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Transformation of amorphous calcium carbonate to rod-like single crystal calcite via "copying" collagen template

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ABSTRACT

Collagen Langmuir films were prepared by spreading the solution of collagen over deionized water, $CaCl_2$ solution and $Ca(HCO_3)_2$ solution. Resultant collagen Langmuir monolayers were then compressed to a lateral pressure of 10 mN/m and held there for different duration, allowing the crystallization of $CaCO_3$. The effect of crystallization time on the phase composition and microstructure of $CaCO_3$ was investigated. It was found that amorphous calcium carbonate (ACC) was obtained at a crystallization time of 6 h. The amorphous $CaCO_3$ was transformed to rod-like single crystal calcite crystals at an extended crystallization time of 12 h and 24 h, via "copying" the symmetry and dimensionalities of collagen fibers. Resultant calcite crystallites were well oriented along the longitudinal axis of collagen fibers. The ordered surface structure of collagen fibers and electrostatic interactions played key roles in tuning the oriented nucleation and growth of the calcite crystallites. The mineralized collagen possessing both desired mechanical properties of collagen fiber and good biocompatibility of calcium carbonate may be assembled into an ideal biomaterial for bone implants.

1. Introduction

Biomineralization, or the formation of inorganic phases by living organisms, is an important phenomenon in nature [1–4]. Biominerals serve a number of useful purposes in organisms, such as mechanical support and protection, gravity and magnetic perception, light amplification and transmission, and/or metabolic energy generation [5–8]. What interests the broad scientific community is that the structure and formation of biominerals are controlled over different length scales by macromolecules such as proteins, lipids, glycoproteins, and polysaccharides [2,4,5]. Scientists have long been fascinated with how organisms produce complex composites that are hierarchically organized in composition and microstructure.

It is generally believed that the water-soluble fraction of bioorganic matrix in biominerals has a strong influence on the morphology and polymorphism of minerals, and that the insoluble matrix is thought to serve as a nucleation surface and predefined mold [9–16]. However, it still remains unclear whether the shape of structural protein (insoluble matrix) plays an important role in controlling the direction of growth. So many biomacromolecules with orientated structure and specific shape, such as collagen fiber [17], silk fibroin [18,19], spider silk [20], and silk sericin [21] have been employed as structural templates for biomimetic mineralization. Among of the biomacromolecules, collagen fiber has been extensively studied [22,23]. Nevertheless, those investigations on growth and morphology of HAP or $CaCO_3$/collagen composites were mostly conducted in mixed solutions [24–28], where disadvantages in relation to the solutions should be unavoidable in terms of the preparation, processing, and transfer of target crystals. This inevitably affects the orientation, phase state and morphology of the target crystals on the surface of organic matrices.

As a major biomineralization protein, the structural details of the most abundant collagen of bone (type-I collagen) are already known [29]. This combination of different polar/nonpolar groups makes collagen molecules thermodynamically stable at the air–water interfaces. Fadeev et al. suggested that the monolayer of collagen Langmuir can be pictured as two-dimensionally organized collagen molecules with orientation [30], and it was constituted from fibrils and fibers at the surface of water [30]. They also studied the spread monolayers of fibril protein collagen at air–water interface and claimed that the spread collagen monolayers at air–water interface were able to form supramolecular structures (fibrils) [31]. Fuller reported that the uniformly oriented collagen protein was formed at air–water interface, which was verified by measuring dichroism spectrum [32].

Therefore, in the present research, we adopt collagen Langmuir monolayers as the substrates to grow calcium carbonate via a biomineralization process so as to get rid of the drawbacks associated with solution routes. The aim of these experiments is to find the effect on the crystallization process of calcium carbonate by collagen Langmuir monolayers. It was found that rod-like single crystal calcite was formed by nano-sized covalent building blocks to millimeter rods. These results provide new

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insights into basic mechanisms of collagen mineralization and can lead to the development of novel bioinspired nanostructured materials.

2. Experiment procedures

2.1. Materials

Analytical grade $CaCO_3$, $CaCl_2$ and acetate buffer were obtained from Tianjin Institute of Biological Products (Tianjin, China). Collagen was purchased from Sigma Company Limited (USA) and used without further treatment. It contains 582 amino acid residues and has a molecular weight of 285 KDa and an isoelectric point of pI 5.7. All solutions were prepared using Millipore-Q water with a resistance of 18 MΩ·cm^{-1} and a pH of 7.0.

2.2. Preparation of collagen solution and LB monolayer as well as $CaCO_3$ crystal

Collagen solution was prepared according to the method reported by Fadeev et al. [30], Collagen concentration was 8.6×10^{-7} M. $CaCl_2$ solution ($[Ca^{2+}] = 5.0$ mM) and $Ca(HCO_3)_2$ solution ($[Ca^{2+}] = 5.0$ mM) were prepared according to the procedures reported by Kitano [33]. Collagen Langmuir monolayers were prepared by spreading the collagen solution over deionized water, $CaCl_2$ solution and $Ca(HCO_3)_2$ solution. Resultant collagen Langmuir monolayer on deionized water was transferred onto holey carbon supported transmission electron microscope (TEM) grids at a surface pressure of 10 mN/m and served for TEM observation. Those on $CaCl_2$ solution and $Ca(HCO_3)_2$ solution were compressed to a lateral pressure of 10 mN/m and held there for different duration, allowing the crystallization of $CaCO_3$. The crystallization process was terminated by transferring on-growing crystals onto glass substrates at different intervals at a lifting speed of 3 mm/min. The pressure–area isotherms were measured using a KSV mini-trough at a compression speed of 3 mm/min and constant temperature of 25 °C.

