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# In Vitro Growth of Nacre-like Tablet Forming: From Amorphous Calcium Carbonate Nanostacks to Hexagonal Tablets

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**ABSTRACT:** The structure and formation mechanism of nacre are hot research topics in biominerization. However, there is no specific conclusion about them. Especially for individual nacre tablet forming, many hypotheses still exist. Here we report an in vitro crystal growth on a nacre surface, by which we monitored in real time the growth process of nacre-like tablets and layers on the fresh nacre surface. The formation of nacre-like tablets was a complex and multistep process, from an amorphous calcium carbonate (ACC) layer, iso-oriented nanostacks to hexagonal tablets. We suggest that the interlamellar organic matrix of nacre absorbed calcium and carbonate in amorphous form, and the ACC layer acted as a temporary storage for aragonite crystals. Aragonite nanostacks grew up easily by two-dimensional crystal growth, and then assembled into hexagonal tablets. This process is fascinating, and the proposed mechanisms of nacre-like tablets forming may have indirect and relevant implications for real nacre tablets forming.

## Introduction

Nacre, also known as mother-of-pearl, is a model system of biomimetic nanocomposite and an inspiration for future research in material science and nanotechnology. Nacre structure is a “brick and mortar” arrangement: the bricks are flat polygonal crystals of aragonite, and the mortar is made out of polysaccharide and proteins mainly. A great deal of investigations have yielded valuable information on the formation mechanism of nacre. Of them, the “flat pearl” experiment introduced by Fritz is effective to analyze biominerization processes in their native dynamic states.<sup>1,2</sup> The growth processes of these layers can be monitored by examining abiotic substrates inserted between the growing edge of the shell and the mantle of live red abalones. Flat pearl displayed the developmental sequence of nacre biominerization spatially and temporally from protein sheet forming, calcite layer forming to *c*-axis-oriented aragonite tablets forming. The stack formation of aragonite tablets over abiotic substrates was similar to the stack mode found in Gastropod nacre.<sup>3</sup> Still, real-time observations about nacre tablets forming have not yet been carried out.

On the formation mechanism of individual nacre tablets, there are three important hypotheses, including (i) single crystal growth, (ii) a coherent aggregation of nanograins, and (iii) phase transformation from amorphous calcium carbonate (ACC) or metastable vaterite to stable aragonite. Previous investigations demonstrated that aragonite tablets are oriented nucleated and grow on preformed organic structural substrates,<sup>4</sup> or immediately grow through mineral bridges.<sup>5,6</sup> In contrast to previous works, several elegant studies testing hypotheses of ACC precursor phase and nanograins have been performed for the formation mechanism of individual nacre tablets. Some organisms are able to form stable ACC as building materials, but most biogenic deposits of ACC are precursor phase used as temporary storage sites for calcium and carbonate.<sup>7–9</sup> Nassif revealed that the aragonite platelets in nacre of *Haliotis laevigata* are covered with a continuous layer of disordered amorphous CaCO<sub>3</sub>.<sup>10</sup> Meanwhile, Rousseau provided evidence of nanocrystals within the aragonite tablet in *Pinctada maxima* nacre.<sup>11,12</sup> However,

the understanding of the aragonite platelets formation process remains superficial.

Inspired by advantages of real-time observation, we sought to constitute a simulated biominerization environment in vitro to investigate the growth process of calcium carbonate on original nacre surfaces, which directly resulted in the nacre-like tablets forming with a complicated spontaneous process. The study demonstrated the morphologies and structures of calcium carbonate deposited at different stages using scanning electron microscope (SEM), X-ray diffraction (XRD), and transmission electron microscope (TEM) with selected area electron diffraction (SAED). Then, the growth mode was analyzed and discussed based on the theory of biominerization mechanism and system energy. We suggest that the formation process of calcium carbonate may be relevant to biologically produced nacre tablets.

