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Rongqing Zhang
School of Life Sciences
Tsinghua University
Beijing, China

Liping Xie
School of Life Sciences
Tsinghua University
Beijing, China

Zhenguang Yan
Chinese Research Academy
of Environmental Sciences
Beijing, China

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Contents

Chapter 1
Molecular Basis of Biomineralization in *Pinctada fucata*

Abstract Biomineralization is the accumulation and formation of minerals regulated by living organisms transforming into biological structures and tissues. This is an extremely widespread phenomenon since we've found many creatures in all six taxonomic kingdoms which could form biominerals, and more than 60 different types of them have been identified, such as silicates in diatoms and algae, carbonates in invertebrates, and carbonates and calcium phosphates in vertebrates. These minerals often form structures like sea shells and the bone in mammals and birds.

The study of biomineralization started at the first half of the twentieth century by the extensive use of optical microscopy, which helped a lot to identify various types and patterns of structures. It was not until the 1960s and 1970s, with the application of transmission electron microscopy (TEM), the scanning electron microscopy (SEM), radioisotope, the biochemical research on membrane transport, the analysis of bone-related organic materials, and the understanding of the ultrastructure of mineralized materials, was the initial idea of the biomineralization formed. Though some progress has been made in revealing the principles of these processes during the past few decades, there are still a lot of unknowns waiting for more exploration. The main content in this book primarily focused on the following aspects: the identification and function characterization of biomineralization-related genes, including those that encode shell matrix proteins and enzymes and participate in calcium metabolism; the cellular regulation such as cell signaling pathways during biomineralization process; and the ecological studies on biomineralization in *Pinctada fucata*.

Keywords *Pinctada fucata* · *Biomineralization* · *Molecular basis* · *Introduction* · *Calcium metabolism*

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1.1 Introduction

Biomineralization is an extremely widespread phenomenon, the process of which is conducted by reaction and precipitation converting ions into solid minerals [1, 2]. Specifically, biomineralization refers to organisms using their synthetic organic macromolecules manipulating the process of the inorganic crystal nucleation, growth, and molecular arrangements, eventually producing minerals with special optical, magnetic, and/or mechanical properties [2, 3]. Biomineralization involves biologists, chemists, and geologists in interdisciplinary studies at the interfaces between Earth and life.

Almost all living creatures, from lower unicellular organisms or the plants to higher animals, are closely associated with the products of biomineralization, biominerals. But the biomineralization mechanisms involved in different biological systems are quite complicated and exhibit a large diversity, and more than 60 different biominerals have been identified, including bones responsible for supporting and movements in vertebrates; shells functioning on protecting soft tissues in bivalves, brachiopods, and foraminifers; otoliths in fishes and mammals for balancing movements; magnetosomes found in magnetotactic bacteria sensing the changes of geomagnetic field; etc. [4]. The main chemical compositions of these biominerals mentioned above contain calcium phosphate, calcium carbonate, and oxidation silicon. Among them, mollusk shells are composed of calcium carbonate or calcium magnesium carbonate, while the bones and teeth in mammals are mainly calcium and magnesium phosphate [4–6].

Biomineralization processes could divided into two fundamentally different categories based upon their degree of biological control, namely, biologically induced biomineralization and biologically controlled biomineralization [4, 6]. Bio-induced mineralization refers to the deposition of inorganic minerals formed by the reaction of the metabolites at the cell wall when they are expelled or into the cell wall, common in protists, alga, and lower prokaryotes. Characterized by the low-degree control of this process, the crystals or biominerals formed during bio-induced mineralization usually lack unique morphologies, most of which are irregular and disorganized. In contrast, in "biologically controlled" mineralization, the organism uses cellular activities to direct the nucleation, growth, morphology, and final location of the minerals which are deposited. While the degree of control varies across species, almost all controlled mineralization processes occur in an isolated environment. The results can be remarkably sophisticated, species-specific products that give the organism specialized biological functions [1, 7, 8]. In conclusion, elucidating the underlying mechanisms of biomineralization will lead to innovative understanding of the field of materials and medicine sciences and also provide significant technical supports for pearl shellfish farming and pearl production.

