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Differences between top-down and bottom-up approaches in mineralizing thick, partially-demineralized collagen scaffolds

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Abstract

Biomaterials exhibit complex hierarchical structures derived from bottom-up self-assembly mechanisms. Type I collagen serves as the building block for mineralized tissues such as bone and dentin. In the present study, 250–300 μm thick, partially-demineralized collagen scaffolds exhibiting a gradient of demineralization from the base to surface were mineralized using a classical top-down approach and a non-classical bottom-up approach. The top-down approach involved epitaxial growth over seed crystallites. The bottom-up approach utilized biomimetic analogs of matrix proteins to stabilize amorphous calcium phosphate nanoprecursors and template apatite nucleation and growth within the collagen matrix. Micro-computed tomography and transmission electron microscopy were employed to examine mineral uptake and apatite arrangement within the mineralized collagen matrix. The top-down approach could only mineralize the base of the partially-demineralized scaffold where remnant seed crystallites were abundant. Minimal mineralization was observed along the surface of the scaffold; extrafibrillar mineralization was predominantly observed. Conversely, the entire partially-demineralized scaffold including apatite-depleted collagen fibrils was mineralized by the bottom-up approach, with evidence of both intrafibrillar and extrafibrillar mineralization. Understanding the different mechanisms involved in these two mineralization approaches is pivotal in adopting the optimum strategy for fabricating novel nanostructured materials in bioengineering research.

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Keywords

biomimetics; bottom-up; collagen; ion-mediated; particle-mediated; mineralization; top-down

Introduction

Nanotechnologies involved in the fabrication of biomaterials may be classified as top-down or bottom-up approaches [1]. The top-down approach starts from a bulk material that incorporates critical nanoscale details. In this approach, a biomaterial is engineered by scaling down a complex entity into its component parts, such as creating small crystals from a bulk mineralized hard tissue via acid-etching. By contrast, the bottom-up approach assembles materials from the nanoscopic scale, such as molecules and atoms, to form larger structures [2]. Examples of the bottom-up approach include self-assembly and molecular patterning [1]. Biomineralization, which involves the spatial regulation of amorphous mineral phases via matrix proteins and organisation of nanoscopic crystalline mineral units into hierarchical structures, represents the perfect example of a bottom-up approach [3,4].

Biominerals found in bone and teeth are carbonated apatites with dimensions that are small enough to fit within the gap zones of type I collagen molecules. This hierarchical arrangement of overlapping platelets can be identified as cross-banded patterns in unstained, non-demineralized ultrastructural sections [5,6]. Partial demineralization of a mineralized collagen matrix by acids derived from bacteria or clastic cells represents an example of a top-down approach in generating seed crystallites [1] as nuclei of heterogeneous nucleation [7]. The orientation of those mineral lattices is determined by the lattice of the seed crystallites [8]. Conventional mineralization strategies often involve the use of metastable calcium and phosphate ion-containing solutions or gels [9,10]. This example of a top-down mineralization approach [3] does not occur by spontaneous nucleation of minerals on the organic matrix but rather by epitaxial growth over existing seed crystallites [11]. Throughout the evolution of biomineralization, matrix proteins play a pivotal role in the regulation of mineral nucleation and growth [12,13]. In the absence of seed crystallites, matrix protein-mineral interactions are responsible for overcoming the thermodynamic barriers in homogeneous nucleation [14].

The precise control of crystal growth at the nanoscale and the creation of natural structures based on bottom-up self-assembly mechanisms have inspired scientists to mimic these non-classical strategies in the fabrication of novel biomaterials [14]. The literature is abound with examples of the use of bottom-up, self-assembly approaches in the fabrication of hybrid nanostructures [15]. A biomimetic mineralization strategy has been developed [16] based on the use of polyanionic molecules to mimic the functions of matrix proteins in biomineralization [17,18]. In this strategy, a polycarboxylic acid-based biomimetic analog is employed as a sequestration agent [19] to stabilize amorphous calcium phosphate (ACP) derived from set Portland cement and simulated body fluid (SBF) in the form of nanoparticles that are moldable enough to infiltrate the water compartments of a collagen fibril. Another phosphorus-based analog mimicking the collagen-binding function of matrix phosphoproteins [20] is used as a template to promote nanoprecursor recruitment to the gap zones of the collagen fibrils, where they nucleate and self-assemble into hierarchically-arranged apatite nanocrystals within the fibril. This example of a particle-mediated [21], bottom-up [4] crystallization strategy differs from the classical top-down crystallization approach in two aspects. Firstly, it recapitulates the progressive dehydration mechanism in natural biomineralization [22] by replacing free and loosely-bound water within the internal compartments of a collagen fibril by apatite crystallites [23]. Secondly, this self-assembly approach proceeds in the absence of apatite seed crystallites and natural matrix

phosphoproteins in a collagen matrix and can be duplicated using reconstituted collagen [24].

The mineral phase in collagenous hard tissues such as bone and dentin is classified as intrafibrillar apatites which are deposited within or immediately adjacent to gap zones of the collagen molecules and extend along the microfibrillar spaces within the fibril; and extrafibrillar apatites which are located within the interstitial spaces separating the collagen fibrils [5,25]. Previous studies have shown that intrafibrillar apatites play a significant role in the mechanical properties of mineralized tissues [26–28]. Thus, biomimetic mineralization must recapitulate both the dimension and hierarchical arrangement of apatites present in natural mineralized tissues [29,30].