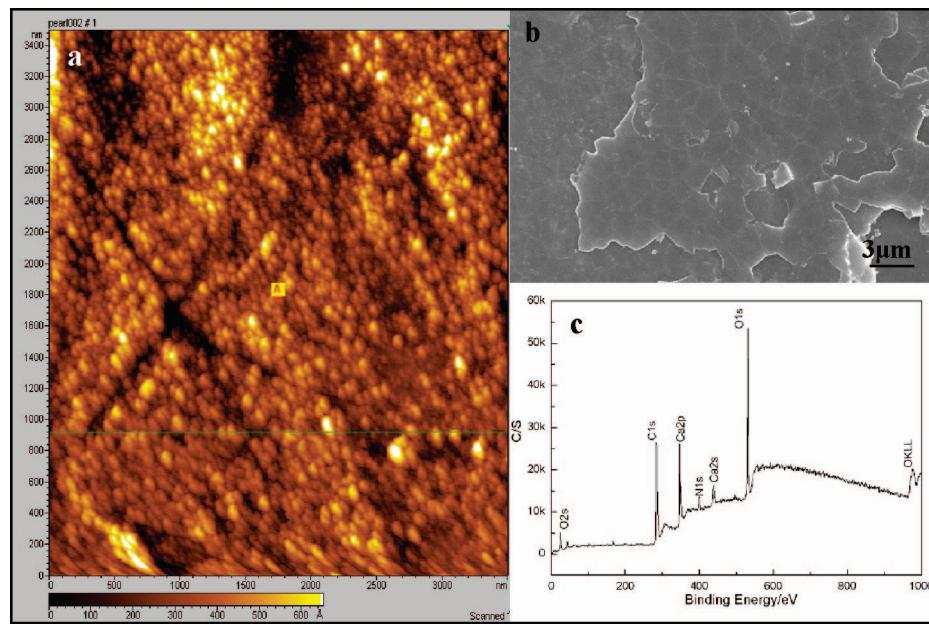
## Experimental Section

Nacre was obtained from freshwater cultured pearls gestated in *Hyriopsis cumingii* Lea. The method of synthesizing crystals was according to the report of Aizenberg.<sup>13</sup> Fresh cleaved surfaces from the inside pearl with the convex side up were put on the bottoms of the culture dishes as substrates for crystal precipitation. Calcium carbonate was deposited by slow diffusion of NH<sub>4</sub>HCO<sub>3</sub> vapor into cell-culture dishes containing 3 mL of 2 mM CaCl<sub>2</sub> solution in a closed desiccator, from 5 min to 10 h at 25 °C. Using this CO<sub>2</sub>-diffusion method, the average supersaturation retains a low level everywhere in the solution. The morphologies and structures of CaCO<sub>3</sub> deposition were observed using scanning electron microscope (SEM, JEOL-1530), X-ray diffraction (XRD, R-AXIS SPIDER) and transmission electron microscope (TEM, JEOL-2011). The original nacre surface was analyzed by atomic force microscopy (AFM, Picoscan) and X-ray photoelectron spectroscopy (XPS, PHI-5300).

## Results

Figure 1a is an AFM micrograph of an original nacre surface showing abundant particles on the nacre layer. Figure 1b is an SEM image of the original nacre surface. Figure 1c is XPS results of the nacre surface, which shows that carbon, oxygen, calcium, and nitrogen were the main surface components. The atomic concentrations of these elements were 56.59 (C), 32.68 (O), 7.33 (Ca), and 3.39 (N), respectively. The Ca atom

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**Figure 1.** Observations of fresh nacre surface. (a) AFM image of the surface; the organic matrix particles with a size of 70 nm were found on the nacre surface; (b) SEM image of nacre surface; (c) XPS results of the nacre surface, which shows the surface element concentrations (atoms): C 56.59, O 32.68, N 3.39, Ca 7.33.

concentration is lower than the C atom concentration, which indicated that this surface was mainly made up of organic matrix. The deposition of calcium carbonate does really occur on this organic surface.

