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Seeded Mineralization in Silk Fibroin Hydrogel Matrices Leads to Continuous Rhombohedral CaCO₃ Films

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Abstract: As many biominerals are formed in gel-like media, hydrogel-mediated mineralization is deemed as paradigms of biomineralization and ideal approaches to synthetic minerals with hierarchical architectures and related functions. Nevertheless, the long diffusion distance in hydrogels makes mineralization a diffusion-limited process, leading to isolated crystals instead of uniform hierarchical architectures. In the current study, seeded mineralization in silk fibroin hydrogel matrices is successful in delivering continuous rhombohedral CaCO₃ films. Though the coverage of hydrogel matrices makes mineralization a diffusion-limited process, the presence of seed layers promotes the growth of uniform overlayers in proper conditions. The regulation of the solid content of hydrogels provides a rational route to rhombohedral architectures with tunable morphologies and thickness. In the course of mineralization, the hydrogel matrices are partially occluded in rhombohedral films as interand intra-crystalline constituents, as confirmed by scanning and transmission electron microscopy. Our study confirms the availability of synthesizing continuous mineralized films with hierarchical architectures and the structural gradient in hydrogel matrices via self-organized mineralization. These films with the occlusion of hydrogel constituents may exhibit significant strength and resilience, and their formation can deepen our mechanistic understanding of biomineralization proceeding in gel-like media.

Keywords: calcium carbonate; silk fibroin; hydrogel; film; biomineralization

1. Introduction

Biomineralization proceeds in a confined space filled with viscous or gel–like media, where the suppression of the mobility of reacting chemicals makes mineralization a self-organized process, leading to hierarchical architectures in far-from-equilibrium conditions [1,2]. The presence of gel-like media has been confirmed in numerous biominerals, including but not confined to coral [3,4], mollusk shells [5–7], otoliths [8], teeth enamels [9], etc. The impact of gel-like media on the formation of the nacreous layer in mollusk shells has been disclosed in detail [10], as the biomineral is among few biogenic forms that can reconcile contradictory strength and resilience by taking advantage of the "brick-and-mortar" architecture [11,12]. It is envisaged that silk fibroin (SF)-like proteins [13]—the gel-like media found in the nacreous layers—form hydrogels prior to biomineralization, filling compartments spaced by two layers of β -chitin [5]. In the course of in situ mineralization proceeding in compartments, gel-like media can mildly inhibit both the nucleation and growth of tablets to fulfill the geometry of layered space, and in the meantime, are pushed to the surface of β -chitin sheets. In a mature mollusk shell, a typical interlamellar layer of the nacreous mineral is therefore characteristic of the

sandwiched architecture, where each sheet of β -chitin is interspaced by the gel-like media comprising both SF-like proteins and glycoproteins [5]. Nevertheless, structural analyses of the gel-like media found in biominerals are technically challenging. Unlike their biomacromolecular counterparts, such as glycoproteins and β -chitin, gel-like media in mollusk shells are a type of multi-component materials comprising SF-like proteins, water, and possibly glycoproteins. Even a dehydration process with care—a prerequisite for structural analyses with electron microscopy, for example—will inevitably deteriorate their pristine networks [14]. Moreover, the understanding of the roles of gel-like media in biomineralization is demanding owing to their inevitable association with both mineralized and biomacromolecular constituents at the microscopic scale [10].