1.2 General Features of Biomineralization in *Pinctada fucata*

1.2.1 *The $CaCO_3$ Crystalline Forms in* Pinctada fucata

There are six different types of natural $CaCO_3$ biominerals, calcite, aragonite, vaterite, $CaCO_3 \cdot H_2O$, $CaCO_3 \cdot 6H_2O$, and amorphous calcium carbonate (ACC), the chemical compositions of which are exactly the same; nevertheless, their crystal structures are totally different. Among these different crystalline forms, calcite and aragonite are thermodynamically stable and most common in mollusk shells [9]. Aragonites could be induced in calcite crystallization system with the addition of 50 mM Mg^{2+}. Vaterite is the metastable status of $CaCO_3$ and the most unstable crystalline. And it could be transformed to calcite or aragonite spontaneously in aqueous solution. Vaterite has not been found in our animal model *P. fucata*, while some exceptions have been reported in other close-related species. A new morphology of vaterite was confirmed in lackluster pearls of *Hyriopsis cumingii* [10]. Moreover, it also formed in the abnormal shells of *Corbicula fluminea* [11]. ACC has been found in plant larvae and the initial stage of shell biomineralization. ACC is usually considered as the precursor in shell formation [12]. In spite of the rapid transformation from ACC to other stable crystals in aqueous solution, it is still relatively stable due to the adhesion of the macromolecules like polysaccharide to its surface. Organisms control the biomineralization process by the regulation of the formation, stabilization, destabilization, and transformation of ACC [13].

1.2.2 *The Structure of the Shell*

Pearl oysters, *Pinctada fucata*, famous as important economical pearl production species, are one of the best studied biomineralization models [14]. The shell can be divided into two distinct layers, the periostracum and the calcified layer (Figs. 1.1 and 1.2).

The periostracum is the organic layer of the hardened organic products on the outer surface of the shell [16]. The hardened part is mainly composed of insoluble Quinone tannin containing dopa [17]. Besides protecting the shell from the destruction of acidic substances and parasites, periostracum also plays important roles during biomineralization. It could block the extrapallial space on the ventral side, isolating the extrapallial fluid from the external environment, delimitating, and sealing the space where the biomineralization takes place thus provides a supersaturation niche for crystal deposition. What's more, the periostracum may also function as a substrate for Ca^{2+} deposition and even affect the formation of prismatic layer [18, 19].

The calcified layer could be subdivided into prismatic layer and nacreous layer, where $CaCO_3$ crystals are deposited as calcite and aragonite, respectively [20–

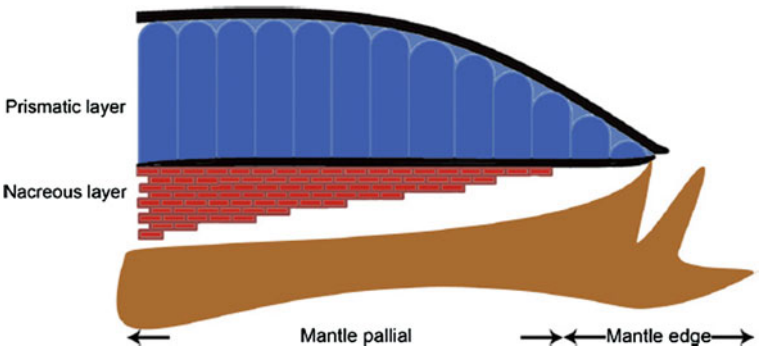

Fig. 1.1 Diagram of the cross section of the shell and the mantle of *P. fucata* [15]