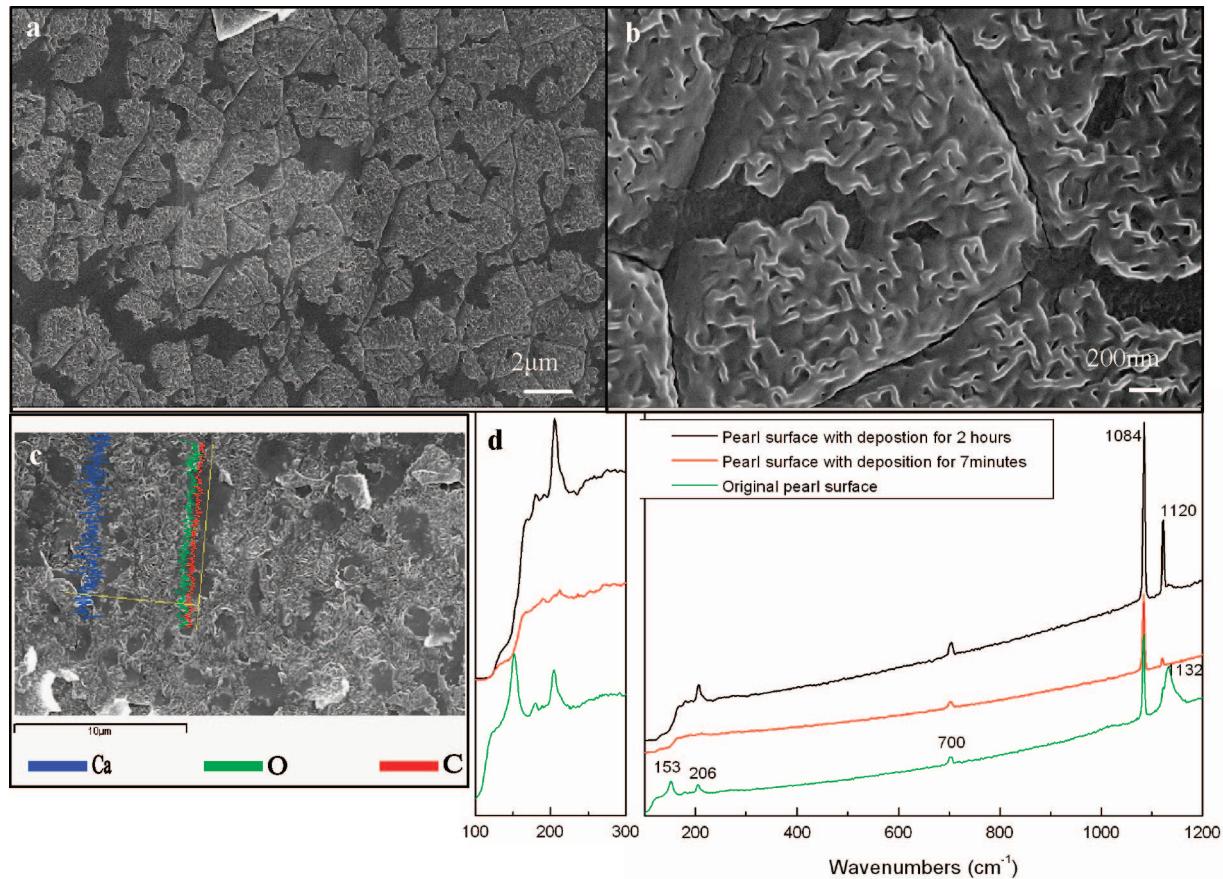
In the first 5 min of deposition, precipitates with a “noodle” shape were formed on the original nacre surface, as shown in Figure 2. These flexuous noodles paved the nacre tablets except the gaps between the tablets. Was the deposition caused by dissolution of organic or precipitation of  $\text{CaCO}_3$ ? A comparison experiment was carried out under similar conditions without supersaturation of  $\text{CO}_2$  vapor from 5 to 30 min, and nothing was observed on the nacre surface. EDS results also show that the main component of the noodle is  $\text{CaCO}_3$ , as shown in Figure 2c, and the Ca content of the noodles is similar to that of the original nacre surface. Figure 2d is the Raman spectra of the nacre surface with different deposition times. Two sharp 153 and 206  $\text{cm}^{-1}$  peaks of aragonite tablets were changed to a broad rise after these noodles  $\text{CaCO}_3$  were deposited on the nacre surface. As a rule, the Raman spectra of stable amorphous calcium carbonate (ACC) are characterized by a broad peak in the lattice frequency region 150–300  $\text{cm}^{-1}$  that occasionally appears as a continuous baseline rise rather than a discrete peak of crystalline.<sup>14</sup> So we confirm that the noodles  $\text{CaCO}_3$  is ACC. After aragonite crystals grew instead of ACC, we cannot detect the existence of ACC using Raman spectroscopy. However, we cannot confirm whether the amorphous-to-aragonite transition occurred or not, because the Raman spectrum of ACC is weak and the ACC layer is thin, and ACC would be easily overlooked when associated with a crystalline form of calcium carbonate.