Noncollagenous matrix proteins which serve as promoters or inhibitors of crystal nucleation or growth play an important role in intrafibrillar mineralization [31]. In the absence of biomimetic analogs of those matrix proteins, there should be no intrafibrillar apatite deposition using the top-down mineralization approach. Thus, the objective in the present study was to examine the difference between the top-down and the bottom-up mineralization approaches in mineralizing thick, partially-demineralized collagen scaffolds. To quantitatively assess the changes of mineral density before and after mineralization, micro-computed tomography (micro-CT) was used to examine the mineralized collagen scaffold non-destructively in three dimensions. Although micro-CT is valuable for longitudinal assessment of mineral uptake, it cannot delineate between intrafibrillar and extrafibrillar apatite deposition within the collagen fibrils. Thus, transmission electron microscopy (TEM) was used to examine the dimension and hierarchy of apatite deposition within the mineralized collagen matrix. The null hypothesis tested was that there is no difference in the extent and quality of mineralization achieved via the top-down vs the bottom-up approaches in thick, partially-demineralized collagen scaffolds.

2. Material and methods

2.1. Partially-demineralized collagen scaffolds

Human dentin was used as a model of a mineralized type I collagen scaffold for two reasons. First, type I collagen in human dentin is highly cross-linked [32] which minimizes their degradation during long-term mineralization procedures. Secondly, dentin is surrounded by a highly radiopaque peripheral rim of enamel, which serves as a natural mineral reference in micro-CT.

Thirty extracted non-carious human third molars were obtained with patient informed consent under a protocol approved by the Human Assurance Committee of the Medical College of Georgia. A 1-mm thick dentin disk was prepared by making two parallel cuts perpendicular to the longitudinal axis of each tooth using a low-speed Isomet saw (Buehler, Lake Bluff, IL, USA) under water cooling. The surface for creating the partially-demineralized collagen scaffold was polished with 1200-grit silicon carbide paper under running water. The other surfaces, together with the circumferential enamel rim and 1 mm of the peripheral dentin of the polished surface (to serve as mineral references) was painted with two coats of an acid-resistant varnish. A 250–300 μm thick layer of partially-demineralized collagen scaffold was created on the uncoated surface by pH-cycling procedure [33] to mimic caries-affected dentin below the excavated surface [34]. After pH-cycling, each disk was sectioned transversely under running water to create a 3-mm wide slab (Fig.1) containing the partially-demineralized collagen scaffold.

2.2 Micro-CT

The demineralization depth and mineral loss from each slab was characterized non-destructively under water using a SkyScan 1174 micro-CT scanner (Micro Photonics, Allentown, PA, USA). Scanning was performed with a spatial resolution of 6.28 μm by placing the specimen into a custom-prepared positioning jig. Projection images were collected at 50 kV and 800 μA using 360° rotation, with 0.6° and 3 sec exposure time per projection step. Averaging of 30 frames was used for each step to improve the signal-to-noise ratio. As the X-ray source is polychromatic, a 1-mm thick aluminum filter was placed in front of the detector to remove low-energy radiation. Flat-field correction, geometric correction and random movement during the acquisition phase, and ring artefact correction during the reconstruction phase were performed to reduce ring artifacts. A 20% beam hardening correction was employed during the reconstruction phase using the NRecon software (Version 1.6.2, Skyscan 1174). Details of the micro-CT scanning and data extraction protocols are included in Section I of the Supplementary Material.

Demineralization depth (μm) and the integrated mineral loss from the partially-demineralized collagen scaffold ($\text{vol}\%.\mu\text{m}$) were recorded as the baseline data for evaluating the efficacy of the two mineralization approaches. Demineralization depth was taken to be the depth where the relative mineral content was 95% of that of the underlying unaltered mineralized tissue. The integrated mineral loss parameter referred to the cumulative mineral loss from the surface of the partially-demineralized collagen scaffold to the point where the relative mineral content was 95% of the underlying unaltered mineralized tissue [35].

2.3. Mineralization media

2.3.1 Top-down mineralization approach—A medium commonly employed in dentistry for mineralization of artificial caries [36] was adopted for this approach. The medium consisted of 1.5 mM CaCl_2 , 0.9 mM KH_2PO_4 , 130 mM KCl, 20 mM HEPES and with 5 mM NaN_3 added to prevent bacterial growth. The pH was adjusted to 7.0 with 1M KOH. The rationale for using this classical medium was that it has been well-reported in the literature for remineralization of caries-affected dentin. As this medium is not appropriate for the biomimetic mineralization technique described below, a more appropriate medium was utilized for that purpose.

2.3.2 Bottom-up mineralization approach—A modified simulated body fluid (SBF) was prepared by dissolving 136.8 mM NaCl, 4.2 mM NaHCO_3 , 3 mM KCl, 1 mM $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 1.5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 2.5 mM CaCl_2 and 0.5 mM Na_2SO_4 , 5 mM NaN_3 in 0.5 M Sørensen's glycine buffer and adjusted to pH 9.0. The SBF acts as the phosphate source. The glycine buffer (pK_a 9.78; effective pH range 8.8–10.6) facilitated direct transformation of amorphous calcium phosphate (ACP) into apatite at $\text{pH} > 9.25$ without passing through an intermediate octacalcium phosphate phase [37]. Set Type I white Portland cement (Lehigh Cement Co., Allentown, PA, USA) was used as the source of sustained calcium and hydroxyl ions release for initial formation of ACP in the presence of phosphate ions derived from the SBF [16].

The aforementioned SBF was used as the negative control with which the two mineralization mechanisms were compared. For the bottom-up mineralization approach, 500 $\mu\text{g/mL}$ polyacrylic acid (Sigma-Aldrich, St Louis, IL, USA) and 200 $\mu\text{g/mL}$ of polyvinylphosphonic acid prepared from free radical polymerization of vinylphosphonic acid [24] were added to the SBF as the respective sequestration and templating analogs of dentin matrix proteins [16].

2.4. Mineralization of thick, partially-demineralized collagen scaffolds

Based on the baseline micro-CT data, 24 slabs were found to have 250–300 μm thick layers of partially-demineralized collagen scaffolds. Specimens with scaffolds that were thinner and thicker than this range were rejected and used for a different purpose (described in the Supplementary Materials). Two slabs with thicker lesions were designated as baseline specimens and processed for light and transmission electron microscopy. The 24 slabs were randomly divided into three groups ($N=8$): 1) top-down approach; 2) bottom-up approach; and 3) negative control.