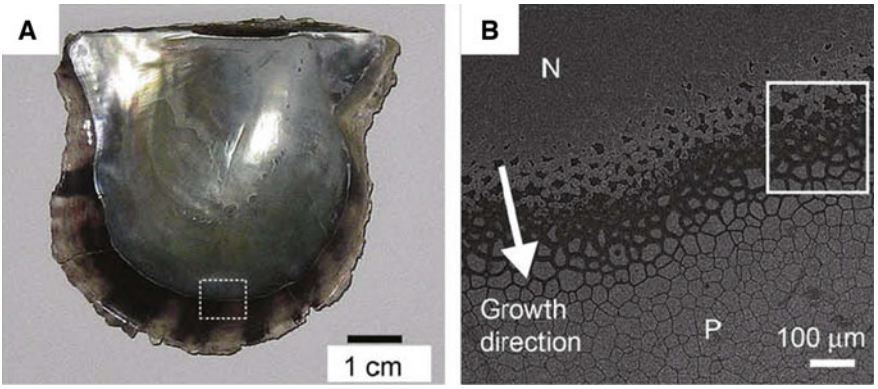

Fig. 1.2 The shells of *P. fucata* [23]
(**a**) The inner shell of *P. fucata* and (**b**) SEM pictures of the inner surfaces. *N* nacreous layer, *P* prismatic layer. The square frame indicated the growth zone

22]. Our concerns on biomineralization studies primarily focused on these two layers (Fig. 1.2). The shell calcification is mainly composed of calcium carbonate crystals with a total weight of more than 95% and much more smaller amount of organic substrates (mainly proteins and polysaccharides) [4].

The initiation of prisms occurs in the proximal region of the outer surface of the outer mantle fold in the pallial space bounded externally by the periostracum [24]. SEM pictures showed that prismatic layer is composed of a great amount of parallel column calcites perpendicular to the shell surface, the cross section of which are regular polygons, and the edges of these "polygons" are surrounded with organic matrix [24, 25] (Fig. 1.3). A lot of studies have proved that the organic matrix could regulate the growth of these calcites in prismatic layer and its main components are proteins and chitin [26, 27]. The cells form mantle edge are commonly believed to be responsible for the formation of prisms [17] (Fig. 1.1).

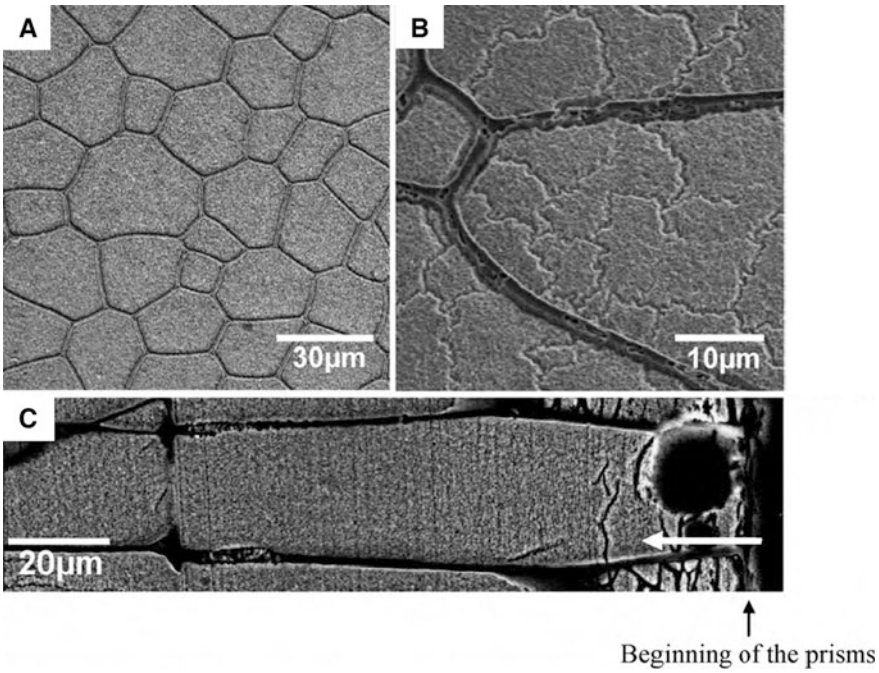

Fig. 1.3 SEM images of the prismatic layer of *P. fucata* etched by EDTA [28]
(**a**) Cross section of the prismatic layer in *P. fucata*, (**b**) enlarged view of the grooves, and (**c**) longitudinal cross section of the prismatic layer. The arrow indicated the growth direction.

