., Garimella, R., Tague, S.E., 2005. The role of matrix vesicles in growth plate development and biomineralization. Front. Biosci. 10, 822–837.
- Bachra, B.N., 1972. Calcification in-Vitro of demineralized bone matrix electronmicroscopic and chemical aspects. Calc. Tiss. Res. 8, 287.
- Beniash, E., 2011. Biominerals-hierarchical nanocomposites: the example of bone. Wires Nanomed. Nanobi. 3, 47–69.
- Beniash, E., Metzler, R.A., Lam, R.S.K., Gilbert, P.U.P.A., 2009. Transient amorphous calcium phosphate in forming enamel. J. Struct. Biol. 166, 133–143.
- Beniash, E., Simmer, J.P., Margolis, H.C., 2005. The effect of recombinant mouse amelogenins on the formation and organization of hydroxyapatite crystals in vitro. J. Struct. Biol. 149, 182–190.
- Beniash, E., Traub, W., Veis, A., Weiner, S., 2000. A transmission electron microscope study using vitrified ice sections of predentin: structural changes in the dentin collagenous matrix prior to mineralization. J. Struct. Biol. 132, 212–225.
- Blumenthal, N.C., Holmes, J.M., Posner, A.S., 1972. Effect of preparation conditions on properties and transformation of amorphous calcium phosphate. Mater Res. Bull. 7, 1181.
- Boonrungsiman, S., Gentleman, E., Carzaniga, R., Evans, N.D., McComb, D.W., et al., 2012. The role of intracellular calcium phosphate in osteoblast-mediated bone apatite formation. Proc. Natl. Acad. Sci. USA 109, 14170–14175.
- Boskey, A.L., 1989. Hydroxyapatite formation in a dynamic collagen gel system effects of Type-I collagen, lipids, and proteoglycans. J. Phys. Chem.-Us 93, 1628– 1633.

- Boskey, A.L., 1998. Biomineralization: conflicts, challenges, and opportunities. J. Cell. Biochem., 83–91.
- Boskey, A.L., Maresca, M., Doty, S., Sabsay, B., Veis, A., 1990. Concentration-Dependent Effects of Dentin Phosphoryn in the Regulation of Invitro Hydroxyapatite Formation and Growth. Bone Miner 11, 55–65.
- Boskey, A.L., Spevak, L., Paschalis, E., Doty, S.B., McKee, M.D., 2002. Osteopontin deficiency increases mineral content and mineral crystallinity in mouse bone. Calcif. Tissue Int. 71, 145–154.
- Bradt, J.H., Mertig, M., Teresiak, A., Pompe, W., 1999. Biomimetic mineralization of collagen by combined fibril assembly and calcium phosphate formation. Chem. Mater. 11, 2694–2701.
- Chapman, J.A., 1974. Staining pattern of collagen fibrils. 1. Analysis of electron micrographs. Connect. Tissue Res. 2, 137–150.
- Chapman, J.A., Hardcastle, R.A., 1974. Staining pattern of collagen fibrils. 2. Comparison with patterns computer-generated from amino-acid sequence. Connect. Tissue Res. 2, 151–159.
- Chen, C.C., Boskey, A.L., Rosenberg, L.C., 1984. The inhibitory effect of cartilage proteoglycans on hydroxyapatite growth. Calcif. Tissue Int. 36, 285–290.
- Chen, J.L., Burger, C., Krishnan, C.V., Chu, B., Hsiao, B.S., et al., 2005. In vitro mineralization of collagen in demineralized fish bone. Macromol. Chem. Phys. 206, 43–51.
- Clark, J.H., 1931. A study of tendons, bones, and other forms of connective tissue by means of X-ray diffraction patterns. Am. J. Physiol. 98, 356–358.
- Crane, N.J., Popescu, V., Morris, M.D., Steenhuis, P., Ignelzi, M.A., 2006. Raman spectroscopic evidence for octacalcium phosphate and other transient mineral species deposited during intramembranous mineralization. Bone 39, 434–442.