2.3. Characterization

The size and morphology of $CaCO_3$ crystals were analyzed using a JSM-5600LV scanning electron microscope (SEM, Jeol. Ltd., Japan, operating at an accelerating voltage of 20 kV). Crystals supported on the glass substrates were mounted on copper sample stubs with conducting carbon tape and sputtered with gold prior to SEM observation. A JEOL 2010 transmission electron microscope (TEM) (JEOL Ltd., Japan, operating at an accelerating voltage of 200 kV) was performed to analyze the microstructure of $CaCO_3$ crystals. The samples for TEM analysis were prepared by horizontally lifting the collagen LB films through the air/subphase interface with holey carbon supported TEM grids to ensure a good contact between the samples and underlying carbon film. The selected area electron diffraction (SAED) patterns of $CaCO_3$ crystals were obtained from the edges of the samples at a low dose of electron beam so as to avoid possible beam-induced damage to the samples. An X'Pero Pro X-ray diffractometer (XRD, Philips Ltd., Holland, Cu-$K\alpha_1$ radiation, $\lambda = 1.5406$ Å) was performed at a scan rate of 0.080° s^{-1}, voltage of 40 kV, and current of 40 mA to analyze the phase structure of $CaCO_3$ crystals.

3. Results and discussion

3.1. XRD results and analysis

It is well known that the reaction time plays an important role in controlling the nucleation and growth of $CaCO_3$ crystal. Thus the effect of crystallization time on the polymorph of $CaCO_3$ crystals was investigated by means of XRD. Fig. 1 shows the XRD patterns of $CaCO_3$ obtained at different crystallization time. Amorphous calcium carbonate (ACC) was obtained at a crystallization time of 6 h (see curve a in Fig. 1), which is consistent with corresponding SAED pattern shown in Fig. 2.

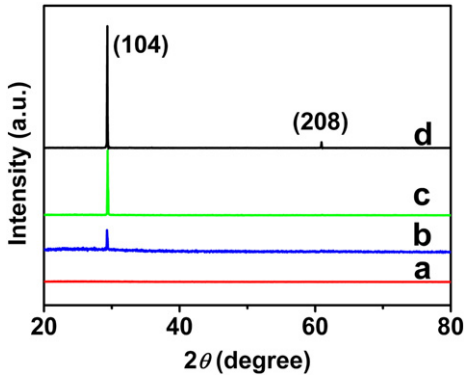

Fig. 1. XRD patterns of calcium carbonate at different intervals, a: 6 h; b: 12 h; c: 24 h; d: 36 h.

Contrary to the above, $CaCO_3$ crystals were obtained at a crystallization time of 12 h and 24 h; and the single sharp diffraction peak (Fig. 1b and c) at a 2θ angle of 29.36° corresponds to (104) plane of calcite (JCPSD No. 83-0578). This indicates that calcite with a high degree of plane orientation is obtained in this case. The above-mentioned XRD analysis confirms that reaction time has a significant effect on the crystallization behavior of calcium carbonate polymorph, and a short reaction time is favorable for the formation of ACC phase in supersaturated $Ca(HCO_3)_2$ solution, which is consistent with the feature of biomineralization process [34]. ACC is thermodynamically the most unstable phase and tends to completely transform into more thermodynamically stable vaterite, aragonite and calcite in aqueous solution in the absence of additives. Therefore, increasing reaction time is to promote the transformation of unstable amorphous calcium carbonate phase into thermodynamically stable calcite in the presence of collagen Langmuir monolayer. In other words, collagen Langmuir monolayer induces the formation of temporarily stabilized ACC in a shortened reaction time, and longer reaction time is favorable for the nucleation of calcite crystal.

3.2. TEM results and analysis

Collagen molecules are able to form nanofibers with a diameter of about 30 nm which is much larger than typical collagen helix diameter (3–5 nm; Fig. 2a) and is due to the self-assembly of collagen molecules. TEM image (Fig. 2b) shows that $CaCO_3$ obtained after growing for 6 h appear as nanoparticles aggregated along the longitudinal axis of collagen fibril. Relevant electron diffraction (ED) pattern (the inset of Fig. 2b) shows that the nanoparticles are amorphous calcium carbonate that acts as an intermediate or even a precursor in biomineralization [34]. After 12 h of crystallization, nanocrystal aggregated along the longitudinal axis of collagen fibril was obtained (Fig. 2c). Corresponding ED pattern (the inset of Fig. 2c) proves that the nanocrystal prepared in this case is calcium carbonate polycrystal. However, the size of the nanocrystal is much smaller than that of the sample obtained after 6 h of crystallization, possibly due to the transformation and dehydration of amorphous calcium carbonate. In other words, a portion of water contained in amorphous calcium carbonate nanoparticles formed at initial process can be dehydrated in crystallization, resulting in reduced size of the nanoparticles. As the crystallization time was further prolonged to 24 h, rod-like $CaCO_3$/collagen particles composed of single crystal calcite phase were obtained (Fig. 2d). Fig. 2e shows the amplified image of the area in rectangle in Fig. 2d, where the rod-like calcite seems to have a diameter of approximately 120 nm which is much bigger than that of collagen fiber without $CaCO_3$ (Fig. 2a). TEM investigations revealed that the rod-like $CaCO_3$/collagen composites consist of intertwined assembly of collagen fibril bundles with a length of more than 1 mm. Each collagen fibril is surrounded by a layer of $CaCO_3$ nanoparticles grown on the surface. Each mineralized bundle of collagen fibrils is much thicker than the self-assembled collagen fibrils, implying