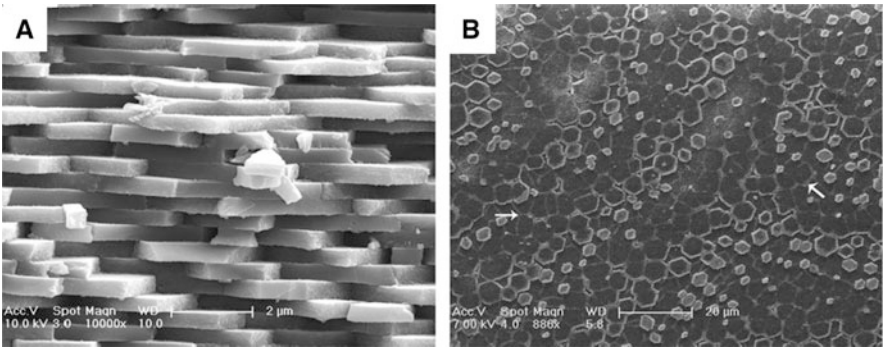

Fig. 1.4 Nacre structure of *P. fucata* [31]
(**a**) The cross section of nacreous layer, composed of continuous parallel lamellae; (**b**) each lamellae is composed of polygonal aragonitic tablets, sealing each other by the intertabular matrix.

The nacreous layer is composed of aragonite tablets, and the aragonite sheet is 0.4 to 0.5 μm in width and 5–10 μm in thickness [29] (Fig. 1.4). These are typical nanostructures. Besides regulating crystal formation and growth, the organic matrix filled in aragonites could spread the force, significantly improving the mechanical

strength of the shell especially when subjected to external forces. The mechanical properties of the nacreous layer, also known as "dense slit brick structure," are approximately 3000 times as strong as the mineral aragonites, thus providing adequate protection for the soft tissue inside the shell [30].

1.2.3 The Process of Shell Formation

The main component of the shell is $CaCO_3$ of course; however, those macromolecules playing crucial roles in the formation of the delicate microstructures during biomineralization only account for less than 5% [32]. Studies on the regulation of matrix proteins during shell formation are one of the current research hot spots. Several different shell formation models were brought up recently [2, 33], but the specific mechanisms have not been clearly elaborated.

The life circle of *P. fucata* could be divided into six stages, including the fertilized egg, trochophore stage, D-shaped stage, umbonal stage, juvenile stage, and adult [34]. The polymorphism of $CaCO_3$ crystals and the shell microstructure changes a lot throughout these stages [35]. Prodissoconch I, forming during early D-shaped stage, is the original form of the shell and is considered to be composed of ACC [34, 35]. Prodissoconch II with a homogeneous structure of aragonite and a thinner layer of calcite appears at the lateral D-shaped stage or umbonal stage. The formation of prismatic and nacreous layer completes while entering the juvenile stage (Fig. 1.5).

Bar, 20 μm in (**a**), (**b**), and (**c**); bar, 200 μm in d. *P1* prodissoconch I shell, *P2* prodissoconch II shell, *D* dissoconch shell, *PL* prismatic layer, *NL* nacreous layer

Classically, the physiology of molluscan shell calcification can be described as a succession of compartments, the most important of which are the inner shell surface, extrapallial space, and outer mantle epithelium. The mantle tissue, coating the inner surface of the shell, with the other compartments being the extrapallial space and the shell, is the critical organ during mollusk shell formation and comprises internal and external epithelium, connective tissue, mantle muscle, and nerve fibers. The ridge between the outer and median folds is the periostracal groove, responsible for the formation of periostracum (Fig. 1.6).