For the top-down approach, each slab was individually placed inside a glass scintillation vial containing 15 mL of the corresponding mineralization medium. For the bottom-up approach, each slab was placed inside a glass scintillation vial with the surface of the partially-demineralized slab in contact with a set Portland cement block (*ca.* 1 g). The vial was filled with 15 mL of the biomimetic analog-containing SBF. A similar procedure was adopted for the negative control, with the exception that the specimen was immersed in 15 mL of SBF without sequestration and templating analogs. The pH was monitored daily to ensure that there was enough calcium ions released from the Portland cement blocks.

Specimens from the three groups were incubated at 37°C for 4 months, with the respective medium replaced twice per month. Each slab was retrieved at monthly intervals (i.e. 1, 2, 3 and 4 months), rinsed briefly with water to remove loose surface precipitates, inserted into the corresponding micro-CT positioning jig, covered with pH-adjusted deionized water (pH 7.0) and scanned with the micro-CT. After scanning, each slab was removed from the positioning jig and returned to the corresponding immersion medium for continuous mineralization.

2.5 Statistical analyses

Demineralization depths and integrated mineral loss (IML) at baseline and after 4 months of mineralization in the three groups were analyzed using two-way repeated measures ANOVAs, with mineralization approach as the independent variable and time period as the repeating factor. As the normality assumption of the data obtained for demineralization depths appeared to be violated, the data were expressed as least square means and their common standard error prior to analysis. As the IML data were normally-distributed (Shapiro-Wilk test) and homoscedastic (Levene test), they were analyzed without further transformation. The mineralization efficacy $[(\text{IML}_{\text{baseline}} - \text{IML}_{4 \text{ months}})/\text{IML}_{\text{baseline}} * 100\%]$ of the control and experimental groups were analyzed using one-way ANOVA as the data were normally distributed and homoscedastic. Pair-wise comparisons were performed after the respective ANOVA analysis using the Tukey-Kramer test. Statistical significance for all analyzes were preset at $\alpha = 0.05$.

As major regional differences between the top-down and bottom-up mineralization approaches were expected to occur along the surface 100 μm of a 250–300 μm thick partially-demineralized collagen scaffold, changes in the relative mineral content along the surface 25 μm , 50 μm , 75 μm and 100 μm levels of the scaffold in the control and experimental groups after 4 months of mineralization were further analyzed. For each specimen, the normalized mineral content determined at baseline from the respective region was subtracted from that determined from the corresponding region after 4 months. As the normality assumption of the data appeared to be violated, they were expressed as least square means and their common standard error and analyzed using two-way ANOVA (mineralization approach as the independent variable and subsurface level as the repeating factor) and Tukey-Kramer multiple comparison test at $\alpha = 0.05$.

2.6 Light Microscopy (LM)

Upon completion of the micro-CT scans, the 4-month old slabs were fixed in Karnovsky's fixative, post-fixed in 1% osmium tetroxide, dehydrated in an ascending ethanol series (50–100%), immersed in propylene oxide as a transitional fluid and embedded in epoxy resin. Undecalcified 1500 nm epoxy-embedded thick sections were prepared with a diamond knife using an ultramicrotome. The sections were stained with Goldner's trichrome, a bone stain, using a modified technique for staining undecalcified resin-embedded sections without dissolving the embedding medium [38]. This differential staining technique allowed discrimination between mineralized tissues, which stained green and exposed collagen in immature or partially-demineralized hard tissues, which stained red. Stained sections were examined using an incandescent light microscope (BX 51, Olympus America, Inc., Melville, NY, USA).

2.7 TEM

Undecalcified 250 nm thick sections of the aforementioned epoxy resin-embedded specimens were prepared for examining the overall extent of mineralization. The sectioning area of the resin blocks was then reduced for preparation of 100 nm thick sections for ultrastructural examination of the manifestations of intrafibrillar and extrafibrillar mineralization of collagen. These sections were examined unstained using a JEM-1230 TEM (JEOL, Tokyo, Japan) at 110 kV. Selected area electron diffraction was performed using centred dark-field imaging. The distribution of Ca and P along selected locations of the TEM thick sections was also examined by performing energy-dispersive X-ray (EDX) analysis line scans using a Tecnai G2-20 TEM equipped with a scanning transmission attachment (FEI Co., Hillsboro, OR, USA).

3. Results

Changes in overall mineral profile over a 4-month period in representative specimens from the control and experimental groups are shown in Fig. 1. There was minimal mineralization in the control group, which occurred predominantly along the surface of the 250–300 μm thick partially-demineralized collagen scaffold (Fig. 1A). Some surface precipitation on top of the scaffold surface could be identified. For the top-down approach, mineralization occurred predominantly along the base of the partially-demineralized scaffold where remnant seed crystallites were located before the specimen was subjected to mineralization (Fig. 1B). There was minimal mineralization of the surface of the collagen scaffold with this approach. The major difference between the top-down and the bottom-up mineralization approaches could be seen along the surface 100 μm of the collagen scaffold (Fig. 1C). For the bottom-up approach, mineralization of this region continued to occur each month. Although mineralization was incomplete in this region by the end of the fourth month, the relative mineral content was much higher than the corresponding region associated with the top-down approach. Mineral precipitation on top of the scaffold surface could also be identified with the bottom-up approach.

Changes in the demineralization depth before and after the 4-month period are shown in Fig. 2A. Significant differences could be detected among the three mineralization approaches ($p < 0.001$) and between the two time periods ($p < 0.001$). The interaction between these two factors was also significant ($p < 0.001$). Multiple comparisons among the different mineralization approaches indicated no difference in the three approaches before mineralization ($p > 0.05$). After mineralization, a significant difference was observed between the control and bottom-up approach ($p < 0.001$). Significant differences were also identified between the control and the top-down approach ($p < 0.001$) and between the top-down approach and the bottom-up approach ($p < 0.001$). For the control group, the

difference in demineralization depth before and after mineralization was not statistically significant ($p = 0.541$). For the two experimental groups, the differences in demineralization depths before and after mineralization were statistically significant ($p < 0.001$ for both groups).

