Templating effect of silk fibers in the oriented deposition of aragonite†

Cheng Cheng,[a] Yuhong Yang,[b] Xin Chen[a] and Zhengzhong Shao*[a]

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Controlled deposition of calcium carbonate crystals can be obtained on degummed *Bombyx mori* silk fibers through the use of a silk fibroin solution; aragonite crystallites are found on the surface of the fiber with consistent orientation along the longitudinal axis; the results indicate that the combination of the ordered surface structure on the silk fiber and the directing-effect of silk fibroin solution are the key factors in the orientative deposition process of the mineral.

Scientists have long been fascinated with how organisms produce complex composites that are hierarchically organized in composition and microstructure. Because of the unique properties of these biocomposites, much effort has been made to investigate and mimic the biomineralization processes, both in the shells of molluscs[1–4] and the bone or dentin tissues of vertebrate.[5–7]

Bone as a composite is a complex assembly composed of type I collagen nanofibrils with oriented hydroxyapatite (HAP) crystals on the surface. The *c*-axis of the HAP is parallel to the longitudinal axis of collagen fibril and the mineralized fibrils are parallel with each other.[6] Similarly, molluscs build their shells based on a highly structured chitinous framework, in which the chitin is in the β-form with parallel chains[8] and has a preferred orientation over a length scale of several microns.[9] Alignment of the crystallographic *a*-axis of aragonite with the chitin fibrils has been observed.[10] It is generally believed that the water-soluble fraction of the organic matrix in biominerals has a strong influence on the morphology and polymorphism of the minerals, whereas the role of the insoluble matrix is thought to serve as a nucleation surface and predefined mold.[11] However, the highly oriented alignment of minerals in both mollusc shells and bones suggests the relationship between the parallel chains of matrix and the orientation of inorganic crystals.

As such, a number of organic materials with orientated structure, such as recombinant collagen fiber,[7] uniaxially deformed gelatin film,[12] and spider silk[13] have been employed as templates for mineralization. Hydroxyapatite or vaterite crystals with preferred nucleation surface, can be formed on these matrices but have yet to show macroscopic alignment. Silkworm silk is an ideal template for investigating whether or not the structure of insoluble matrix is involved in the morphology and orientation control of mineral. This is because of its highly ordered microstructure[14] as well as an interaction between silk protein (fibroin) and inorganic crystals, *e.g.* calcium carbonate ($CaCO_3$).[15] Characterization of *Bombyx mori* silk fibers has shown that silk fibroin contains a crystalline-cross-linked network with a high degree of orientation along the long axis of the fiber with protein crystallites consisting of antiparallel β-sheets.[14] In the present study, we combined degummed silk and regenerated silk fibroin (RSF) solution into a template/additive mineralization system to regulate the crystallization of $CaCO_3$. The apparent parallel alignment of the mineral on the matrices was observed, and we conclude that the oriented β-sheet secondary structure in the silk played a pivotal role in this process.

In general, on simply soaking degummed silk in a supersaturated $CaCO_3$ solution without any additives, mineralization would not occur on the surface even after incubation for two weeks. Through this approach only homogenous nucleated calcite rhombohedra and vaterite florets may occasionally be observed between the silk bundles (image not shown). In the system presented here, degummed silks were incubated in aqueous supersaturated $CaCO_3$ (pH 7.67) in the presence of dissolved silk fibroin (2%, w/w) at room temperature.‡ The surface morphologies of fibers mineralized for different periods of time (5 and 10 days) are shown in Fig. 1. After 5 days, we saw a large amount of mineral deposits which had the appearance of shuttle-shape islands, in parallel to and aligning along the longitudinal axis of the silk fiber (Fig. 1(a)). A longer incubation (10 days) caused an expansion and thickening of these "islands" (Fig. 1(b)). X-Ray diffraction (XRD) (Fig. 2, curve a) of mineralized silk fibers indicated that the polymorph of these crystals growing on the surface of matrices was aragonite, which is not the most thermodynamic stable phase of $CaCO_3$ but coincidentally always found in nacre of mollusc shells.[3]

Fig. 1(c)–(f) show the morphology of crystals deposited on silks soaked for 5 days in the same system but with a series of pH values from 7.92 to 8.60. All the images show the oriented alignment of the $CaCO_3$ crystals. The crystal "islands" grew larger and began to connect with each other as the pH value increased, which was probably due to the accelerated crystallization rate in higher alkali pH conditions. A layer of oriented $CaCO_3$ crystals which covered the surface of silk could be entirely formed when silk fibers were soaked in the silk fibroin containing system with an initial pH value of 8.60 (Fig. 1(f)).