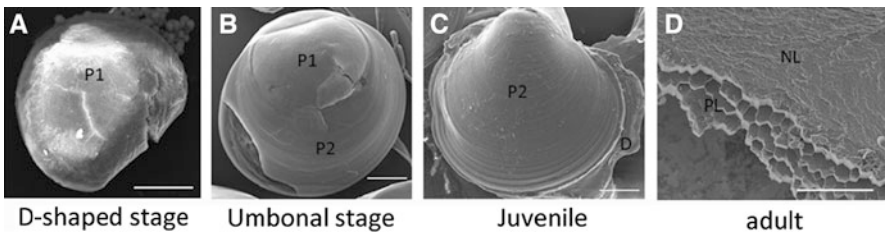

Fig. 1.5 The SEM pictures of three larval development stages in *P. fucata* [36]

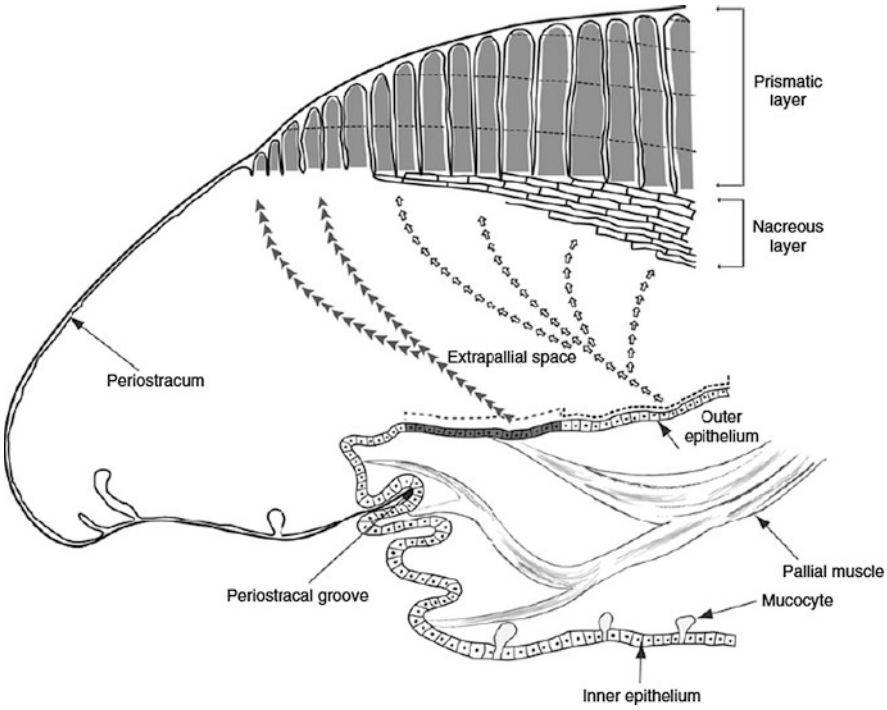

Fig. 1.6 Physiology of the shell calcification in a nacro-prismatic bivalve [17]

Precursor ions, calcium and bicarbonate, are transported via the hemolymph and then directed toward the outer mantle epithelium. After extrusion from the cytosols to extralpallial space, the liquid precursor ions, Ca^{2+} and CO_3^-, are gradually transited to solid $CaCO_3$ on the inner surfaces of the shell under the regulation of matrix macromolecules, especially matrix proteins [17] (Fig. 1.6). But how the organic matrix interacts with the mineral ions to produce this well-designed and fine-structured biomineral is still under debated and more investigation.

Extrapallial fluid (EPF) is a colorless, bloodlike fluid locating in the extrapallial space, where the blood, EPF, and ambient medium (e.g., seawater) carry out the frequent exchanges of substances [37]. The special roles EPF playing during shell formation hasn't gained enough attention initially, fortunately, with the developing of the research, scientists realized its importance. Some evidence suggested that matrix proteins may accomplish self-assembling in extrapallial space, which may explain the formation of shell-organic scaffolds [17, 38]. Whereas, this concept is still debated, and more proofs should be provided for further validation.