Changes in integrated mineral loss (IML) before and after the 4-month period are shown in Fig. 2B. Significant differences could be detected among the three approaches ($p < 0.001$), between the two time periods ($p < 0.001$) and in the interaction between these two factors ($p < 0.001$). Multiple comparison procedures revealed that there were no differences in the IML among the control and experimental groups before mineralization ($p > 0.05$). After mineralization, a significant difference was seen between the control and bottom-up approach ($p < 0.001$). Significant differences were also identified between the control and top-down approach ($p < 0.001$) and between the top-down approach and the bottom-up approach ($p < 0.001$). For each of the three groups, differences in IML before and after mineralization were statistically significant ($p < 0.001$ for all groups). The difference in the extent of mineralization among the three groups was also significant ($p < 0.001$) (Fig. 2C). Multiple comparison procedures revealed significant differences between the bottom-up approach and the control ($p < 0.001$), between the bottom-up approach and the top-down approach ($p < 0.001$) and between the top-down approach and the control ($p < 0.001$).

Statistical analysis of the changes in regional increase in mineral content of the collagen scaffolds revealed significant differences among the three groups ($p < 0.001$), the four subsurface levels ($p < 0.001$) and the interaction of these two factors ($p < 0.001$). For the repeated factor “subsurface level”, although visual differences could be observed from the overall mineral profiles (Fig. 1) and morphologically, pair-wise multiple comparison failed to reveal significant differences at any of the four subsurface levels ($p > 0.05$). The same applied to the results obtained for the bottom-up approach ($p > 0.05$; see also Fig. 6). Regional differences of the six pair-wise subsurface level comparisons associated with the top-down approach were all significantly different ($p < 0.001$ for all comparisons). At the 50 μm , 75 μm and 100 μm levels, the three pair-wise comparisons among groups were all statistically significant for each respective subsurface level ($p < 0.001$ for all comparisons). At the 25 μm level, significant differences could be seen between the bottom-up and top-down approaches ($p < 0.001$) and between the bottom-up and the control ($p < 0.001$). However, there was no difference in the increase in mineral content between the control and the top-down approach ($p = 0.903$).

Figure 3 represents characterization of the partially-demineralized collagen matrix after pH cycling. Light microscopy (Fig. 3A) revealed a 250 μm thick, partially-demineralized layer with exposed collagen (stained red). The mineralized tissue base was stained green. Beneath the exposed collagen zone and the mineralized base was a pale green-stained zone (ca. 100 μm thick) in which the minerals had not been sufficiently removed to permit penetration of the Orange G component of the Goldner's trichrome stain. Thus, the total thickness of the partially-demineralized zone was 350 μm as revealed by the unstained TEM thick section (Fig. 3B). Changes in Ca and P intensities from the fully mineralized base through the partially-demineralized zone are shown in the EDX line-scan profiles in Fig. 3C. Thin sections prepared from the same specimen revealed sparsely-distributed apatite seed crystallites with reduced dimensions along the surface of the partially-demineralized zone (Fig. 3D). Within the body of this zone, apatite seed crystallites were reduced in dimensions but were considerably larger than those present along the surface region (Fig. 3E). Unlike the fully mineralized base of the section (Fig. 3F), intrafibrillar seed crystallites were scanty in most of the collagen fibrils within the body of the partially-demineralized zone. Those collagen fibrils were supported predominantly by extrafibrillar minerals (Fig. 3E).

In the control group, a thin surface mineralized layer was evident from the LM (Fig. 4A), TEM thick section (Fig. 4B), the corresponding EDX line scans (Fig. 4C) and the TEM thin section (Fig. 4D) after 4 months of immersion in the control medium. Nevertheless, the bulk of the partially-demineralized scaffold was poorly mineralized and many collagen fibrils along the surface (Fig. 4E) and body (Fig. 4F) of the scaffold were devoid of intrafibrillar crystallites. In the top-down approach group, there was a decrease in the demineralization depth as mineralization occurred from the base of the original partially-demineralized zone toward the surface. However the surface 100 μm of this zone contained exposed collagen (Fig. 5A) and was poorly mineralized (Figs. 5B and 5C). Although evidence of mineralization of the seed crystallites was evident along the surface (Fig. 5D) and body of the collagen scaffold, this process was achieved via epitaxial growth of seed crystallites along the extrafibrillar compartments, with many crystallites being more than 100 nm long along their C-axis (Fig. 5F). Along the subsurface of the mineralized scaffold, no evidence of intrafibrillar mineralization could be observed (Fig. 5E).

Exposed collagen was not evident from the collagen scaffolds after 4 months of mineralization with the bottom-up approach (Fig. 6A). The surface of the scaffold appeared to be almost fully mineralized at the thick section level (Fig. 6B) and there was only a slight drop in Ca and P intensities along the surface 100 μm of the scaffold (Fig. 6C). At the thin section level, streaks of less heavily mineralized collagen could be identified at the mineralized scaffold surface, along with the presence of surface mineral precipitations (Fig. 6D). At high magnification, these less electron-dense streaks consisted of sparser and less organized minerals. Selected area electron diffraction of the crystallites exhibited ring patterns corresponding to the 002 plane and the three very closely approximated 211, 112 and 300 planes of poorly crystalline apatite (Fig. 6E). Generally, minerals were more hierarchically organized within the body of the mineralized scaffold. However, there were regions with randomly-deposited hexagonal crystallites that were 10–40 nm along their C-axis (Fig. 6F).