- Dahl, T., Sabsay, B., Veis, A., 1998. Type I collagen-phosphophoryn interactions: specificity of the monomer-monomer binding. J. Struct. Biol. 123, 162–168. Dai, L., Qi, Y.P., Niu, L.N., Liu, Y., Pucci, C.R., et al., 2011. Inorganic-organic
- Dai, L., Qi, Y.P., Niu, L.N., Liu, Y., Pucci, C.R., et al., 2011. Inorganic-organic nanocomposite assembly using collagen as a template and sodium tripolyphosphate as a biomimetic analog of matrix phosphoprotein. Cryst. Growth Des. 11, 3504–3511.
- de Jong, W.F., 1926. La substance minerale dans les os. Recl. Clav. Chim. Pays Bas. Belg. 45, 445-448.
- Delak, K., Collino, S., Evans, J.S., 2009. Polyelectrolyte domains and intrinsic disorder within the prismatic asprich protein family. Biochemistry-Us 48, 3669–3677.
- Deshpande, A.S., Beniash, E., 2008. Bioinspired synthesis of mineralized collagen fibrils. Cryst. Growth Des. 8, 3084–3090.
- Deshpande, A.S., Fang, P.A., Simmer, J.P., Margolis, H.C., Beniash, E., 2010. Amelogenin-collagen interactions regulate calcium phosphate mineralization in vitro. J. Biol. Chem. 285, 19277–19287.
- Deshpande, A.S., Fang, P.A., Zhang, X.Y., Jayaraman, T., Sfeir, C., et al., 2011. Primary structure and phosphorylation of dentin matrix protein 1 (DMP1) and dentin phosphophoryn (DPP) uniquely determine their role in biomineralization. Biomacromolecules 12, 2933–2945.
- Dimuzio, M.T., Veis, A., 1978. Phosphophoryns major non-collagenous proteins of rat incisor dentin. Calc. Tiss Res. 25, 169–178.
- Eanes, E.D., Gillesse.Ih, Posner, A.S., 1965. Intermediate states in precipitation of hydroxyapatite. Nature 208, 365.
- Eanes, E.D., Posner, A.S., 1970. A note on crystal growth of hydroxyapatite precipitated from aqueous solutions. Mater. Res. Bull. 5, 377.
- Evans, J.S., 2003. 'Apples' and 'oranges': comparing the structural aspects of biomineral- and ice-interaction proteins. Curr. Opin. Coll. Interface. Sci. 8, 48– 54.
- Fang, P.A., Conway, J.F., Margolis, H.C., Simmer, J.P., Beniash, E., 2011. Hierarchical self-assembly of amelogenin and the regulation of biomineralization at the nanoscale. Proc. Natl. Acad. Sci. USA 108, 14097–14102.
- Fratzl, P., Fratzl-Zelman, N., Klaushofer, K., 1993. Collagen packing and mineralization - an X-ray-scattering investigation of Turkey leg tendon. Biophys. J. 64, 260–266.
- Fratzl, P., Paris, O., Klaushofer, K., Landis, W.J., 1996. Bone mineralization in an osteogenesis imperfecta mouse model studied by small-angle X-ray scattering. J. Clin. Invest. 97, 396–402.
- Frederik, P.M., Sommerdijk, N.A.J.M., 2005. Spatial and temporal resolution in cryoelectron microscopy – A scope for nano-chemistry. Curr. Opin. Coll. Interface Sci. 10, 245–249.
- Freeman, J.W., Silver, F.H., 2005. The effects of prestrain and collagen fibril alignment on in vitro mineralization of self-assembled collagen fibers. Connect. Tissue Res. 46, 107–115.
- Gajjeraman, S., Narayanan, K., Hao, J.J., Qin, C.L., George, A., 2007. Matrix macromolecules in hard tissues control the nucleation and hierarchical assembly of hydroxyapatite. J. Biol. Chem. 282, 1193–1204.
- George, A., Sabsay, B., Simonian, P.A.L., Veis, A., 1993. Characterization of a novel dentin matrix acidic phosphoprotein – implications for induction of biomineralization. J. Biol. Chem. 268, 12624–12630.