Observations using SEM show in detail the crystallization process of calcium carbonate on the fresh nacre surface in  $\text{CaCl}_2$  solution with a supersaturated  $\text{CO}_2$  atmosphere from 10 min to 10 h. At the early stage, ACC phase transitioned the noodle structure into a film coating on the entire nacre surface and even the gaps between tablets, so we observed a successive film shown in Figure 3a. In succession, the initial crystal nucleation occurred during the first 30 min. Figure 3a shows that these incipient crystals were distributed averagely, and grew up continuously to form tabular hexagon. New crystals were

crystallized at the tops of formed nanoplates as old hexagonal plates kept on expanding laterally, just like the stack mode of “nano-Christmas trees”. These nano-Christmas trees were independent and unattached at the original stage. Figure 3b,c shows the crystal growth at 1 and 1.5 h, respectively. The crystals formed at the top of the stacks and then expanded laterally with the growth as more crystals formed on top. Continued lateral growth of formed crystals still maintained the conical shape of the stacks and produced flat tabular crystals. Quickly, these nanostacks spread over the surface of nacre, and spontaneously showed the profiles of each nacre-like tablet, marked by yellow lines in Figure 3c. Figure 3d shows that the bottom crystals platelets have expanded laterally to contact each other and formed a whole as the new crystals continued to form at the tops. When lengthwise growth was restricted or lateral growth exceeded lengthwise growth, lateral expansion ultimately brought adjacent nanostacks into contact, and their lengthwise growth surfaces became smooth. These crystal blocks by aggregation of nanostacks performed a complete single crystal character without holes, gaps, and slits, as shown in Figure 3e. Figure 3f shows the growth state of calcium carbonate on nacre for 10 successive hours. Numerous nanostacks coalesced to form the broad hexagonal tablets with a uniform size between 3 and 4  $\mu\text{m}$ . These hexagonal tablets had a shape and size similar to real nacre tablets. Therefore, we obtained a nacre-like layer composed of calcium carbonate on the original nacre surface.

Figure 4 shows the interface between the nacre surface and the growing  $\text{CaCO}_3$  crystal, which indicated that aragonite nanostacks grew on a kind of flexible substrate. Because of the tiny amount and special location of this flexible medium, it is difficult to confirm what it is exactly ACC or protein.

Figure 5b–f are TEM images of individual nanostacks isolated by sonication of a sample with nanostacks on the nacre surface in acetone. Combining with the top view of stacks shown in Figure 5a, we found that the stack is composed of parallel 12-nm-thickness hexagon plates. The HRTEM image indicates that several neighboring plates in one stack have the same crystal lattices and orientations, just as a perfect single crystal without any lattice distortions and crystal defects, which is an obvious



**Figure 2.** Observations of  $\text{CaCO}_3$  grown on fresh nacre tablets for 5 min. (a) SEM image of the  $\text{CaCO}_3$  deposition on nacre; (b) the amplified observation of (a); (c) EDS results of nacre with “noodles” depositions, which indicated that these noodles were  $\text{CaCO}_3$ ; (d) Raman results of nacre surfaces with and without the early noodles  $\text{CaCO}_3$  depositions.