Moreover, we shouldn't ignore the functions of hemocytes, those free circulating cells of hemolymph, during shell formation and construction. The studies on hemocytes originally focused on its role in immune defense system [39] and tissue reparation process [40]. These functions have been well underlined during the past few decades, but their contribution to biomineralization may be greatly

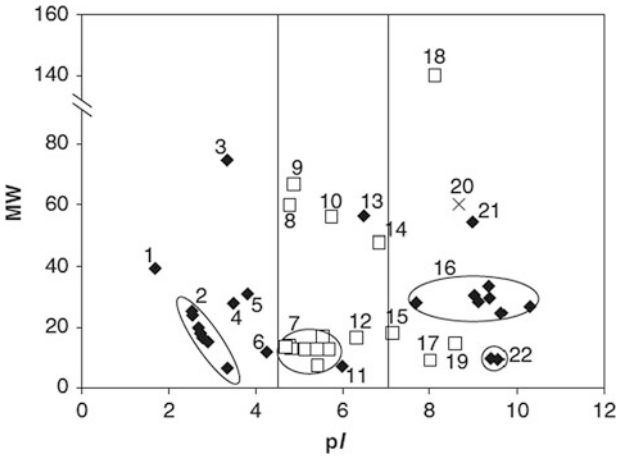

Fig. 1.7 The molecular weight (MW) and isoelectric point (pI) distribution of partial identified shell matrix proteins [17]
The theoretical MW and pI were calculated after identification and removal of the signal peptide. (1, aspein; 2, Asp-rich proteins; 3, ASP-1; 4, ASP-2; 5, MSI31; 6, prismalin-14; 7, N14/N16/pearlin/perline proteins masking AP7 and AP24; 8, MSI60; 9, mucoperlin; 10, nacrein from *P. fucata*; 11, MSI7; 12, dermatopontin; 13, tyrosinase-like 1; 14, nacrein from *T. marmoratus*; 15, perlucin; 16, shematrin proteins; 17, perlustrin; 18, lustrin A; 19, perlwapin; 20, N-66; 21, tyrosine-like2; 22, KRMPs)

underestimated. According to Mount et al., hemocytes could directly release the calcite crystals for successive remodeling at the biomineralization site [41]. But we still need more evidence to confirm whether this is a general metabolic pathway in bivalves or just a particular phenomenon in eastern oyster.

1.2.4 Shell Matrix Proteins

As mentioned in the introduction and detailed later, the organic matrix is a mixture of proteins, glycoproteins, chitin, and lipids. The matrix, especially matrix proteins, interacts with the mineral ions and controls the shape, deposition, and crystalline forms of the produced crystals.

The earliest systematic studies on shell matrix were conducted by Crenshaw et al. in 1972. They used EDTA, a calcium-chelating agent, effective at weak dilute acids to decalcify the shells of *Mercenaria mercenaria*. However, due to the limited conditions, their studies predominantly concentrated on the integral components, amino acid composition of the mixture, or its general functions during shell formation and reparation [42–45]. According to those successfully isolated and identified matrix proteins, we summarized three fundamental features of them (Fig. 1.7):

1. Certain amino acid residues tend to take up extremely high proportions, more commonly are asp, Gly, and Ser, sometimes including pro and Cys. The carboxyl group in side chain of asp may bind with Ca^{2+}, facilitating the formation of $CaCO_3$ crystal lattices. Gly and pro are more likely relevant to the flexibilities of protein structures, for example, proline could form a cycle to the polypeptide backbone. As for cysteine, it contributes to the formation of covalent disulfide bonds to other cysteine residues [46, 47].
2. The sequences of shell matrix proteins (SMPs) are usually composed of different domains. And these domains generally contain modular structures with distinct functions [48, 49]. The first identified matrix protein, nacrein, possesses a carbonic anhydrase domain [38]. Lustrin a contains ten highly conserved cysteine-rich domains and a whey domain [50]. Analyses of these genes of various modular proteins implied that gene assembly may be promoted by intronic recombination, i.E., the proteins were formed by exon shuffling [51, 52].
3. A large amount of matrix proteins have been posttranslationally modified, such as phosphorylated and/or glycosylated [50, 53]. And these posttranslational modifications often associate with the binding properties with Ca^{2+} [54, 55].

