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Multiphase intrafibrillar mineralization of collagen **

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In the past, the two major biom mineralization motifs, biosilicification and biocalcification, have been considered as two discrete processes. However, there is increasing evidence suggesting existence of an inextricable relationship between biosilica and calcium-based biom minerals.^[1] Recent discovery of a unique silica–chitin–aragonite biocomposite in one genus of Demosponges (*Verongida*) further introduces a novel mechanism of multiphase

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Experimental Section Methods summary can be found in Supporting Information S1.

hierarchical biomineralization.^[2] Considerable efforts have been devoted to the development of silica/calcium-based organic-inorganic hybrids;^[3-5] however none of these techniques could demonstrate the composite nature of their natural counterparts. Here, we report a biomineralization scheme that results in intrafibrillar mineralization of collagen with hierarchically-arranged, silica-apatite multiphase minerals via a bottom-up, biomimetic strategy. The mineralization mechanism involves precipitation and crystal growth of polymer-induced amorphous calcium phosphate precursors within the intrafibrillar spaces of hierarchically-silicified collagen. Silicified collagen-templated intrafibrillar apatite formation provides a model for the formation of multiphase-mineralized skeleton in invertebrates, and also results in a biocomposite with increased fatigue resistance and resilience, due to the interpenetrating arrangement of amorphous silica, collagen and crystalline apatite, as well as enhanced bioactivity, biocompatibility and bone defect restoring potential caused by the presence of those multiphase components.^[1,6,7]

Nature has destined each organism to receive one mineral (silica, calcium carbonate or calcium phosphate) as their primary skeletal building block. Nevertheless, genuine multiphase mineralization does exist. Examples include silica–chitin–aragonite skeletons in demosponges, opal–chitin–goethite radula in molluscs, silica–chitin–apatite shells in Brachiopods and silica–chitin–willenite teeth in Copepoda.^[1,2,8-10] These rare, natural multiphase biominerals provide evolutionary insights for biomineralization, and inspiration for the development of novel multiphase-mineralized biocomposites. The unique biocomposite from the order Verongida (*V. gigantea*) is a representative example of natural multiphase mineralization, with a three-dimensional matrix of silicified chitin fibrils infiltrated by regularly-distributed aragonite crystals within the siliceous construct.^[2] We previously reported a multiphase mineralized eggshell membrane (ESM) created by introducing nanostructured calcium phosphate or silica into the different compartmental niches of the biopolymer membrane, using amorphous precursor phases of the corresponding mineral.^[5] The differential distribution of calcium phosphate and silica in the ESM is attributed to the different organic composition of the fiber cores and mantles, which represents a different phenomenon from the multiphase mineralization in Verongida, in which two different minerals are hierarchically-arranged within one organic template. As both biomimetic intrafibrillar calcification and intrafibrillar silicification of type I collagen have recently been achieved *in vitro*,^[11,12] examination of the possibility of intrafibrillar hierarchical multiphase mineralization of type I collagen via a biomimetic strategy may result in novel multiphase biocomposites.

In the present multiphase biomimetic mineralization scheme, *in vitro* intrafibrillar silicification and calcification of type I collagen are respectively achieved using poly(allylamine) hydrochloride-stabilized silicic acid (PAH-SA) and poly(aspartic acid) stabilized-amorphous calcium phosphate (PAsp-ACP), via step-wise bottom-up approaches (Supplementary S2).^[11,12] Collagen sponges were silicified with PAH-SA for 2 days and then incubated in PAsp-ACP for 7 days (Figure 1). During the second phase (intrafibrillar apatite deposition) of multiphase mineralization, apatite crystallites formed around the intrafibrillar silica by the fourth day, and both intrafibrillar platelike apatite and the banding characteristics of silicified collagen can be distinguished (Figure 1C). After 7 days, elongated electron-dense crystallites occupy the entire volume of the intrafibrillar spaces, masking the banding characteristics of silicified collagen. Scanning transmission electron microscopy-energy dispersive X-ray analysis revealed the multiphase feature of the mineralized collagen scaffold, as indicated by the Si-banding structure caused by selective condensation of silica inside the collagen fibril, and intrafibrillar deposition of calcium phosphate-based minerals (Figure 2). Similar to the multiphase skeleton of Verongida, a three-dimensional matrix is produced, consisting of intrafibrillarly-distributed apatite crystallites within the siliceous collagen matrix. In the absence of PAsp as a calcium

phosphate stabilization agent, large crystals are formed only on the surface of the silicified collagen fibrils (Supplementary S3).