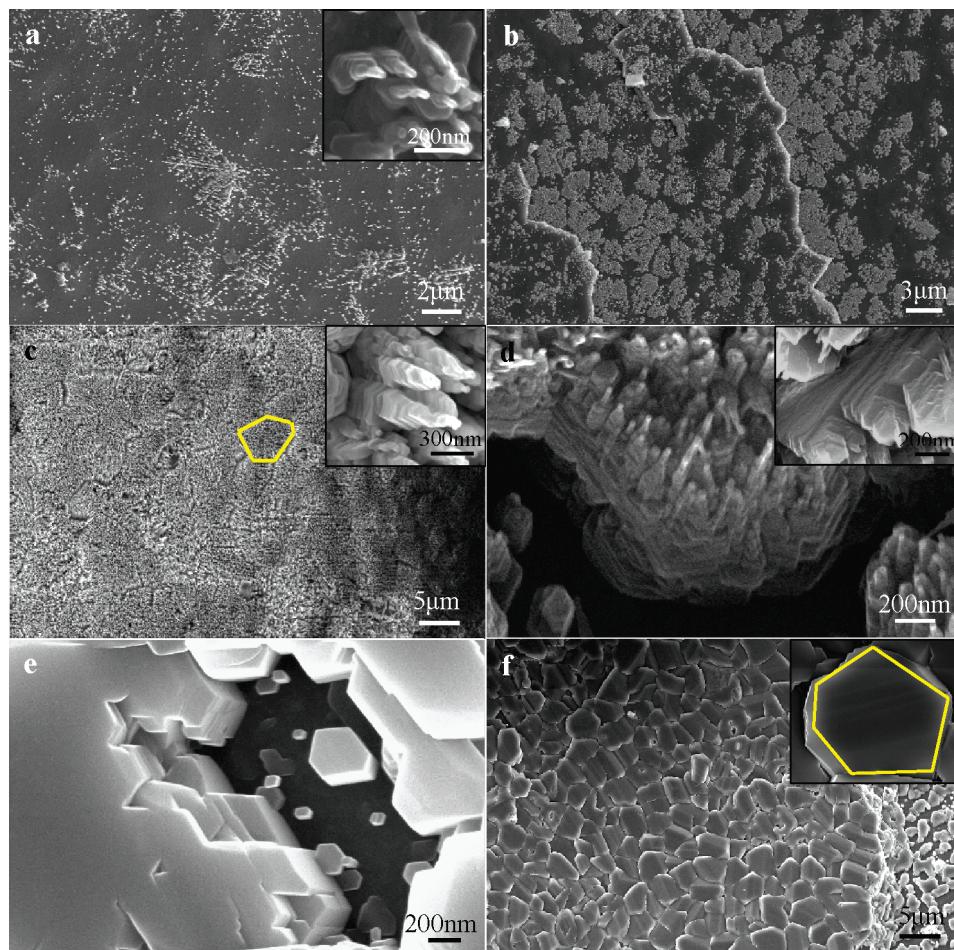
two-dimensional nucleation on flat crystal surface. SAED patterns, the inset of Figure 5d, show that the observed surface is aragonite ( $\bar{1}10$ ), its normal direction is perpendicular to the growth direction toward the spire of the stacks. Other HRTEM images, as shown in Figure 5e,f, offer similar information that two adjacent stacks have consistent crystal lattices and orientations, and the observed surface is aragonite (010) whose normal direction is also perpendicular to the growth direction of stacks. Therefore, the growth direction toward spire is aragonite [001] direction according to vector multiplication operation.