Therefore, hydrogel-mediated mineralization has been used as paradigms of biomineralization [14]. In hydrogel matrices, reacting agents are required to transport a long diffusion distance for mineralization, causing the elongation of induction periods and accumulation of supersaturation levels to an extremely high level before the appearance of the first crystals [15]. Subsequently, mineralization aiming for the rapid depletion of reactants produces mineral particles characteristic of being few in number and large in size. It is worth moting that the shape of the mineralized products is mainly determined by the permeation properties of the hydrogels used. Numerous studies have nicely shown that a diffusion-controlled growth microenvironment can be achieved by the selection of hydrogels with good affinity to mineral targets [16,17], increase of hydrogel concentrations [18–20], increase of the supersaturation levels in the stock solution [21,22], or use of extra additives [23,24]. The appearance of complex morphologies exemplified by spherulites, dendrites, branching morphologies, and networks is in accordance with crystallization theories, that is, structural mismatch starts to show up when the local supersaturation level exceeds the threshold value [25,26]. As a result of crystal growth at high supersaturation levels, the hydrogel matrices can be occluded in growing crystals, and their spatial distribution at the nanoscale can be disclosed with advanced tools like transmission electron microscopy coupled with tomographic accessories [27]. The hydrogel constituents functioning as interand intra-crystalline constituents in the crystalline matrices in turn give rise to the resilience of the synthetic hybrids, which is also a universal biomineralization strategy of reconciliating contradictory mechanical properties [10]. Nevertheless, the reduction of the nucleation rate is accompanied by the limitation of the maintenance of the growth rate of crystals, and the synergistic effect of both factors impedes the formation of continuous crystalline architectures. Falini et al. took the lead in establishing mineralization model systems to study the synergistic effect of insoluble matrices, soluble additives, and hydrogels [28]. Nevertheless, films comprising individual CaCO₃ crystals instead of continuous structures were collected on chitin films, which was ascribed to the inhibition effect caused by the elongation of the diffusion distance in hydrogel matrices [14]. It is envisaged that the introduction of active sites on insoluble matrices can promote heterogeneous nucleation, giving rise to the formation of mineralized films with structural uniformity [6]. As a proof of concept, Estroff and coworkers introduced a nacre specific peptide—n16N on chitin substrates to guide heterogenous crystallization in SF hydrogel matrices, still obtaining individual CaCO₃ microcrystals [29]. In short, existing mineralization systems in hydrogel matrices have the limitation of delivering crystalline films with structural uniformity, which is an indispensable factor for the integration of numerous biominerals with regards to body support and defense against predators.

Hereby, we introduce the concept of seeded growth to hydrogel-mediated mineralization, aiming for growing continuous crystalline $CaCO_3$ films with distinct mesostructural character. Though it remains unknown how biological systems deliver continuous architectures with structural regularity in a confined space filled with gel-like media, seeded growth is a widely recognized practical approach to uniform columnar architecture [30]. The method is advantageous for facilitating the formation of continuous columnar films in the course of overgrowth. Moreover, it is worth noting that seeded growth is accompanied by competition between adjacent growing structural units, leading to crystalline films with a structural gradient and orientational preference [30]. For instance, seeded mineralization opens the door for obtaining prismatic-type $CaCO_3$ films with a vertical orientational

preference [31–33]. Owing to the high packing density and uniform alignment of prisms, these films can exhibit comparable mechanical performance to their biogenic counterparts [31]. In principle, the application of underlying granular seeds can effectively promote heterogenous nucleation in hydrogel matrices, so that mineralization in proper conditions can give rise to the delivery of uniform films.

In this paper, we studied the synergistic effect of seeded growth and SF hydrogel-mediated mineralization on the structural control of CaCO₃ films. We mainly investigated the influence of the solid contents of SF hydrogels on the structural characters of the CaCO₃ overlayers, including the uniformity, thickness, morphology, etc. A further aim was to relate the structural changes of the CaCO₃ overlayers to the porosity and permeation properties of the mineralization media relative to the solid contents of SF hydrogels.

2. Experimental Section

2.1. Materials

The cocoons of *Bombyx mori* silkworm were purchased from Fu'an cocoon silk Co., Ltd (Dongtai, Jiangsu, China). Chitosan, with a weight-average molecular weight of 3.0×10^5 g mol⁻¹ and a degree of deacetyl of 95%, was purchased from Aoxing Biotechnology Co., Ltd. (Yuhuan, Zhejiang, China). Calcium chloride (powders, AR) and (NH₄)₂CO₃ (powders, AR) were purchased from Sinopharm Chemical Reagent (Shanghai, China). Poly (acrylic acid) solutions (PAA; $M_w = 2.0 \times 10^3$ g mol⁻¹, 50 wt% in water) were purchased from Sigma-Aldrich Co. LLC (Shanghai, China). Ethanol was purchased from Zhenxin Chemicals Factory (Shanghai, China). All chemicals were purchased and used as received without further purification if not specified. Degassed purified water was obtained from Direct-Q®3 UV Water Purification System, and boiled for 0.5 h to remove CO₂ before use.