With the wide use of new technologies, over 40 different kinds of matrix proteins have been identified from different mollusks. But we still cannot illustrate the molecular mechanisms of shell formation; plenty of questions remain unsolved. The isolation of SMPs lays the foundation for its further function characterization. We went through three stages on this process:

1. As mentioned above, EDTA, ethylic acid, and water were frequently used to dissolve the shell, among which EDTA is the most widely used agent. This decalcification procedure yields two organic fractions, EDTA-soluble matrix (ESM) and EDTA-insoluble matrix (EISM). ESM and EISM have been proved to play different roles during shell formation. EISM often functions as the organic scaffold, providing crystal nucleation sites for $CaCO_3$ deposition, while ESM usually takes control of the crystal form and their morphologies [56–58]. Matrix protein Narein [59] MIS60 [60] lustrin A [50] MSI7 [50] N16 [61] N14 [47] p10 [62] etc. were isolated in this way. The main disadvantage of this method is that only the proteins with high abundancy could be identified, while those exhibiting a relative lower expression level but still crucial in biomineralization are excluded.
2. Then the search for new members benefited a lot from the major technical advances in molecular biology based on the cDNA sequences and primary structures of identified ones. PCR and rapid amplification of complementary DNA ends (RACE) were broadly applied, obtaining matrix protein KRMP [63] and Prisilkin-39 [64]et al. The principal factor limiting this method was that we could only get matrix proteins similar to previously identified ones in both sequences and functions.
3. Another strategy successfully developed was cDNA expression library. In most cases, genes encoding matrix proteins exhibit high expression levels in mantle tissue. With the help of the mantle cDNA library, the construction of the

suppression subtractive hybridization (SSH) library paves the way for the bulk cloning and characterization of new genes involved in biomineralization [35, 65]. Our group constructed three SSH libraries that represented genes expressing at three key points during shell formation in *P. fucata* in 2011 (Fig. 1.8) [35]. Finally, we obtained 227 unigenes in U–D library, 89 unigenes in J–U libraries, and novel matrix protein PfN23 [15], PfN44 [66], and DT252 (unpublished data). Since the fragments identified from SSH library were not long (shorter than 330 bp on average), we couldn't get much information for the establishment of the biomineralization model in mollusks. Much more efforts should be put to screen new matrix protein candidates.

In addition to the above methods, our group have identified 72 unique SMPs using liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of proteins directly extracted from the shells of *P. fucata* combined with a draft genome (Fig. 1.9). These proteomic data not only increase the repertoires of the SMPs but also give us new inspiration on shell formation which may involve tight regulation of cellular activities and the extracellular microenvironment [21]. Moreover, based on mantle transcriptome and microarray, the gene expression profiles during larval development, 5 novel potential matrix protein candidates were identified for further function analysis, brought global perspectives to the relationship between gene expression profiles and larval shell development (Fig. 1.10) [36].

1.3 Calcium Metabolism During Shell Formation

The studies of shell and pearl formation can be divided into two aspects: the isolation, identification, and functional analysis of SMPs and calcium metabolism process, including Ca^{2+} absorption, transportation, storage, deposition, etc [67] However, more focus has been dragged to the purification and characterization of SMPs previously, while the mechanism of oyster calcium metabolism has been comparatively "neglected." Acting as a universal second messenger in various cells, calcium is an indispensable regulator during the act of fertilization and development of the whole life in all kinds of creatures [68]. Numerous functions of all types of cells are regulated by Ca^{2+} to a greater or lesser degree [69]. In a word, biomineralization implies the transport and control of calcium ions [67].