## Discussion

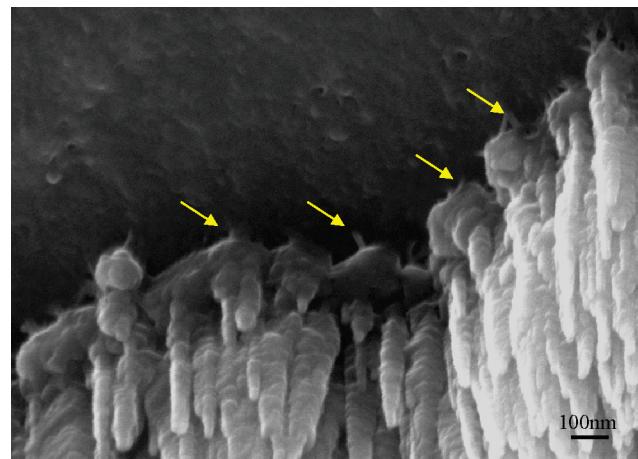
ACC in its pure form is highly unstable that may form in supersaturated solutions of calcium carbonate, but transforms within minutes into the stable crystalline phase. The biogenic depositions of ACC are used as stable building materials, or as temporary storage sites for calcium and carbonate as a precursor. In biological environments, ACC is stabilized by specialized macromolecules rich in hydroxyamino acids, glycine, glutamate, phosphate, and polysaccharides.<sup>15</sup> Some calcite crystals in certain biological systems formed through the transformation of transient ACC.<sup>16</sup> But the formation and transition of amorphous precursor  $\text{CaCO}_3$  in nacre have been still discussed. Weiss investigated the shell formation process of mollusk bivalve and considered that ACC is the precursor form of aragonite.<sup>17</sup> Nassif's study first reveals that the aragonite platelets in nacre of *Halimeda laevigata* are covered with a continuous layer of disordered amorphous  $\text{CaCO}_3$ .<sup>10</sup> Most of ACC particles produced in in vitro experiments are spherical.<sup>4,18</sup>

We first found that amorphous precursor  $\text{CaCO}_3$  formed special “noodles” shapes and then grew into a film on the nacre surfaces in this study. However, we can not confirm whether the amorphous-to-aragonite transition occurred or not during the crystal growing process. In fact, ACC is difficult to be detected when associated with a crystalline form of calcium carbonate; its presence may well have been overlooked. Nassif found a continuous layer of disordered amorphous  $\text{CaCO}_3$  with aragonite platelets in nacre. Contrarily, Aizenberg also found ACC before calcite single crystals forming during the in vitro experiment of crystal growth on templates, and she suggested that the calcite was formed during the amorphous-to-crystalline transition.<sup>19</sup> Some previous studies suggested the transition did occur using FTIR or Raman. We also used Raman spectroscopy to test, but it may be not enough evidence to confirm the transition. However, ACC can serve as a precursor and storage of calcium and carbonate at least.

If ACC did not disappear completely, the ACC layer not only stores calcium and carbonate, but also serves as the nucleation sites of iso-oriented aragonite crystals. Nassif considered that oriented growth of aragonite tablets in ACC layer are not in contradiction to the observed [001] orientation of the aragonite platelets within the organic matrix, see the detailed explanation in ref 10. Volkmer observed that highly oriented single and multiple layers of calcite crystals are grown epitaxially on the underlying polycrystalline thin film, and he thought that highly organized crystal architectures such as nacre or the foliated and prismatic layers may grow spontaneously and continuously once a first crystal layer has been deposited, requiring no further



**Figure 3.** SEM images of  $\text{CaCO}_3$  grown on nacre surface for different times. (a) 10 min; (b) 30 min; (c) 1 h; (d) 2 h; (e) 3 h; (f) 10 h.



