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Non-invasive optical characterization of biomaterial mineralization

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Abstract

Current approaches to study biomaterial mineralization are invasive and prevent dynamic characterization of this process within the same sample. Polarized light scattering spectroscopy (LSS) may offer a non-invasive alternative for assessing the levels of mineralization as well as some aspects of the organization of the mineral deposits. Specifically, we used LSS to characterize the formation of hydroxyapatite deposits on three types of silk films (water-annealed, methanol-treated and polyaspartic acid (PAA)-mixed) following 1, 3, 5 and 7 cycles of mineralization. We found that the total light scattering intensity provided a quantitative measure of the degree of mineralization as confirmed by thermal gravimetric analysis (TGA). The PAA-mixed silk films yielded the highest level of mineral deposition and the water-annealed ones the least, consistent with the β sheet content of the films prior to the onset of mineralization. The wavelength dependence of the singly backscattered light was consistent with a self-affine fractal morphology of the deposited films within scales in the range of 150–300 nm; this was confirmed by Fourier analysis of scanning electron microscopy (SEM) images of the corresponding films. The deposits of minerals in the water-annealed films were predominantly flake-like, with positively correlated density fluctuations (Hurst parameter, $H > 0.5$), whereas methanol-treated and PAA-mixed silk films resulted in densely-packed, bulk mineral deposits with negatively correlated density fluctuations ($H < 0.5$). Therefore, LSS could serve as a valuable tool for understanding the role of biomaterial properties in mineral formation, and, ultimately, for optimizing biomaterial designs that yield mineral deposits with the desired organization.

Keywords: Silk; Fibroin; Tissue engineering; Polarization; Light scattering

1. Introduction

Nature synthesizes hierarchical, self-assembled, organic/biomineral complex composites under ambient conditions with superior mechanical properties. These composite systems provide a rich ground for insight into mechanisms of biomineralization and novel material design [1]. In general, biomineralization can be divided in two categories: biologically induced mineralization, in which an organism modifies its local microenvironment to establish conditions suitable for the chemical precipitation of extracellular mineral phases, or boundary organized biomineralization, in which inorganic particles are grown within or on a matrix generated by an organism [2,3]. The biological substances which produce biologically induced mineralization do not strictly control the crystallization process, resulting in mineral particles without unique morphology and with a broad particle size distribution [4]. In contrast, boundary organized biomineralization provides better control over size, morphology and crystallographic orientation of the mineralized particles [5]. The central tenet in the regulation of mineral deposition in biological systems is that organic matrices control the nucleation and growth of the inorganic structure. This control is exerted through the use of organic macromolecules that provide sites of nucleation and dictate crystal orientation and crystal morphology; however, these events are not well characterized.

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A better understanding of the mineralization process and crystal formation in biocomposites may provide a way to engineer new types of high performance materials in laboratories. Further, these types of mineralized systems have significant relevance and impact in areas of biomaterials and bone repair/regeneration treatments. These biominerals often have nanoscale crystal morphologies at the beginning, but their orientation, size and shape change at different stages of mineralization. Scanning electron microscopy (SEM) has been traditionally used as a characterization technique to study the detailed surface topography and crystal morphology of the mineral deposits [6]. However, SEM is an invasive technique, as are most of the other commonly used methods to study mineralized samples, such as X-ray diffraction, X-ray photoelectron spectroscopy (XPS), and transmission electron microscopy (TEM). Therefore, time dependent measurements at different stages of mineralization on the same sample are not possible. Fourier transform infrared (FTIR) spectroscopy is a non-invasive technique and has been used for molecular characterization of the mineralized samples, but it lacks the ability to provide morphological information and its use is limited in samples with high water content. Further, in biomedical imaging for bone formation, micro computed tomography or X-ray analysis are most often used to assess bone density and mineral distribution. These methods are effective for biomaterial and tissue assessments. However, their spatial resolution is insufficient to characterize the detailed mineral microstructure, and their sensitivity is not adequate to monitor early stages of mineralization. These approaches are also destructive, thus tissues cannot be monitored on a real time basis.

Light scattering spectroscopy (LSS) has been used extensively in biomedical research to find small scale morphological changes in human tissues and cells [7–10]. LSS has also been performed in vivo to detect non-invasive changes in nuclear morphology in pre-cancerous lesions in the esophagus, colon, oral cavity, bladder and the cervix [8–10]. This approach relies on the fact that the intensity of the light scattered off the structures that have a different refractive index from their surroundings varies as a function of wavelength and scattering angle in a manner that depends on the size, shape and refractive index of the scatterer. Therefore, we sought to assess the use of LSS as a non-invasive means to characterize the amount and organization of minerals deposited on silk films.