2.2. Preparation of Regenerated Silk Fibroin (SF) Solutions

The degumming of *Bombyx mori* silk fibroin and preparation of regenerated SF aqueous solutions were conducted following the established procedures [34]. The *B. mori* silkworm cocoons were cut into small pieces, and boiled in 0.5 wt% Na₂CO₃ aqueous solution for 60 min, and then rinsed thoroughly with abundant deionized water to remove the glue-like sericin proteins. After drying completely, degummed silk fiber was obtained. After that, the degummed silk fibroin was dissolved in 9.3 mol L⁻¹ LiBr aqueous solution for 4 h at 60 °C, and then, the SF solution was dialyzed continuously against deionized water for 2 days at room temperature by using a semi-permeable membrane (MEMBRA-CEL, 12,000–14,000 MWCO). The resulting SF solution was stored at 4°C for further use. The concentration of the resulting regenerated SF aqueous solution was determined by the gravimetric method, and further diluted to a given concentration with deionized water for the preparation of CaCO₃ films.

2.3. Substrates Coating

A volume of 20 μ L 1.5 wt% SF solution was transferred on a clean cover glass (1 cm × 1 cm, pretreated with sodium hydroxide solution), followed by a spin-coating process using a spin rate at 5000 rpm for 45 s. Afterwards, ethanol steam was used to treat the SF thin films for a period of 2 days, aiming for the promotion of the formation of β -sheets [28].

2.4. Deposition of Seed Layers

The mineralization was performed by the slow diffusion of CO_2 (based on the slow decomposition of $(NH_4)_2CO_3$ into a volume of 4 mL 50 mM CaCl₂ in the presence of 0.01 g L⁻¹ PAA as the additive in a closed desiccator for about 36 h [32]. SF thin films after the ethanol treatment were positioned at the bottom of the reacting mother liquor for mineralization of the seed layer. Subsequently, the hybrid thin film together with the substrate was removed and rinsed twice with degassed purified water before being used for characterization or overgrowth.

2.5. Deposition of SF Hydrogel Layers on Seed Layers

The SF solutions were dialyzed to the concentration of 1 wt%, 4 wt%, and 6 wt%, respectively, beforehand. Then, a volume of 20 μ L above solutions was transferred onto the surface of the seed layers, followed by a spin-coating process using a spin rate at 1000 rpm for 10 s. Afterwards, ethanol steam was used to transform SF solutions to hydrogels in a period of 2 days.

2.6. Growth of Overlayers

The seed layers coupled with SF hydrogels were carefully positioned at the bottom of plastic petri dishes containing a volume of 4 mL of CaCl₂-SF solution for the deposition of overlayers. Growth of the overlayers was conducted in the presence of an appropriate amount of 0.2% (w/w) SF monomers and 20 mM CaCl₂ via the standard CO₂ vapor diffusion method. The mineralization was performed in ambient conditions for 48 h.

2.7. Characterization

Scanning electron microscopy (SEM) images were obtained by using a Hitachi SU–70 scanning electron microscope. Samples were prepared by sticking fronted and fractured thin films onto a double-sided conducting adhesive tape. Subsequently, to increase the conductivity, a thin layer of platinum was coated onto samples by using a JFC-600 sputter coater for 30 s (i.e., for SEM acquisition at 15 kV). Optical microscopy (OM) and polarized optical microscopy (POM) images were collected by using an Olympus BX53 OM equipped with polarizers. The atomic force microscopy (AFM) (Multimode 8, Bruker, USA) images were obtained at room temperature to determine the surface features and image topographic information. Confocal laser scanning microscopy (CLSM) analysis was carried out by using a VK-X200K device (Keyence, Japan). A focused ion beam-transmission electron microscopy (FIB-TEM) sample was prepared by using a Hitachi NB5000. A bar of SiO₂ was deposited on the sample by using gas-assisted deposition to protect the surface during milling and lift-out. The sample was polished to the final thickness (~50–80 nm) using a 50 pA current after lift-out and transfer onto the TEM grid. HR-TEM and selected area electron diffraction (SAED) tests were also carried out by using a JEM-2100 device (200 kV). Attenuated total reflection Fourier transform infrared (FT-IR) spectra from 4000 to 1200 cm⁻¹ were recorded at room temperature by using a Nicolet iS10 spectrometer in attenuated total reflection mode. Rheology measurements were performed by using an MCR 302 Rheometer (Anton Par, Austria). The X-ray diffraction (XRD) patterns were obtained by using an X'pert PRO, PANalytical, X-ray diffractometer equipped with Cu Kα radiation. Diffraction patterns were generated with the instrument operation at 40 mA and 40 kV with a step size at 0.016°.