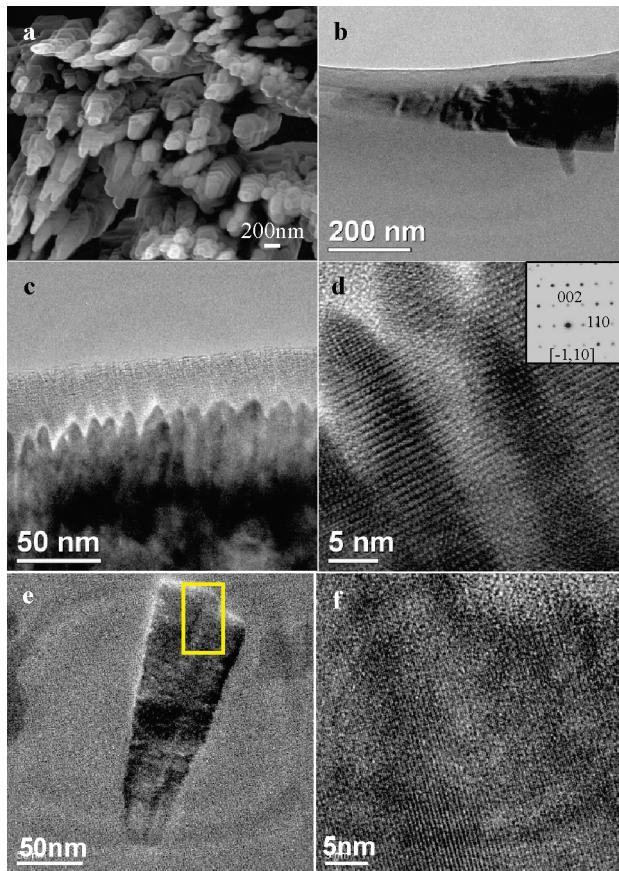
**Figure 4.** SEM images of the interface between the nacre surface and  $\text{CaCO}_3$  crystal, which indicated that aragonite nanostacks grew on a kind of flexible substrate (ACC or protein).

elaborate control mechanisms.<sup>20</sup> So if ACC did not disappear, oriented growth of aragonite nanostack might occur spontaneously. Besides, for most of biological systems, the existence of ACC has its own specific functions, for example, enhance interface toughness, release interface lattice distortion and structure stress, and expel impurities and macromolecules from the crystalline phase.

The stack mode of biogenic  $\text{CaCO}_3$  occurs in gastropod nacre, and was also found in bionic simulation processes using the

“flat pearl” method. Wise suggested that the stack mode of deposition in Gastropod nacre is functionally useful to the animal because it increases the number of crystals that can form and develop on the growth surface at one time.<sup>3</sup> This stack structure of nacre tablet reported in previous studies was confirmed to be caused by a mineral bridge. However, the stack structure observed in this study occurred in the inner of the nacre tablet, and was caused by multiple two-dimensional (2D) nucleation and layer-by-layer growth. Within the Burton-Cabrera-Frank (BCF) formalism and its later modifications, growth by 2D nucleation at low supersaturations is quite difficult due of the large activation energy for formation of a nuclear whose radius exceeds that of the critical nucleus. However, some improved reports show that 2D nucleation would take place easily at low supersaturations in the presence of biomacromolecule. In the present work, it is possible that the fresh soluble organic matrix of nacre was dissolved in solution to induce 2D nucleation and layer-by-layer growth of aragonite crystals. Pokroy found similar 2D growth of aragonite in inorganic substrate without protein.<sup>21</sup> In our opinion, the mechanism of 2D growth is complex and has many control factors; protein is probably one of them.

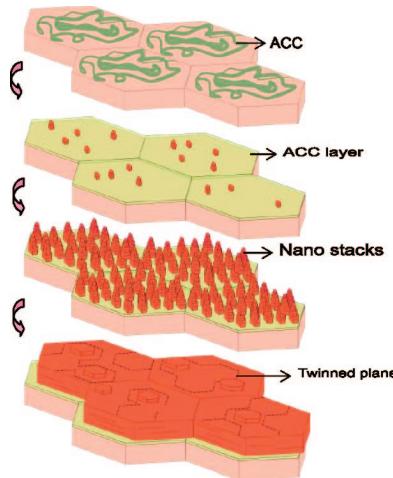
With regard to the nanostructure of individual nacre tablets, Rousseau provided evidence that the individual aragonite tablet is composed of “hybrid composites” of nanoaragonite crystals and organic matrix.<sup>11</sup> Bruet and Takahashi’s observations indicated the existence of nanostructure in nacre directly.<sup>22,23</sup> But the reasons for the formation of iso-oriented nanoparticles are still unknown. Self-organization arrangement of mesocrystals is an important supposition in biominerization to explain the