Attenuated total reflection-Fourier transform infrared spectroscopy of the multiphase mineralized collagen scaffold (Supplementary S4) reveals both the Si-O-Si bonds vibrational modes of silica (TO_3 , TO_2 and TO_1), and the O-P-O bending modes of apatite ($\nu_3\text{PO}_4$, $\nu_4\text{PO}_4$, $\nu_2\text{PO}_4$ and $\nu_1\text{PO}_4$).^[5,11] The $\nu_3\text{C-O}$ stretching mode at 1413 cm^{-1} is indicative of carbonate substitution of the apatite lattice.^[5] Inorganic phases within the multiphase mineralized collagen scaffold are identified using $^1\text{H} \rightarrow ^{29}\text{Si}$ cross polarization-magic angle spinning solid-state nuclear magnetic resonance spectroscopy (CP-MAS NMR) and ^{31}P MAS NMR (Figure 3). The broad peak between -100 to -125 ppm in the $^1\text{H} \rightarrow ^{29}\text{Si}$ CP-MAS NMR is deconvoluted to reveal the Q_3 ($\text{Si}(\text{OSi})_3(\text{OH})$) and Q_4 (SiO_4) peaks at -100 and -110 ppm, respectively,^[13] which is indicative of the condensation of a hydrated silica phase within the biocomposite. The ^{31}P MAS spectrum reveals three ^{31}P resonances, PO_4^{3-} , HPO_4^{2-} , and P-O-Si at 3.2, 0.78 and -6.17 ppm, respectively, with an intensity ratio of 100:17.07:3.43.^[13-15] The presence of a minute amount of P-O-Si moiety indicates the reaction between the silanol group in the silicified collagen with HPO_4^{2-} or PO_4^{3-} ions.^[15-17]

Powder X-ray diffraction of the multiphase mineralized collagen scaffold (Figure 4) reveals a broad background spectrum from 13° to 37° that may be attributed to amorphous silica. Distinct fractions of hydroxyapatite [$\text{Ca}_5(\text{PO}_4)_3(\text{OH})$] (JCPD# 09-0432) can be identified.^[18] The spectrum also shows diffraction corresponding to nagelschmidite [$\text{Ca}_7(\text{SiO}_4)_2(\text{PO}_4)_2$] (JCPD# 11-0676), which is not normally formed under physiologic conditions.^[19] Thermogravimetric analysis reveals a 78.7 wt% mineral content in the multiphase mineralized collagen scaffold (Supplementary S5). The derivative weight loss curve indicates three major episodes at 20–200, 300–500, and 700–900 °C. The first weight loss represents elimination of physisorbed water which is reflected by the peaks at 41.3, 121.3 and 189.0 °C. The second weight loss is reflected by peaks centered around 350.2 and 486.9 °C, which are due to decomposition of collagen molecules. The third weight loss from 772.7–830.2 °C is attributed to combustion of the organic matrix and release of CO_2 from carbonated apatite.^[20]

To further delineate the spatial relationship between amorphous silica and crystalline apatite within the collagen fibril, the multiphase biocomposite was selectively dissolved in an acidic or an alkaline solution to remove apatite or silica, respectively (Supplementary S6). These results confirm the co-existence of siliceous and apatitic intrafibrillar phases within the collagen fibril. Analogous to the proposed mechanism for multiphase mineralization in the silica-chitin-aragonite biocomposite of *V. gigantean*,^[2] synthesis of silica-collagen-apatite biocomposite in the present study represents a novel nanotechnology that comprises organization of apatite crystallites in the presence of polymeric silica, with the latter functioning as niduses for apatite growth. As the PAH-stabilized, “soluble” silicic acid precursors infiltrate the collagen fibrils, they condense at specific sites along the collagen triple helix.^[11,21] The hierarchically-silicified collagen fibrils containing multiple negative silanol groups further sequester calcium from fluidic PAsp-stabilized ACPs that are small enough to infiltrate the silicified collagen. This results in deposition of calcium phosphate phases over the original amorphous silica.^[12,22] During the later stages of biocalcification, crystalline apatite phases fill the entire intrafibrillar spaces of the amorphous silicified collagen fibril, masking the original silica phase.^[23]

