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Initial formation stage and succedent biomineralization of pearls

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ABSTRACT

The initial formation stage and succedent biomineralization of pearls were studied using scanning electron microscopy, Raman spectroscopy, transmission electron microscopy and atomic force microscopy. A new initial formation phase with needle-like structure which is found to be nanocrystallites of aragonite was discovered. As a result, two possible formation modes are proposed to describe the initial formation stage of pearls. As for the succedent mineralization of "brick and mortar" structure, nanostripes were first discovered inside the "brick" (aragonite platelet), compared with the foregoing finding of nanograins. The various nanostructures of aragonite platelet allow us to reconsider the role of the inter- and intracrystalline organic material surrounding $CaCO_3$, and a possible biomineralization mechanism was proposed.

1. Introduction

Mollusk shell and pearls are well-known examples of biomaterials that exhibit an orders-of-magnitude increase in toughness and strength over their predominant constituent material: $CaCO_3$ [1,2]. These remarkable properties have compelled scientists to study the architecture and nucleation mechanism of nacre. Although some endeavors have been made to explore the microstructure of mollusk shell in the past [3–5], the nucleation and growth mechanism of pearls are still not clear. For this reason, in the present work we have focused on the initial nucleation and succedent growth mechanism of pearls.

In regard to the initial formation stage of $CaCO_3$, some experiments have been done in the past. By deposition of a supersaturated $Ca(HCO_3)_2$ solution on a template of monolayer stearic acid, Pouget et al. [6] confirmed a template-controlled initial nucleation mechanism for $CaCO_3$. Similar experiments have also been done in vitro by other authors [7–9]. Therefore, a template-controlled formation mechanism is reasonable to illustrate the nucleation of $CaCO_3$ in biomimetic systems. It is believed that the initial formation phase for the nucleation of shell is amorphous calcium carbonate (ACC) in a cluster structure [8,9]. However, the growth of pearls and shell in vivo is complex, and their actual initial formation is likely to be different from the above-mentioned model. Further research is required.

The succedent mineralization after the initial formation stage of pearls is another issue. The main debates concerning nacre construction can be divided into three categories: a) the organic matrix compartment model [10–12]; b) the mineral bridge hypothesis [13–17]; and c) the hetero-epitaxial growth theory [9,18,19]. Both the compartment model and hetero-epitaxial

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growth theory agree that the aragonite platelets are absolutely spaced by an organic matrix. But this proposal cannot explain the consistent of crystal orientation of the aragonite platelets. The mineral bridge hypothesis proposed by Wada [13] in 1972 has attracted many more supporters. This hypothesis was firmly supported by Schäffer et al. [14], who found that the organic layer through which the "mineral bridges" could grow was porous. Later on, the nanostructure of the biomineral forming the mineral bridge was directly observed in the organic matrix layers of nacre by transmission electron microscopy (TEM) and scanning electron microscopy (SEM) [15]. However, the evidence of mineral bridges was mainly presented for gastropods, not for bivalves [20]. Here, we present observations on pearls produced by the *Hyriopsis cumingii* oyster after almost complete decalcification and examine the structure of the organic matrix surrounding the mineral bridges. It has been pointed out that the identification of the true structural details of these intricate and highly ordered materials requires nanoscale imaging without fixation or the use of organic solvents [14]. For this purpose, we used only EDTA solution to decalcify the samples in order to avoid artifacts.

2. Sample and Methods

A five year old *H. cumingii* oyster was sacrificed, and its pearls were washed with pure water before drying. Cross-sections of the pearls were obtained by cutting and lapping. Subsequently, these samples were almost completely decalcified for 20 h by EDTA (pH = 10) containing 200 mL water, 20 mL ethylenediamine and 7.4 g disodium ethylenediamine tetraacetic acid and then washed in de-ionized water by ultrasonic cleaning.

The cross-section morphology of the pearls was observed using a Hitachi S-4800 scanning electron microscopy at an acceleration voltage of 5 kV. The samples were coated with Au for high-resolution scanning electron microscopy observation.