3. Results and Discussion

The protocol of the deposition of rhombohedral CaCO₃ films contains multiple steps (Scheme 1). First, continuous seed layers comprising granular CaCO₃-PAA constituents were deposited on insoluble SF matrices, according to our previous work [32]. Afterwards, a thin layer of SF solution of different solid contents was coated on the seed layer for gelation, following a standard procedure reported by Falini et al. [28]. Finally, the seed layers coated with a layer of SF hydrogels were used as receiving substrates for the overgrowth of CaCO₃ films in the presence of monomeric SF additives. The morphological control of the overlayers was executed by varying the concentrations of SF solution of SF hydrogels therein were characterized by using electron microscopic tools like scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Additionally, a novelty of the study lies in the synergistic effect of multiple soft constituents of the same proteinous structural units, namely monomeric additives, insoluble matrices, and hydrogels, which are all based on regenerated SF. The reason for the selection of multiple SF forms in one mineralization is two-fold. First, SF is an analogue of silk-like proteins in nacreous layers of mollusk shells in terms of the amino acid sequence and the secondary structure [10].

SF hydrogel-mediated mineralization may resemble the biomineralization process in mollusk shells, and meanwhile, SF matrices and monomeric additives mimic the roles of chitin and glycoproteins, respectively. As a comparison, previous studies have used the individual forms of SF, such as soluble monomers [35], films [36], and hydrogels [28,29], in the formation of CaCO₃ products. Second, SF is among few proteins that can be produced in a massive manner. It is therefore practically important to couple the SF constituents in biocompatible hybrids with bottom-up approaches, where they also play an active role in determining the mesostructural form of the products [37].



Scheme 1. Schematic showing the protocol used to grow rhombohedral CaCO₃-silk fibroin (SF) hybrid films. Note that SF hydrogels of different concentrations have a direct impact on the morphologies of rhombohedral films.

The formation of continuous seed layers on insoluble SF matrices is pivotal for obtaining uniform rhombohedral films. A marked dependence of the continuity of the seed layers on the crystallinity of the insoluble SF matrices underneath was confirmed in a pair of comparison mineralization experiments. It is known that the alcohol treatment can promote the conformational transformation of SF matrices from the amorphous form and α -helices to β -sheets [28]. An Fourier transform infrared (FT-IR) spectrum with peaks at 1620 (amide I), 1515 (amide II), and 1255 cm⁻¹ (amide III) verifies the dominance of the β -sheet form in ethanol-treated SF matrices (Figure 1a, the red curve) [38]. As a comparison, its pristine counterpart exhibits FT-IR peaks at 1653 (amide I), 1539 (amide II), and 1239 cm⁻¹ (amide III), which are assigned to the conformation of random coils and α -helices (Figure 1a, the blue curve). An AFM image shows that the SF matrices after the ethanol treatment are smooth at the nanoscale (Figure 1b). The mineralization results showed that the SF matrices with high crystallinity were successful in delivering continuous seed layers (Figure 1c), while their amorphous counterparts led to sporadic polycrystalline domains (Figure S1). As the crystallinity of the SF matrices shows direct impact on the structural uniformity of the seed layer, we assume that there exists a structural match between β -sheets of SF and calcitic CaCO₃ [35,36].

Continuous seed layers deposited on crystalline SF matrices were characterized with multiple techniques (Figure 1c–f). The structural uniformity and polycrystalline nature of the seed layers were verified by POM (Figure 1c). A zoomed-in SEM image confirms that each polydomain is composed of raspberry-shaped nanodomains 100–200 nm in size (Figure 1d). The granular nature of the nanodomains was next confirmed by AFM imaging (Figure 1e). The rough topography of the seed layers deposited on SF matrices is in contrast with the flat counterparts disclosed on PVA [31] and chitosan [32], respectively. We assume that the appearance of raspberry-shaped nanodomains is ascribed to the structural inhomogeneity of the crystalline SF matrices at the nanoscale. The XRD pattern verifies that the seed layer is calcite CaCO₃ in polymorphic form (Figure 1f), and the low intensity reflects the moderate crystallinity of the seed layers.