The multiphase biomineralization strategy is further confirmed by using rat tail tendon collagen as a naturally-occurring soft tissue model substrate (Supplementary S7). Because of the co-existence of different intrafibrillar minerals, changes in the mechanical properties of

the biocomposites are to be expected. Thus, nanoscopy dynamic mechanical analysis indentations were used to investigate the effect of biosilicification, biocalcification or multiphase mineralization on the biomechanical properties of rat tail collagen, using a triboindenter system over hydrated samples. The complex modulus (total elastic and dampening capacity of a material), storage modulus (elastic response of a material), and loss modulus (viscous response of a material) of silicified rat tail collagen were significantly lower than the corresponding properties derived from multiphase mineralized rat tail collagen, and calcified rat tail collagen ($P < 0.05$; Figure 5). This is because the modulus of elasticity of amorphous siliceous minerals is lower than that of crystalline mineral phases. $\tan \delta$ values (ability of a material to absorb energy) of the three types of mineralized rat tail collagen revealed significant differences in their damping properties, in the order: silicified collagen > multiphase mineralized collagen > calcified collagen ($P < 0.05$). This step-wise decrease in damping properties is a reflection of the increase in crystalline mineral phase and decrease in amorphous mineral phase within those collagen matrices, with the multiphase mineralized biocomposites representing a balance between stiffness and resilience properties. These nanoscopy mechanical properties derived from rat tail collagen are further confirmed by analysis of compressive stress-strain responses (tangent modulus and modulus of toughness) in macroscopic specimens of hydrated biosilicified, biocalcified and multiphase-mineralized collagen sponges (Supplementary S8).

In the silica-chitin-aragonite biocomposite of *V. gigantean*, a chitinous template is used for deposition of aragonite crystalline aggregates over silicified chitin.^[2] Because type I collagen is widely distributed in both invertebrates and vertebrates, possesses high tensile strength, low antigenicity and controllable biodegradation properties, it was chosen as the template for biomimetic multiphase mineralization in the present study.^[1,23] Silica and carbonated apatite were chosen as the multiphase mineral components in our mineralization model because of their wide distribution in the biological world. The so-formed silica-collagen-apatite biocomposite indicates that two separate minerals, one in amorphous form and the other in crystalline form, may be simultaneously introduced into the intrafibrillar spaces between tropocollagen molecules.

The multiphase mineralization scheme was further confirmed using demineralized bovine trabecular bone matrix as a naturally-occurring hard tissue model substrate. Micro-computed tomography showed homogeneous mineralization of multiphase-mineralized bone specimens (Supplementary S9). It is speculated that the novel biomaterial will combine the biocompatibility and osteoinductivity of a demineralized bone matrix,^[24] the collagen synthesis and neovascularization stimulating effect of silica,^[25] and the osteoconductive and integrative properties of calcium phosphate minerals.^[26] Biocompatibility of the remineralized specimens was investigated using a green fluorescent protein-expressing mouse mesenchymal stem cell line (mMSC). The results demonstrate that multiphase-mineralized bovine trabecular bone scaffolds are highly biocompatible and suitable for adhesion and infiltration of mMSCs into the scaffolds (Supplementary S10). Further work has to be performed to examine the osteoinductivity of these novel scaffolds.

The current multiphase mineralization process also contributes to understanding the effect of silicon on bone formation, a phenomenon that has been studied since the 1970s. Carlisle *et al.* showed that silicon is present in active calcification sites in young mouse and rat bone. In animal trials, silicon deficiency results in abnormal bone formation with decreased deposition of both the extracellular matrix and bone mineral (apatite).^[6,7] Positive association between dietary silicon intake and bone mineral density has also been found in a human study.^[27] It is generally thought that silicon stimulates human osteoblasts and osteoblast-like cells to secrete type I collagen and other biochemical markers of osteoblast maturation and bone formation.^[28] Although bone formation and metabolism are mainly

controlled by different type of cells in a living organism, the presence of silica along the calcification front may enhance the role of collagen as a template for intrafibrillar apatite deposition, with the polysilanol groups of the amorphous silica serving as niduses for apatite growth. Thus, we speculate that a similar multiphase silica-collagen-apatite structure may exist during the initial process of natural bone formation. Differences in mechanical properties among silicified collagen, multiphase mineralized collagen and calcified collagen may help explain a phenomenon that occurs with aging - mature bone tissues contain a higher apatite concentration (i.e. becoming more brittle) and a lower silicon concentration (i.e. decreased resilience) than newly formed bone. This may result in increasing the risk of bone fracture with aging.^[29]