- George, A., Veis, A., 2008. Phosphorylated proteins and control over apatite nucleation, crystal growth, and inhibition. Chem. Rev. 108, 4670–4693.
- Glimcher, M.J., Muir, H., 1984. Recent studies of the mineral phase in bone and its possible linkage to the organic matrix by protein-bound phosphate bonds. Philos Trans. R. Soc. London, B 304, 479–508.
- Gower, L.B., 2008. Biomimetic model systems for investigating the amorphous precursor pathway and its role in biomineralization. Chem. Rev. 108, 4551–4627.
- Habraken, W. J. E. M., Tao, J., Brylka, L. J., Friedrich, H., Bertinetti, L., et al., 2013. Ionassociation complexes unite classical and non-classical theories for the

biomimetic nucleation of calcium phosphate. Nature Communications 4, Article number 1507.

- He, G., Dahl, T., Veis, A., George, A., 2003. Nucleation of apatite crystals in vitro by self-assembled dentin matrix protein, 1. Nat. Mater. 2, 552–558.
- He, G., George, A., 2004. Dentin matrix protein 1 immobilized on Type I collagen fibrils facilitates apatite deposition in vitro. J. Biol. Chem. 279, 11649–11656.
- He, G., Gajjeraman, S., Schultz, D., Cookson, D., Qin, C.L., et al., 2005a. Spatially and temporally controlled biomineralization is facilitated by interaction between self-assembled dentin matrix protein 1 and calcium phosphate nuclei in solution. Biochemistry-Us 44, 16140–16148.
- He, G., Ramachandran, A., Dahl, T., George, S., Schultz, D., et al., 2005b. Phosphorylation of phosphophoryn is crucial for its function as a mediator of biomineralization. J. Biol. Chem. 280, 33109–33114.
- Hodge, A. J. and Petruska, J. A., 1963. Recent studies with the electron microscope on ordered aggregates of the tropocollagen macromolecule, in Aspects of Protein Structure, pages 289–300, ed. Ramachandran, G. N., Academic Press, New York.
- Hunter, G.K., Goldberg, H.A., 1993. Nucleation of hydroxyapatite by bone sialoprotein. Proc. Natl. Acad. Sci. USA 90, 8562–8565.
- Hunter, G.K., O'Young, J., Grohe, B., Karttunen, M., Goldberg, H.A., 2010. The flexible polyelectrolyte hypothesis of protein–biomineral interaction. Langmuir 26, 18639–18646.
- Hunter, G.K., Poitras, M.S., Underhill, T.M., Grynpas, M.D., Goldberg, H.A., 2001. Induction of collagen mineralization by a bone sialoprotein-decorin chimeric protein. J. Biomed. Mater. Res. 55, 496–502.
- lijima, M., lijima, K., Moriwaki, Y., Kuboki, Y., 1994. Oriented growth of octacalcium phosphate crystals on Type-I collagen fibrils under physiological conditions. J. Cryst. Growth 140, 91–99.
- Jackson, S.F., 1957. The fine structure of developing bone in the embryonic fowl. Proc. R. Soc. London, B 146, 270–280.
- Jee, S.S., Kasinath, R.K., DiMasi, E., Kim, Y.Y., Gower, L., 2011. Oriented hydroxyapatite in turkey tendon mineralized via the polymer-induced liquidprecursor (PILP) process. Crystengcomm 13, 2077–2083.
- Jee, S.S., Thula, T.T., Gower, L.B., 2010. Development of bone-like composites via the polymer-induced liquid-precursor (PILP) process. Part 1: influence of polymer molecular weight. Acta Biomater. 6, 3676–3686.
- Johnsson, M.S.A., Nancollas, G.H., 1992. The role of brushite and octacalcium phosphate in apatite formation. Crit. Rev. Oral Biol. M 3, 61–82.
- Katz, E.P., Li, S., 1973. Structure and function of bone collagen fibrils. J. Mol. Biol. 80, 1–15.
- Kawska, A., Hochrein, O., Brickmann, A., Kniep, R., Zahn, D., 2008. The nucleation mechanism of fluorapatite-collagen composites: ion association and motif control by collagen proteins. Angew. Chem.-Int. Ed. 47, 4982–4985.