The Raman spectrum of the central part of the pearls was measured at room temperature. The 532.2 nm line of the Nd:YAG laser was used as the excitation source with a laser power of 100 mW and a laser spot size of about 2 μm × 2 μm. The backscattered light was dispersed by a grating with 1800 grooves/mm. For all measurements with visible excitation, slits were set at 200 μm and a 100× objective was used. The specimens were positioned at the focal point of the spectral collection apparatus with an automatic X–Y–Z translation stage. The acquisition time was set to 30 s in order to achieve maximum intensity without causing detector saturation.

Imaging and analysis of the pearl center were performed with a FEI Tecnai F20 X-Twin transmission electron microscope at a 200 kV acceleration voltage. The specimens were specially prepared using the following process: 1) cut transversely into thin slices of 0.3 mm in thickness from the center of the pearl; 2) mechanically grind the thin slices to 30 μm in thickness with 2000# and 5000# grit wet abrasive paper, and ensure that the center parts remain; 3) form a tiny hole at the center of the sample with a dimpler; 4) retain the circular specimen, glue it onto a single-hole Ni grid and perform ion milling.

Atomic force microscopy (AFM) was used to characterize the structure of the decalcified pearls. The microscopy was a Dimension 3100 connected to a Nanoscope IIIa controller manufactured by Veeco Metrology, Inc. The vertical and transverse resolutions were respectively 0.05 nm and 0.15 nm. The tapping mode of operation was used to obtain both a height topograph and phase information. The tapping mode was originally developed specifically to minimize damage to soft, easily damaged samples [21]. The resonance frequency for the commercial cantilevers used in this study was between 279 and 363 kHz. The scanning resolution was 256 × 256 and the scanning frequency 1 Hz. The sharp probe is made of SiNi with a tip radius of about 7 nm. Data were collected in air at room temperature.

3. Results

Fig. 1 illustrates the central morphology of two *H. cumingii* pearls with increasing magnification. Fig. 1(f) exhibits sample pearl-1 (left one) and sample pearl-2 (right one) and their cross-sectional images. The central pattern of sample pearl-1 shows an amorphous cluster structure with granules of size ranging from 100 nm to 1 μm, as seen in Fig. 1(a–b). Such a cluster structure is similar to the morphology of the amorphous carbonate calcium phase (ACC) discovered by other authors [6–9,22]. Subsequently, the amorphous cluster partially transforms into an aligned "brick and mortar" structure of aragonite platelets. There is no well-defined boundary between the inner aragonite layer and the amorphous cluster. It is noteworthy that there is no prismatic layer of calcite beneath the aragonite platelet formation in the *H. cumingii* pearl, a result which is different from many other seashells such as *Pinctada fucata* [20], *Mercenaria* and *Crassostea gigas* [22], and abalone shell [23].

In contrast, sample pearl-2 has a different pattern in its central part, although it was also cultured from the same *H. cumingii*, as illustrated in Fig. 1(c–e). The initial mineral phase of this pearl is radially distributed just like a chrysanthemum, as seen in Fig. 1(c). Figs. 1(d) and (e) present the details of the heart of the "chrysanthemum" and the subsequent aragonite platelets. Such a "chrysanthemum" pattern is composed of needle-like mineral. It can be seen that this pearl sample does not initially nucleate in the ACC phase, but in a needle-like crystal structure. It should be noted that the thickness of the initial aragonite platelets is about 500 nm, which is much thicker than the platelets that are far away from the center.

Fig. 2 presents the Raman spectra of the central part of pearl-1 and pearl-2 in the range of 100–1700 cm^{-1}. It is generally accepted that aragonite contains four peaks, in which 151 cm^{-1} and 205 cm^{-1} correspond to the translational lattice modes of the carbonate ion; the peak at 1084 cm^{-1} and the doublet peaks at about 699 cm^{-1} and 704 cm^{-1} correspond respectively to the $\nu 1$ symmetric and the $\nu 2$ in-plane bending vibrational mode of the carbonate ion (CO_3^{2-}) [22,24,25]. This is compatible with the Raman spectrum of the central part of the pearl-2 sample, so the mineral phase of the needlelike structure is actually aragonite. However, the Raman spectrum of the central part of the pearl-1 sample shows the characteristics of the family of ACC phases, which only contains the $\nu 1$ symmetric (1084 cm^{-1}) peak of CO_3^{2-} [22,26]. Thus, it is confirmed that the amorphous cluster at the