4. Discussion

From a crystallization perspective, there are two mechanisms involved in the growth of a crystal: the classical ion-mediated and the non-classical particle-mediated crystallization pathways [39]. The former pathway utilizes primary building blocks such as calcium and phosphate ions. Conversely, the particle-mediated crystallization pathway involves self-assembly of metastable or amorphous precursor particles and their subsequent mesoscopic transformation [40]. Thus, two different mineralizing solutions were employed respectively for the two approaches examined in the present study.

Results from the micro-CT mineral profiles showed that there were significant differences between the top-down and bottom-up approaches, both in the location of mineral deposition and the final mineral uptake. The partially-demineralized collagen scaffolds exhibited a gradient of demineralization from the surface to the base of the scaffold, with approximately 8–8.5 vol% mineral content along the surface 25 μm of the scaffold (data not shown). These minerals were almost exclusively located in the extrafibrillar compartments of the collagen matrix. In the top-down approach, the overall increase in mineralization was $56.4 \pm 6.9\%$ after 4 months (Fig. 2C). However, mineralization occurred predominantly along the base of the scaffold that contained more seed crystallites (Fig. 3E of the control lesion before mineralization). This result is consistent with the findings from a previous study [41], in that the mineral distribution of the demineralization surface layer influences the characteristics of subsequent mineralization, including the location and density of mineral deposition. Conversely, in the bottom-up approach, the overall increase in mineralization was $93.8 \pm 3.1\%$ after 4 months (Fig. 2C). It is important to note that these results were achieved in the

absence of fluoride; the latter has never been implicated as a functional motif in biomineralization [13]. Minerals were deposited over the entire partially-demineralized scaffolds. Even along the scaffold surface where very few seed crystallites were present, there was more extensive mineralization compared with the top-down approach, although the density and hierarchy of those intrafibrillar apatite crystallites (Fig. 6E) were not as dense or as highly-ordered as those present in the underlying unaltered mineralized base (Fig. 3F of the control lesion before mineralization). This probably explained why the overall normalized mineral density along the surface of the mineralized scaffolds in the bottom-up group (Fig. 1C) did not attain the normalized 50 vol% of the original mineralized dentin. The presence of seed crystallites could have sterically prevented a more highly-ordered crystallite arrangement to be achieved. Nevertheless, such extensive mineralization of a thick, partially-demineralized collagen scaffold has not been reported, particularly with adjunctive evidence of intrafibrillar apatite growth. For example, microradiography results reporting the success of mineralization of artificial carious lesions in dentistry were not supported by ultrastructural data showing evidence of intrafibrillar mineralization of the collagen matrix [36,41,42].

The baseline TEM images clearly indicated that intrafibrillar minerals were scanty especially toward the surface of the scaffold. Even within the body of partially-demineralized scaffold, the collagen matrix was supported predominantly by extrafibrillar minerals. Thus, we hypothesize that the intrafibrillar minerals etch faster than the extrafibrillar minerals during pH-cycling. This is contrary to the result reported by Balooch *et al.* [28]. This may be attributed to the different methods of demineralization employed in the two studies. With the top-down approach, there was no mineral deposition in the intrafibrillar compartments along the subsurface of the mineralized scaffold. However, in the base of the mineralized scaffold, the newly precipitated mineral phase could not be distinguished from the existing seed crystallites by conventional TEM [43]. Thus, a thick, fully-demineralized collagen scaffold was created to confirm that in the absence of biomimetic analogs, no additional intrafibrillar mineralization could be observed using the top-down approach (Supplementary Material – Section II). Conversely, fully-demineralized scaffolds mineralized with the bottom-up approach exhibited intrafibrillar mineralization of the collagen matrix within the surface 30–40 μm of those scaffolds (Supplementary Material – Section II). The increase in mineral density and reduction of demineralization depth via the top-down approach (Figs. 5A, 5B and 5C) may be attributed to the epitaxial growth of remnant seed crystallites, with the latter acting as templates for the deposition of calcium and phosphate ions [11]. Although direct evidence of epitaxial growth was absent from the present study, many of the crystallites deposited within the body of the mineralized scaffold were longer than 100 nm along their C-axis (Fig. 5F). At this length scale, it is unlikely that those crystallites can be fitted intrafibrillarly into the gap zones of the collagen molecules (*ca.* 40 nm wide). Although the micro-CT results indicated increase in radiopacity of the scaffolds mineralized using the top-down approach, the overall lack of intrafibrillar mineralization in this group challenges the use of mineral density alone as the end-point for assessing the success or failure of mineralization [27]. This is because heterogeneous precipitation of extrafibrillar apatites contributes minimally to the mechanical properties of mineralized collagen fibrils [26,44]. Nanodynamic mechanical analysis of the mineralized scaffolds should be performed in future studies to compare the dynamic mechanical properties of collagen matrices mineralized with the top-down and the bottom-up approaches.

Amorphous calcium phosphate (ACP) can be formed by the use of type I white Portland cement as the source of sustained calcium and hydroxyl ions and SBF as the source of phosphate ions [24]. In the absence of a sequestering analog such as polyacrylic acid or polyaspartic acid, large ACP globules up to 2 μm in diameter were initially formed, which

were then transformed to large apatite crystallites that are 200–250 nm along their c-axis [16]. Theoretically, these large apatite crystallites can not fit in the gap zones between collagen molecules. In the control group of the present study, although the bulk of the scaffold was poorly mineralized, there was evidence of surface mineralization after 4 months. These are not surface precipitations as the use of a model containing dentinal tubules clearly indicated mineralization of the intertubular dentin adjacent to the tubules, as complementary illustrated by the LM, TEM and EDX results (Figs. 4A, 4B and 4C). This mineralization may be induced by remaining non-collagenous proteins in the partially-demineralized scaffold [45]. Some of these remaining non-collagenous proteins may act as sequestration agents for controlling the dimension of ACPs, and as templates for guiding the nucleation of the stabilized ACPs deposited along the collagen fibrils. Nevertheless, exposed collagen was identified in the underlying scaffold (Fig. 4A). As the mineralization mechanisms in the control and the top-down groups were incapable of generating enough intrafibrillar minerals to mask the exposed collagen, the observation of almost fully mineralized scaffolds with the bottom-up approach could not be solely attributed to the presence of remnant noncollagenous proteins. In the present study, we did not use Ca and P ratios derived from TEM-EDX to determine the type of calcium phosphate phase produced after mineralization as quantitative analysis of EDX data derived from biologic specimens is not accurate in the absence of appropriate standards for ZAF correction [46]. We employed selected area electron diffraction of the TEM sections instead. Analysis of the *hkl* indices from ring patterns obtained from the mineralized scaffolds indicated that the newly formed minerals were apatite.