Figure 1. Characterization of SF matrices and seed layers. (**a**) Fourier transform infrared (FT-IR) spectra of SF matrices with (red) and without (blue) ethanol treatment. (**b**) atomic force microscopy (AFM) image of an SF matrices after ethanol treatment. (**c**–**f**) polarized optical microscopy (POM) (**c**), SEM (**d**), AFM (**e**), and XRD pattern (**f**) of CaCO₃-PAA seed layers.

Calcium cations and other reacting agents are required to transport through the SF hydrogels for mineralization. The properties of SF hydrogels are largely dependent on the solid contents of the SF solutions used. Three SF solutions with different solid contents of 1 wt%, 4 wt%, and 6 wt% were used for the fabrication of SF hydrogels. Figure 2a shows the rheological profiles of the three corresponding

SF hydrogels, where in each pair of curves, the storage moduli are distinctively higher than the loss ones. Thus, the elastic response is predominant in each SF network. The data also show that the mechanical strength of SF hydrogels becomes stronger with the increase of the solid content. The comparison of SEM images collected on the lyophilized SF hydrogels with different solid contents (Figure 2b–d) clearly indicates that the solid content of SF strongly influences the microstructures of the hydrogels. With the increase of solid contents, the pore size of hydrogels decreases progressively. For instance, a 1 wt% SF hydrogel shows an average pore size of over 10 μ m, whereas the value in its 4 wt% counterpart is less than 5 µm. With the further increase of solid contents, spiderweb-like structures appear in the 6 wt% SF hydrogel, where the pore size is decreased to about 1 µm. Moreover, large pores are characteristic of sheet-like structures with thick pore walls (Figure 2b), while spiderweb-like structures are comprised of thin walls interspacing adjacent pores (Figure 2d). Thus, SF hydrogels of different solid contents have different mechanical performances and microstructures. As SF has the calcium cation-binding capacity ascribed to the presence of carboxyl groups, SF hydrogels can exhibit cationic permeation capabilities [39], and consequently, their presence can turn overgrowth into diffusion-limited processes [15]. It is therefore anticipated that SF hydrogels of different solid contents can exhibit different degrees of inhibition in the course of overgrowth and have a direct impact on the structural outcome of the overlayers.



Figure 2. (a) Rheology curves of SF hydrogels at the [SF] values of 1 wt% (black), 4 wt% (red), and 6 wt% (blue). SEM images of lyophilized SF hydrogels by using 1 wt% (b), 4 wt% (c), and 6 wt% (d) monomeric solutions.

It is extremely imperative to understand the correlation between the solid content of the SF hydrogels and the structural character of the overlayers. The morphological variation with a change of the structures of SF hydrogels is displayed in Figure 3a–i. When the SF solid content was set at 4 wt%, continuous CaCO₃ overlayers with clear birefringent contrast were obtained. The iridescent birefringent contrast confirms the apparent polycrystalline nature of the overlayers. Interestingly, the uniform birefringent contrast at a scale of several micrometers hints that adjacent structural units within the same crystalline polydomain can exhibit a close shape and orientation. Both the top- and side-view SEM images depict that the overlayers are comprised of densely packed rhombohedral microcrystals

with an approximate 3D-orientation, verifying the correctness of the assumption. Therefore, polarized light transporting through these rhombohedra with close alignment shares the same route, which is reflected by the uniform birefringent contrast under polarizers.



Figure 3. Structural characterization of rhombohedral CaCO₃-SF hybrid films in 4 wt% (**a**,**d**,**g**) and 1 wt% SF hydrogels (**b**,**e**,**h**) and in the absence of SF hydrogels (**c**,**f**,**i**). Corresponding POM images (**a**–**c**), side- (**d**–**f**) and top-view (**g**–**i**) SEM images of rhombohedral films are listed in the left, middle, and right columns.