Microstructural characterization of the silk fibers has revealed the oriented β-sheet crystal structure along the axis of the fiber and the conversion of this high ordered silk II structure back to silk I can be accomplished by dissolving the fibroin in a polar solvent such as aqueous lithium bromide

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[a] *Key Laboratory of Molecular Engineering of Polymers of Ministry of Education, Advanced Materials Laboratory, Department of Macromolecular Science, Fudan University, Shanghai, 200433, People's Republic of China. E-mail: zzshao@fudan.edu.cn*
[b] *The Center of Analysis and Measurement, Fudan University, Shanghai, 200433, People's Republic of China*

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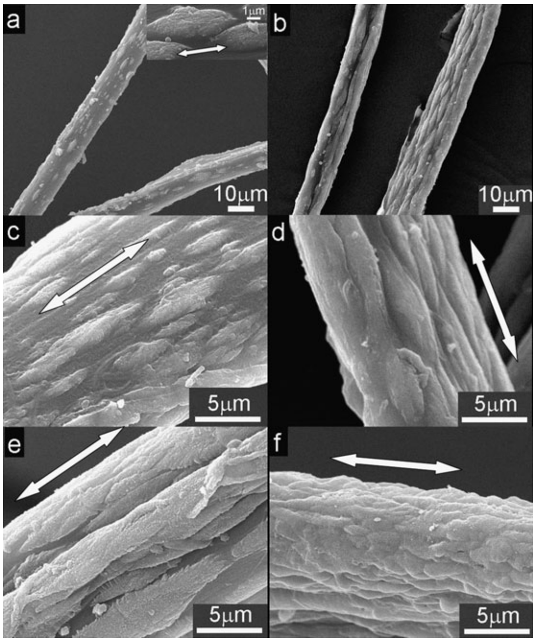


Fig. 1 SEM images of silk fibers after mineralization in the presence of silk fibroin: (a) 5-day soaked silks at pH 7.67 with magnified figure (inset); (b) 10-day soaked silks at pH 7.67; (c), (d), (e) and (f) 5-day soaked silks at pH 7.92, 8.14, 8.35 and 8.60, respectively. White arrows show longitudinal orientation of the silks.

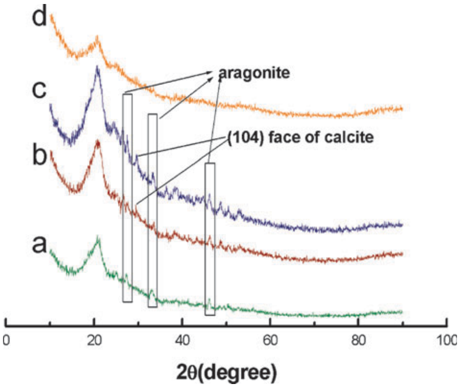


Fig. 2 XRD patterns of 5-day mineralized silks: (a) untreated degummed silk; (b), (c) degummed silk treated with LiBr solution (6 mol L^{-1}) for 10 and 30 min, respectively; (d) degummed silk treated with LiBr solution (9 mol L^{-1}) for 10 min.

(LiBr).[14] As our results implied that the oriented structure of the silk fiber may have a templating effect on the orientation of mineral aggregation, a series of comparative experiments were conducted. We used aqueous LiBr with different concentrations (6 and 9 mol L^{-1}), to treat the degummed silk over different times,‡ to alter the structure and orientation of molecules on the surface of the silk fibers, and then the mineralization was carried out by using the same procedure.