Silk fibroin is a fibrous protein with high mechanical strength and elasticity, which has been used as biomaterial for scaffolds in tissue engineering to produce bone, ligaments and skin in-vitro [11–13]. Unlike native silk fibroin, whose structure includes a high percentage of aligned β sheets and α helices, regenerated silk fibroin adopts a random coil conformation in solution and is intrinsically amorphous when formed into solid materials. A number of processing approaches have been developed to enhance formation of β sheet and α-helical domains in these materials, in order to influence the solubility, biocompatibility, thermal and mechanical properties of the material [14,15]. This can be accomplished by physicochemical treatment such as application of mechanical forces (stretching, shearing, rolling, spinning or compressing), thermal treatment, and immersion in selected organic solvents such as methanol, which causes dehydration of the hydrated structure, leading to crystallization, i.e. higher β sheet content [16–21]. Silk fibroin protein has been used as an organic macromolecule to regulate mineralization in silk films [22]. It has also been shown that co-processing of the silk with polyaspartic acid leads to an increase in the control and level of mineral deposits on silk fiber mats [6].

In the present work, we examined the use of LSS as a method to assess the progression of mineralization in three different types of silk films (water-annealed, methanol-treated and polyaspartic acid (PAA)-mixed) exhibiting different levels of crystallinity. To gain a better understanding of the origins of the LSS signals, we compared the LSS analysis results with standard thermal techniques and SEM. We demonstrate that LSS may serve as a useful non-invasive tool to assess not only the amount, but also the organization of mineral deposits. Further, it is a technique that can be used at early stages of mineralization, offering early insight in dynamic processes at organic–inorganic interfaces. As such, it offers a novel approach that could result in improved monitoring, understanding and control of biomineralization.

2. Materials and methods

2.1. Silk film preparation

To extract the silk fibroin protein, *Bombyx mori* silkworm cocoons were boiled for 30 min in an aqueous solution of 0.02 M Na_2CO_3 and rinsed thoroughly with water to extract the glue-like sericin proteins. The extracted silk was then dissolved in 9.3 M LiBr solution at 60 °C, yielding a 20% (w/v) solution. This solution was dialyzed in water using Slide-a-Lyzer dialysis cassettes (Pierce, MWCO 3500). The final concentration of aqueous fibroin solution was 8.0% w/v, which was determined by weighing the remaining solid after drying. Pure silk films were cast by pouring the silk fibroin solution into polystyrene petri dishes and allowing them to dry at ambient temperature in a hood for 2 days. To increase the β sheet content, the films were kept in a water-filled vacuumed (less than 10^{-3} mmHg) desiccator for approximately 24 h (water annealed). To further induce β sheet content some films were also immersed in a 90% methanol solution for about 10 h (methanol-treated). Polyaspartic acid (PAA) films were prepared by adding 0.2% PAA in the aqueous fibroin solution (PAA-mixed). The thickness of all films was approximately 90 ± 5 μm as assessed using a Leica DMIRE2 microscope equipped with a spectral confocal TCS SP2 scanner (Wetzlar, Germany).

For each mineralization cycle the films were kept in a 0.2 M $CaCl_2$ solution for 20 min and then allowed to dry at room temperature. Subsequently, the films were moved to a 0.12 M aqueous Na_2HPO_4 solution for 20 min [6]. Di-calcium phosphate ($CaHPO_4$) mineral deposits were formed on the surface of silk fibroin films after each mineralization cycle. Each film was subject to seven mineralization cycles.

2.2. Light scattering spectroscopy

The LSS system used in this study has been described in detail previously [23]. Briefly, the set-up acquired the scattering angle- and wavelength-dependent intensity of light scattered in the backward direction. Light in the 450–700 nm region from a 500 W Xenon lamp was collimated and linearly polarized before illuminating the sample at 45° from the surface normal in order to avoid detecting specular reflections. The backscattered light was detected through an analyzer that was placed either parallel (I_{par}) or perpendicular (I_{perp}) to the polarizer. Singly scattered photons maintained their initial polarization, while multiply scattered photons are depolarized and consist of equal amounts