It is prudent to mention that exposed collagen unprotected by intrafibrillar minerals can undergo degradation by endogenous matrix metalloproteinases [47]. The polyvinylphosphonic acid employed as the templating analog in the bottom-up approach is an effective inhibitor of matrix metalloproteinases [48]. This could have protected the seed crystallite-sparse collagen fibrils along the superficial part of the scaffold from degradation before they could be mineralized.

5. Conclusions

The TEM findings in the present study supported the results derived from quantitative assessment of mineral densities by micro-CT. Such a complementary approach has seldom been utilized in previous studies. Based on these results, the null hypothesis that there is no difference in the extent and quality of mineralization achieved via the top-down vs the bottom-up approaches in thick, partially-demineralized collagen scaffolds has to be rejected. The mechanisms involved in the classical top-down and non-classical bottom-up mineralization approaches appear to be different. The former probably relies on epitaxial growth of seed crystallites within the collagen fibrils. The latter involves transformation of ACP nanoparticles into apatite crystallites in presence of biomimetic analogs.

The biomimetic bottom-up mineralization strategy based on the use of calcium and hydroxyl ion-releasing set Portland cement and phosphate-containing SBF in the presence of sequestration and templating analogs of matrix proteins was able to mineralize thick, partially-demineralized collagen scaffolds, with evidence of both intrafibrillar and extrafibrillar mineralization. However, in the absence of biomimetic analogs in the top-down mineralization approach, there was no evidence of intrafibrillar mineralization in the partially-demineralized collagen fibrils. Interactions between type I collagen fibrils and biomimetic analogs in the bottom-up approach produce mineralized collagen fibrils that approximate the dimension and hierarchy of the apatites found in natural mineralized tissues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Silva GA. Neuroscience nanotechnology: progress, opportunities and challenges. *Nat Rev Neurosci* 2006;7:65–74. [PubMed: 16371951]
2. Zhang S. Fabrication of novel biomaterials through molecular self-assembly. *Nat Biotechnol* 2003;21:1171–1178. [PubMed: 14520402]
3. Niederberger M, Cölfen H. Oriented attachment and mesocrystals: non-classical crystallization mechanisms based on nanoparticle assembly. *Phys Chem Chem Phys* 2006;8:3271–3287. [PubMed: 16835675]
4. Wong TS, Brough B, Ho CM. Creation of functional micro/nano systems through top-down and bottom-up approaches. *Mol Cell Biomech* 2009;6:1–55. [PubMed: 19382535]
5. Traub W, Arad T, Weiner S. Three-dimensional ordered distribution of crystals in turkey tendon collagen fibers. *Proc Natl Acad Sci USA* 1989;86:9822–9826. [PubMed: 2602376]
6. Weiner S, Wagner HD. The material bone: structure-mechanical function relations. *Ann Rev Mater Sci* 1998;28:271–298.
7. Jiang H, Liu XY. Principles of mimicking and engineering the self-organized structure of hard tissues. *J Biol Chem* 2004;279:41286–41293. [PubMed: 15192103]
8. Nelson DG, Barry JC. High resolution electron microscopy of nonstoichiometric apatite crystals. *Anat Rec* 1989;224:265–276. [PubMed: 2672890]
9. Klont B, ten Cate JM. Remineralization of bovine incisor root lesions in vitro: the role of the collagenous matrix. *Caries Res* 1991;25:39–45. [PubMed: 2070381]
10. Silverman L, Boskey AL. Diffusion systems for evaluation of biomineralization. *Calcif Tissue Int* 2004;75:494–501. [PubMed: 15455184]
11. Koutsoukos PG, Nancollas GH. Crystal growth of calcium phosphates – epitaxial considerations. *J Crystal Growth* 1981;53:10–19.
12. Mann S. The chemistry of form. *Angew Chem Int Ed Engl* 2000;39:3393–3406.
13. Boskey AL. Biomineralization: an overview. *Connect Tissue Res* 2003;44(Suppl 1):5–9. [PubMed: 12952166]
14. Xu A-W, Ma Y, Cölfen H. Biomimetic mineralization. *J Mater Chem* 2007;17:415–449.
15. Mann S. Self-assembly and transformation of hybrid nano-objects and nanostructures under equilibrium and non-equilibrium conditions. *Nat Mater* 2009;8:781–792. [PubMed: 19734883]
16. Tay FR, Pashley DH. Guided tissue remineralisation of partially demineralised human dentine. *Biomaterials* 2008;29:1127–1137. [PubMed: 18022228]
17. Gajjeraman S, Narayanan K, Hao J, Qin C, George A. Matrix macromolecules in hard tissues control the nucleation and hierarchical assembly of hydroxyapatite. *J Biol Chem* 2007;282:1193–1204. [PubMed: 17052984]
18. Gericke A, Qin C, Sun Y, Redfern R, Redfern D, Fujimoto Y, et al. Different forms of DMP1 play distinct roles in mineralization. *J Dent Res* 2010;89:355–359. [PubMed: 20200415]
19. Tsortos A, Nancollas GH. The role of polycarboxylic acids in calcium phosphate mineralization. *J Colloid Interface Sci* 2002;250:159–167. [PubMed: 16290647]
20. George A, Veis A. Phosphorylated proteins and control over apatite nucleation, crystal growth, and inhibition. *Chem Rev* 2008;108:4670–4693. [PubMed: 18831570]