- Kirkham, J., Brookes, S.J., Shore, R.C., Bonass, W.A., Robinson, C., 1995. The effect of glycosylaminoglycans on the mineralization of sheep periodontal ligament in vitro. Connect. Tissue Res. 32, 345–351.
- Knott, L., Bailey, A.J., 1998. Collagen cross-links in mineralizing tissues: a review of their chemistry, function, and clinical relevance. Bone 22, 181–187.
- Kuijpers, A.J., Engbers, G.H.M., Krijgsveld, J., Zaat, S.A.J., Dankert, J., et al., 2000. Cross-linking and characterisation of gelatin matrices for biomedical applications. J. Biomat. Sci.-Polym. E 11, 225–243.
- Landis, W.J., Hodgens, K.J., Arena, J., Song, M.J., McEwen, B.F., 1996. Structural relations between collagen and mineral in bone as determined by high voltage electron microscopic tomography. Microsc. Res. Tech. 33, 192–202.
- Landis, W.J., Moradianoldak, J., Weiner, S., 1991. Topographic imaging of mineral and collagen in the calcifying Turkey tendon. Connect. Tissue Res. 25, 181–196.
- Landis, W.J., Silver, F.H., 2002. The structure and function of normally mineralizing avian tendons. Comp. Biochem. Phys. A 133, 1135–1157.
- Landis, W.J., Silver, F.H., 2009. Mineral deposition in the extracellular matrices of vertebrate tissues: identification of possible apatite nucleation sites on Type I collagen. Cells Tissues Organs 189, 20–24.
- Landis, W.J., Song, M.J., 1991. Early mineral deposition in calcifying tendon characterized by high-voltage electron-microscopy and 3-dimensional graphic imaging. J. Struct. Biol. 107, 116–127.
- Landis, W.J., Song, M.J., Leith, A., Mcewen, L., Mcewen, B.F., 1993. Mineral and organic matrix interaction in normally calcifying tendon visualized in 3 dimensions by high-voltage electron-microscopic tomography and graphic image-reconstruction. J. Struct. Biol. 110, 39–54.
- Lausch, A.J., Quan, B.D., Miklas, J.W., Sone, E.D., 2013. Extracellular matrix control of collagen biomineralization, in press. http://dx.doi.org/10.1002/adfm. 201203760.
- Li, Y.P., Thula, T.T., Jee, S., Perkins, S.L., Aparicio, C., et al., 2012. Biomimetic mineralization of woven bone-like nanocomposites: role of collagen cross-links. Biomacromolecules 13, 49–59.
- Liu, Y., Kim, Y.K., Dai, L., Li, N., Khan, S.O., et al., 2011. Hierarchical and nonhierarchical mineralisation of collagen. Biomaterials 32, 1291–1300.
- Luo, G.B., Ducy, P., McKee, M.D., Pinero, G.J., Loyer, E., et al., 1997. Spontaneous calcification of arteries and cartilage in mice lacking matrix GLA protein. Nature 386, 78–81.
- MacDougall, M., Gu, T.T., Luan, X.G., Simmons, D., Chen, J.K., 1998. Identification of a novel isoform of mouse dentin matrix protein 1: spatial expression in mineralized tissues. J. Bone Miner. Res. 13, 422–431.
- Mahamid, J., Aichmayer, B., Shimoni, E., Ziblat, R., Li, C., et al., 2010. Mapping amorphous calcium phosphate transformation into crystalline mineral from the cell to bone in zebrafish fin rays. Proc. Natl. Acad. Sci. USA 107, 6316– 6321.

- Mahamid, J., Sharir, A., Addadi, L., Weiner, S., 2008. Amorphous calcium phosphate is a major component of the forming fin bones of zebrafish: indications for an amorphous precursor phase. Proc. Natl. Acad. Sci. USA 105, 12748–12753.
- Mahamid, J., Sharir, A., Gur, D., Zelzer, E., Addadi, L., et al., 2011. Bone mineralization proceeds through intracellular calcium phosphate loaded vesicles: a cryoelectron microscopy study. J. Struct. Biol. 174, 527–535.