Raman spectroscopy was used to characterise the conformation as well as the orientation of silk protein,[16] and the polarised amide I bands (around 1665 cm^{-1}) and amide III bands (around 1230 cm^{-1}) in the spectra confirmed that the β-sheet and partial ordered regions in the silk fibers were oriented along the fiber axis.[16–18] The polarization of these bands for LiBr-treated silks, was much weaker than that of those in the untreated silks (see Fig. 1 ESI†). Therefore, it may be concluded that orientation of molecules on the surface of treated silks was apparently disrupted by the LiBr.

Fig. 3 shows the surface morphology of the mineralized silks, both untreated and treated with LiBr. Although $CaCO_3$ was successfully deposited on all three kinds of treated silk filaments, the amount of the crystals was appreciably smaller than those of untreated silks. Moreover, the aligned crystals growing on the untreated silk, contrasted to the random deposits growing on the silks treated with 6 mol L^{-1} LiBr solution for 10 and 30 min (Fig. 3(b) and (c)). This observation provided the evidence that the preferential aggregation of $CaCO_3$ was controlled by the oriented aligned β-sheet structure or some partially ordered regions on the silk. In contrast to the 6 mol L^{-1} LiBr solution which only affected the surface structure of silk fibroin, 9 mol L^{-1} LiBr solution readily dissolved the *Bombxy mori* silk after 30 min. Therefore, the larger crystal "islands" (indicated as black arrows in Fig. 3(d)) deposited on the silks treated with 9 mol L^{-1} LiBr solution showed alignment which could due to the exposure of oriented inner part of the fiber after dissolving of the first layer. However, the aggregates became fewer than those on other silks, presumably since the ordered structure on the silk was partly damaged. The polymorph of the crystals deposited on the LiBr treated silks was slightly different from the aragonite crystals on the untreated silks. The appearance of a peak at 29.5° in XRD patterns (Fig. 2, curve (b) and (c)), corresponding to the (104) plane of calcite indicated that the crystals on the 6 mol L^{-1} treated silks were composed of calcite and aragonite. Unfortunately, the amount of crystals on the 9 mol L^{-1} treated silks was too low to be detected (Fig. 2, curve (d)).

Considering that the alignment of $CaCO_3$ aggregates appeared only on the highly oriented surface of fibers in the presence of dissociated silk fibroin as additive, it is believed that the templating effect of the ordered region of silk fibroin on the matrices and the selective binding of Ca^{2+} on the additive proteins both contributed to the oriented deposition of $CaCO_3$ on the silk fiber. Our previous study on the regulating effect of aqueous silk fibroin on the crystallization of $CaCO_3$[15] has revealed that some of silk fibroins existed as clusters which had partial orientation and served as nucleation centers at

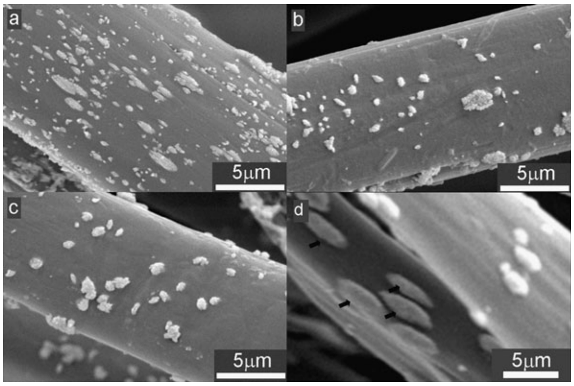
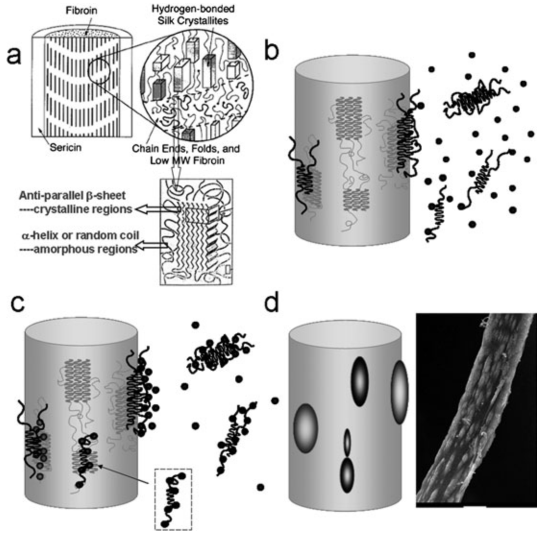