As a comparison, blur birefringent contrast was detected in the CaCO₃ films obtained either in 1 wt% SF hydrogels or in the absence of SF hydrogels (Figure 3b,c). SEM images show that structural units are superstructures comprising multiple rhombohedral external faces. Therefore, polarized light is scattered when transporting in the overlayers comprising superstructures complex in shape, causing the appearance of blur contrast. The formation of superstructures with the exposure of rhombohedral faces suggests that the interactions between both SF hydrogels and growing calcitic microcrystals should be weak. Similarly, the rhombohedral habit of calcitic crystals was also preserved in agarose hydrogels that had weak interactions with calcite [19,21,40,41]. Otherwise, the rhombohedral shape will not be retained by using hydrogels that have strong interactions with calcite [16,17]. Moreover, the formation of superstructures reflects the relatively high supersaturation levels in the course of mineralization, which generate the appearance of embryonic calcite on the exposed (104) facets of existing rhombohedral calcite [42,43]. As a comparison, moderate supersaturation levels cause the growth of crystals via ionic attachments on edges and vertices. Crystal growth was retarded in very concentrated SF hydrogels, which was ascribed to the increasing difficulty of diffusion of the reacting reagents therein. For instance, no continuous film was obtained when the solid content of SF hydrogels was increased to a value of 6 wt%, as the mass diffusion through the hydrogel layer was largely blocked (Figure S2). Besides the morphological difference, the solid contents of SF hydrogels had a direct impact on the total thickness of the rhombohedral films. For instance, the confocal laser scanning microscopy (CLSM) images show the thickness of rhombohedral films decreased with the increase of the solid contents of SF hydrogels (Figure 4a–d), verifying that the inhibition effect of SF hydrogels was increasingly prominent with the increase of their solid contents.



Figure 4. Confocal laser scanning microscopy (CLSM) images of rhombohedral CaCO₃-SF hybrid films obtained in 4 wt% (**a**) and 1 wt% (**b**) SF hydrogels and in the absence of SF hydrogels (**c**). The thickness statistics based on the above CLSM images is summarized in the chart (**d**), where 30 positions each were used for statistics.

In addition to SF hydrogels, the presence of monomeric SF also played a crucial role in delivering continuous rhombohedral films, as its absence led to sporadic microcrystals (Figure S3). Additionally, the exposure of (1 0 4) faces of rhombohedral calcite suggests relatively weak interactions between monomeric SF and growing crystals, and the surface roughening of rhombohedra should be ascribed to the adsorption of monomeric with growing facets via steric, hydrogen bonding, and van der Waals interactions. As both SF monomers and hydrogels have weak interactions with growing crystals and the concentration of monomeric SF is constant, the crystal shape is solely controlled by the diffusion of reacting reagents through the hydrogel matrices.

Structural detail was disclosed with electron microscopic tools including TEM and SEM. The overlayer shows an apparent structural gradient on the vertical direction with the appearance of cavities. Rhombohedra positioned close to the seed layer are about tens of nanometers, while those in the exterior layer are hundreds of nanometers in size. The increase of sizes accompanying the decreased number density of the rhombohedra reflects the gradual decrease of supersaturation levels in the course of overgrowth [44] alongside the "competition for space" pathway [30]. With time, crystal growth in moderate supersaturation levels proceeded via ionic attachments on the edges and vertices, leading to the formation of rhombohedral calcitic blocks with the dominance of (1 0 4) faces. The vertical structural gradient in rhombohedra films is similar to that found in prismatic architectures of biogenic [45–48] and synthetic [31–33] minerals. Additionally, rhombohedra grew right on top of the seed layer with the presence of multiple cavities (Figure 5a,b). Owing to fast growth rates in the course of overgrowth, the open space between adjacent rhombohedra could be blocked permanently because of the stoppage of mass consumption. These cavities can potentially be used to store drug compounds, which will be studied separately.

A close scan at an exemplary rhombohedral film discloses additional structural information, which gives rise to a mechanistic understanding of overgrowth. An overview TEM image shows that the rhombohedra are comprised of nanocrystalline structural subunits (Figure 5c). A zoomed-in image confirms that adjacent nanocrystals are not directly connected but separated by nanofibrillar architectures, assumedly SF hydrogels (Figure 5d). Moreover, a selected area electron diffraction pattern shows the single crystalline nature of an exemplary rhombohedron (Figure 5c). The global crystallographic orientation distribution of nanocrystalline structural units indicates the mesocrystalline nature of the rhombohedron [49]. It is also worth noting that these defined diffraction spots are slightly smeared, which indicates that the nanocrystalline subunits deviate slightly from the main orientational direction. This is in line with the current understanding of biominerals and their synthetic counterparts, which show a single crystalline nature at the nanoscale but are statistically oriented at the micro-and macro-scopic scales [16,48]. The formation of overlayers proceeding in SF hydrogels could be a stepwise crystal growth [19], but the probability of the sequential attachment of nanostructural units should not be excluded completely [50,51].