**Figure 5.** Morphologies of aragonite stacks. (a) SEM image of many stacks grown on nacre; (b) TEM image of the section of an individual stack; (c) the amplified observation of (b) showing many parallel plates; (d) HRTEM image of several plates that have the same crystallographic orientation. The inset of (d) is the SAED patterns of this region; (e, f) TEM images of two neighboring nanostacks, which indicates that neighboring stacks have the same crystal orientation.

“single crystal” character of aggregate crystals.<sup>24</sup> In this study, we propose a new mode to explain the aggregation of iso-oriented nanoparticles to form crystallographically continuous particles in in vitro or biomineralization processes. It is readily understood that iso-oriented growth of nanocrystals on certain templates can form a pseudo crystals via the incessant growth, just like what was observed in this study. This nanostacks mode by 2D nucleation occurred in other material systems,<sup>25</sup> but was used in biomineralization process for the first time. The nano cluster of biocrystals and organic matrix existing in nacre was observed frequently in previous studies.<sup>26–28</sup> However, Nudelman’s study shows an independent nucleation site for each crystal tablet.<sup>29</sup> So the conclusions about the forming mechanism of nacre tablets remain unclear.

Twinned crystals of aragonite have been reported previously in the ligament, nacreous structure of mollusc shells, and the crossed lamellar structure of bivalves.<sup>30–32</sup> Both Marsh and Iwao considered that this twinned structure is a growing defect, and the existence of the organic matrix may be important for the formation of twinned aragonite crystals.<sup>30,32</sup> This twinning mainly occurs on the {110} system of aragonite. In this study, [001] directions of nanostacks are toward spires, the six side surfaces of hexagon plates are aragonite {110} planes. It is imaginable that {110} twinning of aragonite occurs easily when the side surfaces of neighboring nanostacks contact each other. The twinned crystals in nacre-like tablets still can be considered as growing defects but is irrelevant with organic matrix.



**Figure 6.** A schematic representation of the growth sequence of  $\text{CaCO}_3$  on nacre. From ACC, nanostacks to hexagonal nacre-like tablets.

The unattached hexagonal nacre-like tablets, instead of the entire aragonite layer, were formed via the nanostacks assembling. There are three possible reasons for this: (1) the large misorientation angles between adjacent nacre-like tablets; but this cannot explain why the shapes and dimensions of nacre-like tablets are uniform and the boundaries between them are straight and smooth; (2) nanostacks are hex-prisms, so the tablets easily form the hexagonal shape; (3) the presence of hexagon structure follows the lowest energy principle, such as a snowflake, honeycomb, and Giant’s Causeway in Ireland. Different from this process of nacre-like tablets forming, real nacre tablets are separated by organic matrix. But it is still unclear whether the wrap of organic matrix exerts the action on the final shapes of nacre tablets or not.

## Conclusion

In conclusion, we observed in real time the growth process of aragonite on the fresh surface of nacre in supersaturated  $\text{CaCO}_3$  solution. The process is as follows (Figure 6): the deposition of ACC, the iso-oriented growth of nanostacks, and the nacre-like tablets forming. These hexagonal tablets with 3–4  $\mu\text{m}$  in diameter have a strong texture and the *c*-axis is perpendicular to the tablet surface. These nacre-like tablets were aligned closely to form a layer that is similar to a real nacre layer. In this process, interlamellar organic matrix absorbs calcium and carbonate, and ACC serves as a precursors phase and plays an important role in nanostack forming. This complex self-assembled process is interesting and may offer new assumptions for nacre forming.

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