Fig. 3 SEM images of $CaCO_3$ aggregates deposited on (a) untreated degummed silk; (b), (c) degummed silk treated with LiBr solution (6 mol L^{-1}) for 10 and 30 min, respectively; (d) degummed silk treated with LiBr solution (9 mol L^{-1}) for 10 min.



Scheme 1 Proposed deposition process of $CaCO_3$ on the silk fiber. (a) The model of *Bombyx mori* silk showing that the protein nanocrystals are preferentially oriented along the longitudinal axis of fiber.[14] (b) The absorbance of dissociated aggregated protein on the crystal region of silk while the amorphous $CaCO_3$ nucleated in solution. (c) Binding of $CaCO_3$ on the silk fibroin aggregates. (d) Model of mineralized silk after the process compared to the experimental image.

pH 8.6. The amorphous $CaCO_3$ particles absorbed on the surface of these clusters were organized along the orientation of the core and the cooperative effect of the cluster structure and the oriented chains finally led to the rice-like orientative aggregation of the minerals. In combination with such a crystal growth mechanism and the template effect of the matrices, we propose a possible formation pathway of the orientative aligned crystal "islands" on the silks, represented in Scheme 1. Initially, the amorphous $CaCO_3$ particles form *via* homogenous nucleation in the supersaturated solution system,[19] and then the strong interaction between Ca^{2+} and the charged amino acid residues (of the regenerated silk protein fragments) caused the binding of the nanocrystals and the silk fibroin chains in the form of partially oriented clusters. Some clusters then act as nuclei for rice-like particles in solution (which has been reported in previous work[15]), with the remaining clusters tending to absorb on the highly oriented crystalline region of silks because of the nucleation-dependent aggregation of β-sheets.[20] The cooperative effect of the dissociated silk fibroin clusters to "mediate" and the ordered molecule chains on the silk to "template" would finally lead to the orientative deposition of $CaCO_3$ on the silk fibers.

The pure aragonite polymorph of orientative aligned $CaCO_3$ crystals growing on the untreated degummed silk in the present system supports the hypothesis that the oriented chains involved in the crystal formation may reduce the energy barrier for nucleation of aragonite.[21] This is further supported by the fraction of calcite in the crystals with no consistent orientation on the LiBr treated silk. The oriented aragonite layer on the matrix which has an ordered structure is analogous to the biomineralization of nacre in mollusc shells, and the contribution of the structure of matrix on the aragonite formation in the present system is interesting and worthy of further investigation.

In summary, in the presence of dissociated silk fibroin as an additive, aragonite crystals could be nucleated and grown on degummed silk to form a mineral layer with a uniform orientation along the longitudinal axis of the fiber. We propose that the cooperative effect of the dissociated silk fibroin clusters "mediate" and the ordered molecules on the silk "template" this process, finally leading to the orientated deposition of $CaCO_3$. The mineralization-inducing effect of silk fibroin may broaden its application for bone-substitution materials, however it might be also an undesirable character of the protein when applied to the field of tissue engineering.

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Notes and references

‡ The degumming and dissolving process of *Bombyx mori* silk followed established procedures.[22] Some of the silks were treated with aqueous LiBr solution and then thoroughly washed with deionized water to remove the salt. Degummed silk and LiBr-treated silks were incubated in the supersaturated system at room temperature for mineralization. The supersaturated $CaCO_3$ solution with regenerated silk fibroin as additive was obtained by mixing the $CaCl_2$ solution (20 mmol L^{-1}) into an equal volume of $NaHCO_3$ solution (20 mmol L^{-1}), which contained 2% (w/w) silk fibroin aqueous solution at different pH values adjusted with 1 mol L^{-1} NaOH.

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