It is also important to disclose the spatial distribution of the SF constituent for a better understanding of the occlusion mechanism in the course of overgrowth. SEM images of the overlayers formed in SF hydrogels depict the presence of intercrystalline nanofibers. For instance, the rhombohedral microcrystals deposited in 4 wt% SF hydrogels are connected by a thin layer of networks (Figure 3d), while their counterparts in 1 wt% are linked by nanofibrillar architectures (Figure 3e). As a comparison, overgrowth in the absence of SF hydrogels led to rhombohedral superstructures with the absence of apparent soft constituents, hinting that these visible structures should belong to SF hydrogels that were deteriorated in the course of mineralization. In addition to intercrystalline constituents, a close scan of rhombohedra confirms the presence of matrix-like voids assumedly ascribed to the occlusion of intracrystalline constituents (Figure 5c). We deduce that these voids were formed owing to the presence of SF hydrogels instead of their monomeric counterparts, as no cavity existed in rhombohedral CaCO₃-SF hybrid films grown in the absence of SF hydrogels in our previous report [33]. It is worth noting that both individual and interconnected voids have been disclosed in numerous biogenic [52] and synthetic minerals [53–55] and deemed as carbon-rich constituents. The results of the current study provide unambiguous evidence that the voids should be ascribed to the presence of occluded nanostructures instead of their monomeric counterparts. The presence of intracrystalline constituents, in principle, can give rise to the mechanical performance of individual rhombohedron by decreasing the possibility of the propagation of microcracks. In short, both inter- and intra-crystalline constituents are beneficial for the reconciliation of high strength and resilience, which are otherwise contradictory properties in traditional engineering materials [11,12]. The simultaneous occlusion of inter- and intra-crystalline biomacromolecular networks is a universal strategy of biological systems for the increase of toughness in biogenic minerals [10,11,56,57].



Figure 5. TEM (**a**,**b**) and high-resolution TEM (**c**,**d**) images of rhombohedral films prepared in 1 wt% SF hydrogel matrices. The selected area electron diffraction (SAED) pattern of the film is alongside an HR-TEM image (**c**). The sample for TEM measurements was treated with the focused ion beam technique. (**e**) XRD patterns of rhombohedral films obtained in the presence of 4 wt% SF hydrogels and in the absence of SF hydrogels, respectively, where the blue curve shows the standard XRD pattern of calcitic CaCO₃.

4. Conclusions

Seeded mineralization led to continuous architectures in hydrogel matrices, which has been otherwise technically difficult to achieve. The success lies in the synergistic effect of hydrogels and seed layers in proper mineralization conditions. While the presence of hydrogel matrices makes mineralization a diffusion-limited process, seeded mineralization guarantees the formation of continuous architectures by the promotion of overgrowth on the seed layers. It is also worth noting that unlike its prismatic counterparts, the rhombohedral architecture is not an existing biogenic form that has been disclosed so far. Nevertheless, the formation of both architectures passes through the competition growth pathway, where the gradual decrease of supersaturation levels in the hydrogel matrices causes the appearance of a structural gradient in the vertical direction. Therefore, the mechanistic understanding collected in this synthetic system can inform the universal strategies used in biomineralization. Moreover, mineralization in hydrogel matrices leads to the occlusion of nanofibrous constituents in the mineralized matrices across multiple length scales, as the presence of both inter- and intra-crystalline constituents are disclosed using electron microscopic tools. The occlusion can give rise to the resilience of rhombohedral films, which mimics the toughening mechanism of biominerals. In short, the rational design based on the dynamic association of multiple soft constituents can make mineralization a controllable self-organized process, delivering mineralized products with a preferred architecture and emerging structure-related functions in far-from-equilibrium conditions.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4352/10/3/166/s1, Figure S1: POM image showing the seed layer deposited on amorphous silk fibroin matrices. Figure S2: POM image showing the deposition of discontinuous CaCO₃-SF hybrid films in 6 wt% SF hydrogels. Figure S3: POM image showing the deposition of rhombohedral single crystals on the seed layer in the absence of monomeric SF as additives. All authors have read and agreed to the published version of the manuscript.

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