21. Gower LB. Biomimetic model systems for investigating the amorphous precursor pathway and its role in biomineralization. *Chem Rev* 2008;108:4551–4627. [PubMed: 19006398]
22. Wehrli FW, Fernández-Seara MA. Nuclear magnetic resonance studies of bone water. *Ann Biomed Eng* 2005;33:79–86. [PubMed: 15709708]
23. Kim YK, Mai S, Mazzoni A, Liu Y, Tezvergil-Mutluay A, Takahashi K, et al. Biomimetic remineralization as a progressive dehydration mechanism of collagen matrices - Implications in the aging of resin-dentin bonds. *Acta Biomater* 2010;6:3729–3739. [PubMed: 20304110]
24. Kim YK, Gu L-S, Bryan TE, Kim JR, Chen L, Liu Y, et al. Mineralisation of reconstituted collagen using polyvinylphosphonic acid/polyacrylic acid templating matrix protein analogues in the presence of calcium, phosphate and hydroxyl ions. *Biomaterials* 2010;31:6618–6627. [PubMed: 20621767]
25. Landis WJ, Hodgins KJ, Arena J, Song MJ, McEwen BF. Structural relations between collagen and mineral in bone as determined by high voltage electron microscopic tomography. *Microsc Res Tech* 1996;33:192–202. [PubMed: 8845518]
26. Jäger I, Fratzl P. Mineralized collagen fibrils: a mechanical model with a staggered arrangement of mineral particles. *Biophys J* 2000;79:1737–1746. [PubMed: 11023882]
27. Kinney JH, Habelitz S, Marshall SJ, Marshall GW. The importance of intrafibrillar mineralization of collagen on the mechanical properties of dentin. *J Dent Res* 2003;82:957–961. [PubMed: 14630894]
28. Balooch M, Habelitz S, Kinney JH, Marshall SJ, Marshall GW. Mechanical properties of mineralized collagen fibrils as influenced by demineralization. *J Struct Biol* 2008;162:404–410. [PubMed: 18467127]
29. Sanchez C, Arribart H, Guille MM. Biomimetism and bioinspiration as tools for the design of innovative materials and systems. *Nat Mater* 2005;4:277–288. [PubMed: 15875305]
30. Weiner S. Biomineralization: A structural perspective. *J Struct Biol* 2008;163:229–234. [PubMed: 18359639]
31. Wiesmann HP, Meyer U, Plate U, Höhling HJ. Aspects of collagen mineralization in hard tissue formation. *Int Rev Cytol* 2005;242:121–156. [PubMed: 15598468]
32. Kuboki Y, Mechanic GL. Comparative molecular distribution of cross-link in bone and dentin collagen. Structure-function relationships. *Calcif Tissue Int* 1982;34:306–308. [PubMed: 6809294]
33. ten Cate JM, Buijs MJ, Damen JJ. pH-cycling of enamel and dentin lesions in the presence of low concentrations of fluoride. *Eur J Oral Sci* 1995;103:362–367. [PubMed: 8747671]
34. Neves Ade A, Coutinho E, Vivan Cardoso M, Jaecques SV, Van Meerbeek B. Micro-CT based quantitative evaluation of caries excavation. *Dent Mater* 2010;26:579–88. [PubMed: 20347481]
35. Gelhard TBFM, Arends J. Microradiography of *in vivo* remineralized lesions in human enamel. *J Biol Buccale* 1984;12:59–65. [PubMed: 6584426]
36. ten Cate JM. Remineralization of caries lesions extending into dentin. *J Dent Res* 2001;80:1407–1411. [PubMed: 11437209]
37. Eanes ED. Amorphous calcium phosphate. *Monogr Oral Sci* 2001;18:130–147. [PubMed: 11758445]
38. Gruber HE. Adaptations of Goldner's Masson trichrome stain for the study of undecalcified plastic embedded bone. *Biotech Histochem* 1992;67:30–34. [PubMed: 1617000]
39. Meldrum FC, Cölfen H. Controlling mineral morphologies and structures in biological and synthetic systems. *Chem Rev* 2008;108:4332–4432. [PubMed: 19006397]
40. Cölfen H, Antonietti M. Mesocrystals: inorganic superstructures made by highly parallel crystallization and controlled alignment. *Angew Chem Int Ed Engl* 2005;44:5576–5591. [PubMed: 16035009]
41. Kawasaki K, Ruben J, Tsuda H, Huysmans MC, Takagi O. Relationship between mineral distributions in dentin lesions and subsequent remineralization *in vitro*. *Caries Res* 2000;34:395–403. [PubMed: 11014906]
42. Preston KP, Smith PW, Higham SM. The influence of varying fluoride concentrations on *in vitro* remineralisation of artificial dentinal lesions with differing lesion morphologies. *Arch Oral Biol* 2008;53:20–26. [PubMed: 17920030]

43. Kim J, Arola DD, Gu L, Kim YK, Mai S, Liu Y, et al. Functional biomimetic analogs help remineralize apatite-depleted demineralized resin-infiltrated dentin via a bottom-up approach. *Acta Biomater* 2010;6:2740–2750. [PubMed: 20045745]
44. Bertassoni LE, Habelitz S, Kinney JH, Marshall SJ, Marshall GW Jr. Biomechanical perspective on the remineralization of dentin. *Caries Res* 2009;43:70–77. [PubMed: 19208991]
45. Saito T, Yamauchi M, Crenshaw MA. Apatite induction by insoluble dentin collagen. *J Bone Miner Res* 1998;13:265–270. [PubMed: 9495520]
46. Vajda EG, Skedros JG, Bloebaum RD. Errors in quantitative backscattered electron analysis of bone standardized by energy-dispersive x-ray spectrometry. *Scanning* 1998;20:527–535. [PubMed: 9857528]
47. Pashley DH, Tay FR, Yiu C, Hashimoto M, Breschi L, Carvalho RM, et al. Collagen degradation by host-derived enzymes during aging. *J Dent Res* 2004;83:216–221. [PubMed: 14981122]
48. Tezvergil-Mutluay A, Agee KA, Hoshika T, Tay FR, Pashley DH. The inhibitory effect of polyvinylphosphonic acid on functional MMP activities in human demineralized dentin. *Acta Biomater* 2010;6:4136–4142. [PubMed: 20580949]

Fig.1. Changes in overall mineral profile over time. A representative example each is shown for **A.** the control group, **B.** the top-down mineralization approach, and **C.** the bottom-up mineralization approach. Difference in mineral profile along the surface 100 μm of the collagen scaffold is highlighted in gray.