- McKee, M.D., Nanci, A., 1996. Osteopontin at mineralized tissue interfaces in bone, teeth, and osseointegrated implants: ultrastructural distribution and implications for mineralized tissue formation, turnover, and repair. Microsc. Res. Tech. 33, 141–164.
- Mckee, M.D., Nanci, A., Landis, W.J., Gotoh, Y., Gerstenfeld, L.C., et al., 1991. Effects of fixation and demineralization on the retention of bone phosphoprotein and other matrix components as evaluated by biochemical analyses and quantitative immunocytochemistry. J. Bone Miner. Res. 6, 937–945.
- Meyer, J.L., Eanes, E.D., 1978. Thermodynamic analysis of amorphous to crystalline calcium-phosphate transformation. Calcif. Tissue Res. 25, 59–68.
- Nanci, A., Zalzal, S., Lavoie, P., Kunikata, M., Chen, W.Y., et al., 1998. Comparative immunochemical analyses of the developmental expression and distribution of ameloblastin and amelogenin in rat incisors. J. Histochem. Cytochem. 46, 911– 934.
- Nassif, N., Gobeaux, F., Seto, J., Belamie, E., Davidson, P., et al., 2010. Self-assembled collagen-apatite matrix with bone-like hierarchy. Chem. Mater. 22, 3307–3309.
- Nudelman, F., Bomans, P.H.H., George, A., de With, G., Sommerdijk, N.A.J.M., 2012. The role of the amorphous phase on the biomimetic mineralization of collagen. Faraday Discuss. 159, 357–370.
- Nudelman, F., de With, G., Sommerdijk, N.A.J.M., 2011. Cryo-electron tomography: 3-dimensional imaging of soft matter. Soft Matter 7, 17–24.
- Nudelman, F., Pieterse, K., George, A., Bomans, P.H.H., Friedrich, H., et al., 2010. The role of collagen in bone apatite formation in the presence of hydroxyapatite nucleation inhibitors. Nat. Mater. 9, 1004–1009.
- Okamoto, Y., Hidaka, S., 1994. Studies on calcium-phosphate precipitation effects of metal-ions used in dental materials. J. Biomed. Mater. Res. 28, 1403–1410.
- Olszta, M.J., Cheng, X.G., Jee, S.S., Kumar, R., Kim, Y.Y., et al., 2007. Bone structure and formation: a new perspective. Mater. Sci. Eng. R 58, 77–116.
- Omelon, S., Georgiou, J., Henneman, Z. J., Wise, L. M., Sukhu, B., et al., 2009. Control of Vertebrate Skeletal Mineralization by Polyphosphates. Plos One 4.
- Orgel, J.P.R.O., Miller, A., Irving, T.C., Fischetti, R.F., Hammersley, A.P., et al., 2001. The in situ supermolecular structure of type I collagen. Structure 9, 1061–1069.
- Orgel, J.P.R.O., Irving, T.C., Miller, A., Wess, T.J., 2006. Microfibrillar structure of Type I collagen in situ. Proc. Natl. Acad. Sci. USA 103, 9001–9005.
- Papagerakis, P., MacDougall, M., Hotton, D., Bailleul-Forestier, I., Oboeuf, M., et al., 2003. Expression of amelogenin in odontoblasts. Bone 32, 228–240.
- Price, P.A., Toroian, D., Lim, J.E., 2009. Mineralization by inhibitor exclusion: the calcification of collagen with fetuin. J. Biol. Chem. 284, 17092–17101.
- Rey, C., Combes, C., Drouet, C., Glimcher, M.J., 2009. Bone mineral: update on chemical composition and structure. Osteoporos. Int. 20, 1013–1021.
- Roberts, J.E., Bonar, L.C., Griffin, R.G., Glimcher, M.J., 1992. Characterization of very young mineral phases of bone by solid-state P-31 magic angle sampling spinning nuclear-magnetic-resonance and X-ray-diffraction. Calcif. Tissue Int. 50, 42–48.

- Robinson, R.A., Watson, M.L., 1952. Collagen-crystal relationships in bone as seen in the electron microscope. Anat. Rec. 114, 383–409.