In summary, the present study demonstrates a novel multiphase intrafibrillar mineralization scheme which is important for understanding the diversities of biomineralization. Further study will focus on the biological application of this biocomposite in hard tissue regeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. TEM images of unstained multiphase mineralized collagen sponge

A, the collagen sponge is heavily silicified after incubation in poly(allylamine) hydrochloride-stabilized silicic acid (PAH-SA) for 2 days. Electron-dense intrafibrillar minerals replicate the cross-banding and microfibrillar architecture of fibrillar collagen (bar = 200 nm). Inset: selected area electron diffraction reveals the amorphous nature of the infiltrated minerals. B, during the initial stage of multiphase mineralization (2 days), poly(aspartic acid)-stabilized amorphous calcium phosphate (PAsp-ACP) particles (arrow) infiltrate into the collagen fibril and nucleate along the preformed intrafibrillar silica (bar = 100 nm). The banding structure of the silicified collagen fibrils can still be clearly distinguished at this stage. C, with further incubation in PAsp-ACP solution (4 days), transformation of ACP into nano-sized apatite crystallites can be identified (arrow head) (bar = 100 nm). Elongated growth of the intrafibrillar apatite consumes more intrafibrillar space and gradually masks the banding structure of silicified collagen. D, after 7 days of incubation in PAsp-ACP solution, the apatite crystallites organized along the longitudinal-axis of the collagen fibrils and filled all the intrafibrillar spaces (double-headed arrow). The banded structure in the silicified collagen cannot be distinguished (bar = 100 nm). Inset: selected area electron diffraction indicates the crystalline feature of the newly formed minerals, with diffraction patterns that are characteristic of apatite.

Figure 2. Elemental analysis (scanning transmission electron microscopy-energy dispersive X-ray analysis) of an non-osmicated, unstained section of a collagen fiber confirms the multiphase feature of the mineralized collagen sponge

A and B, bright and dark field images reveal electron-dense minerals deposited predominantly within the collagen fibrils (bar = 500 nm). C-F, element mappings indicate that silicon, oxygen calcium and phosphate are simultaneously present within the collagen fibrils (bar = 500 nm). Silicon mapping shows regular collagen D-spacings that are attributed to the presence of intrafibrillar silicon. Manifestation of calcium and phosphorus signals inside the collagen fibrils is indicative of the co-existence of intrafibrillar calcification.

Figure 3. Solid-state NMR spectra of multiphase mineralized collagen sponge

A, $^1\text{H} \rightarrow ^{29}\text{Si}$ CP-MAS spectrum exhibits two signals at -100 and -110 ppm, corresponding to Q₃ (Si(OSi)₃(OH)) and Q₄ (SiO₄). No Q₁ (SiO(OH)₃) and Q₂ (SiO₂(OH)₂) signals at -88 and -91 ppm can be identified. B, In the ^{31}P MAS spectrum, three ^{31}P resonances are identified at 3.2, 0.78 and -6.17 ppm, corresponding to PO_4^{3-} , HPO_4^{2-} , and P-O-Si moieties, respectively.

Figure 4. X-ray diffraction spectrum of multiphase mineralized collagen sponge
The broad background is due to the amorphous silica. The crystalline parts of the hybrid material are assigned to hydroxyapatite (black triangle) and nagelschmidite (star).

Figure 5. Box plots summarizing the nanoscopy dynamic mechanical properties of silicified, calcified, and multiphase mineralized rat tail collagen

A, Complex modulus; B, Storage modulus; C, Loss modulus; D, Tan δ . Each box plot represents the minimum value, 25th percentile, median, 75th percentile, and maximum value of a data set (N=10). For each parameter, groups designated by the same letter are not statistically different (One-way ANOVA and Holm-Sidak post-hoc multiple comparisons; $P > 0.05$).