Fig.2.

A. Changes in the demineralization depths after 4 months of mineralization. Values are least square means $\pm$ common standard error. For the baseline subgroups, columns with the same upper case letters are not significantly different ($p > 0.05$). For the 4 months subgroups, columns with different lower case letters are significantly different ($p < 0.05$). For each mineralization group, time periods connected by a black horizontal bar are significantly different ($p < 0.05$). **B.** Changes in integrated mineral loss (IML) at baseline and after 4 months. Values are means $\pm$ standard deviations. Labels of statistical significance were the same as described in Fig. 3A. **C.** Differences in the mineralization efficacy $[(\text{IML}_{\text{baseline}} - \text{IML}_{4 \text{ months}})/\text{IML}_{\text{baseline}} \times 100\%]$ among the control and experimental groups. Values are means $\pm$ standard deviations. Groups with different upper case letters are significantly different ($p < 0.05$). **D.** Changes in relative mineral content (i.e. 4 months – baseline) at different subsurface levels of the partially-demineralized collagen scaffolds in the three groups. Values are least square means $\pm$ common standard error. For the control, top-down and bottom-up subgroups, columns with different lower case letters, upper case letters and numerical labels, respectively, are significantly different ($p < 0.05$). For each level, groups connected by a crossed horizontal bar are not significantly different ($p > 0.05$).

Fig.3.

Characterization of a partially-demineralized collagen scaffold created after pH cycling (baseline). **A.** Light microscopy. Mineralized collagen was stained green. Note the presence of a thick layer of exposed collagen (red). **B.** Unstained TEM thick section. “D”, “E” and “F” represent the regions from which Figs. 3D, 3E and 3F were taken from a subsequently prepared thin section of the same specimen. **C.** TEM-EDX showing the distribution of Ca and P along the white horizontal line in Fig. 3B. **D.** Minerals are sparse and very much reduced in size along the surface of the scaffold (i.e. top-down reduction into seed crystallites). **E.** Intrafibrillar minerals were sparsely observed in many collagen fibrils within the body of the partially-demineralized scaffold (between open arrows). Those fibrils were surrounded by a peripheral rim of extrafibrillar minerals. Some collagen fibrils still contained intrafibrillar minerals (pointer). **F.** Intrafibrillar minerals were present in a hierarchical order in collagen fibrils (between open arrows) derived from the underlying mineralized base.

Fig.4.

Mineralization of a partially-demineralized collagen scaffold in the control group after 4 months. **A.** Light microscopy. A thin mineralized surface layer (green) could be seen above the layer of exposed collagen (red). **B.** Unstained TEM thick section. Open arrow: surface layer with increased electron density. **C.** TEM-EDX line scans of Ca and P distribution along the white line indicated in Fig. 4B. Open arrow depicts increase Ca and P intensities along the surface of the scaffold. **D.** Thin section showing increase in mineral deposition along the surface. E: epoxy resin; T: dentinal tubules. **E.** High magnification of Fig. 4D showing many collagen fibrils that are devoid of intrafibrillar mineral deposition (open arrowheads). E: epoxy resin. **F.** Mineralization was sparse within the body of the scaffold. Intrafibrillar minerals were absent from most collagen fibrils and only extrafibrillar minerals could be identified (between open arrows).

Fig.5.

Mineralization of a partially-demineralized collagen scaffold with the top-down approach after 4 months. **A.** Light microscopy. Note layer of exposed collagen (red). **B.** TEM thick section showing that the surface 100 μm of the scaffold was poorly mineralized. **C.** TEM-EDX line scans of Ca and P distribution along the white line indicated in Fig. 5B. **D.** Thin section showing the poorly mineralized surface of the collagen scaffold. E. epoxy resin; T: dentinal tubules. **E.** High magnification of a region at approximately 25 μm from the scaffold surface showing poorly mineralized fibrils devoid of intrafibrillar minerals (between open arrows). **F.** Minerals deposited by epitaxial growth in the body of the mineralized scaffold (approximately 150 μm from the surface) were longer than 100 nm.

Fig.6. Mineralization of a partially-demineralized scaffold with the bottom-up approach after 4 months. **A.** Light microscopy. Note absence of red-stained exposed collagen. **B.** Unstained TEM thick section. **C.** TEM-EDX line scans of Ca and P distribution along the white line indicated in Fig. 6B. **D.** Thin section showing a layer of precipitate (pointer) on top of the mineralized scaffold surface. Arrow: some of the dentinal tubules were filled with precipitate. **E:** epoxy resin. **E.** High magnification of a region at approximately 25 µm from the scaffold surface. Despite the extent of mineralization, there were areas that were not as heavily mineralized. This provides the ultrastructural evidence for the incomplete surface mineralization shown in the overall mineral profiles in Fig. 1C. Selected area electron diffraction of the minerals indicated that they were poorly crystalline apatite deposited along the longitudinal axis of the collagen fibrils. Ring patterns instead of arc-shaped patterns were seen in the 002 diffraction plane due to the 3-D orientation of the mineralized collagen matrix. **F.** High magnification of a region from approximately 150 µm away from the scaffold surface. Some of the intrafibrillar minerals were not as hierarchical as those identified from the mineralized tissue base (Fig. 3F).