- Saito, T., Arsenault, A.L., Yamauchi, M., Kuboki, Y., Crenshaw, M.A., 1997. Mineral induction by immobilized phosphoproteins. Bone 21, 305–311.
- Silver, F.H., Freeman, J.W., Horvath, I., Landis, W.J., 2001. Molecular basis for elastic energy storage in mineralized tendon. Biomacromolecules 2, 750–756.
- Silver, F.H., Landis, W.J., 2011. Deposition of apatite in mineralizing vertebrate extracellular matrices: a model of possible nucleation sites on Type I collagen. Connect. Tissue Res. 52, 242–254.
- Silverman, L., Boskey, A.L., 2004. Diffusion systems for evaluation of biomineralization. Calcif. Tissue Int. 75, 494–501.
- Sodek, J., Ganss, B., McKee, M.D., 2000. Osteopontin. Crit. Rev. Oral Biol. M 11, 279– 303.
- Stetler-Stevenson, W.G., Veis, A., 1983. Bovine dentin phosphophoryn composition and molecular-weight. Biochemistry-Us 22, 4326–4335.
- Termine, J.D., Conn, K.M., Eanes, E.D., Gelman, R.A., 1980. Modulation of Apatite Crystal-Formation and Collagen Fibril Self-Assembly by Phosphoproteins and Calcium. J Dent Res 59, 502–502.
- Termine, J. D., Posner, A. S., 1966. Infrared Analysis of Rat Bone Age Dependency of Amorphous and Crystalline Mineral Fractions. Science 153, 1523-&.
- Termine, J.D., Posner, A.S., 1970. Calcium phosphate formation in-vitro.1. Factors affecting initial phase separation. Arch. Biochem. Biophys. 140, 307–317.
- Thula, T.T., Svedlund, F., Rodriguez, D.E., Podschun, J., Pendi, L., et al., 2011. Mimicking the naostructure of bone: comparison of polymeric processdirecting agents. Polymers 3, 10–35.
- Toroian, D., Lim, J.E., Price, P.A., 2007. The size exclusion characteristics of Type I collagen – implications for the role of noncollagenous bone constituents in mineralization. J. Biol. Chem. 282, 22437–22447.
- Traub, W., Arad, T., Weiner, S., 1989. 3-Dimensional ordered distribution of crystals in Turkey tendon collagen-fibers. Proc. Natl. Acad. Sci. USA 86, 9822–9826.
- Traub, W., Arad, T., Weiner, S., 1992a. Origin of mineral crystal-growth in collagen fibrils. Matrix 12, 251–255.
- Traub, W., Jodaikin, A., Arad, T., Veis, A., Sabsay, B., 1992b. Dentin phosphophoryn binding to collagen fibrils. Matrix 12, 197–201.
- Tye, C.E., Hunter, G.K., Goldberg, H.A., 2005. Identification of the Type I collagenbinding domain of bone sialoprotein and characterization of the mechanism of interaction. J. Biol. Chem. 280, 13487–13492.
- Veis, A., 1997. Collagen fibrillar structure in mineralized and nonmineralized tissues. Curr. Opin. Solid State M 2, 370–378.
- Veis, A., Perry, A., 1967. Phosphoprotein of Dentin Matrix. Biochemistry-Us 6, 2409-&.
- Wang, Y., Azais, T., Robin, M., Vallee, A., Catania, C., et al., 2012. The predominant role of collagen in the nucleation, growth, structure and orientation of bone apatite. Nat. Mater. 11, 724–733.
- Wu, Y.T., Glimcher, M.J., Rey, C., Ackerman, J.L., 1994. A Unique Protonated Phosphate Croup in Bone-Mineral Not Present in Synthetic Calcium Phosphates – Identification by P-31 Solid-State Nmr-Spectroscopy. J. Mol. Biol. 244, 423– 435.
- Zeiger, D.N., Miles, W.C., Eidelman, N., Lin-Gibson, S., 2011. Cooperative calcium phosphate nucleation within collagen fibrils. Langmuir 27, 8263–8268.