1.3.1 Calcium Absorption

The mechanism of Ca^{2+} metabolism could be depicted in Fig. 1.11. Precursor ions, Ca^{2+} and HCO_3^-, are taken up from the ambient medium with the help of the body epithelium, or gill, which can also originate from the food in *P. fucata*. Then these ions are transited by hemolymph, soon afterward directed toward the outer mantle

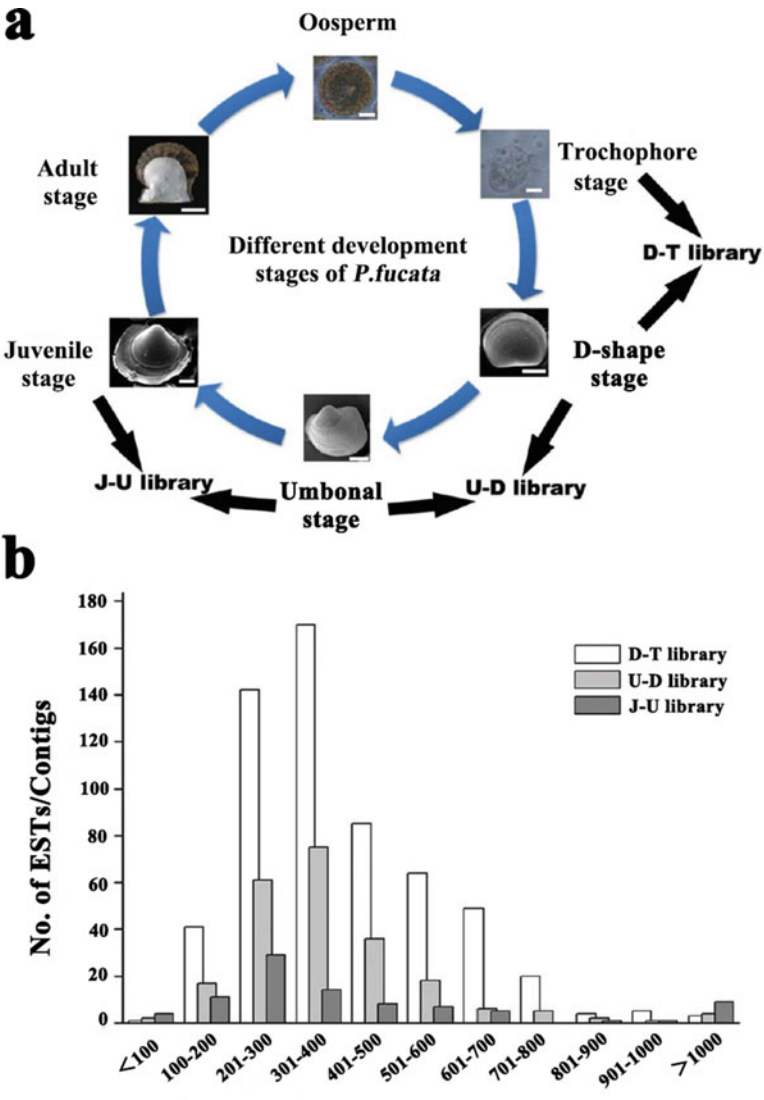

Fig. 1.8 Overview of the three SSH libraries [35]
(**a**) Schematic of SSH library construction. The D–T library was constructed using D-shaped stage larval cDNA as the tester and trochophore stage larval cDNA as the driver. The U–D library was constructed using umbonal stage larval cDNA as the tester and D-shaped stage larval cDNA as the driver. The J–U library was constructed using juvenile stage larval cDNA as the tester and umbonal stage larval cDNA as the driver. Bar = 15 mm in oosperm, 15 mm in trochophore and D-shaped stages, 40 mm in umbonal and juvenile stages, and 4 cm in the adult stage. (**b**) Length distribution of unigenes in the three SSH libraries. The white columns represent unigenes in the D–T library, gray columns represent unigenes in the U–D library, and the dark gray columns represent unigenes in the J–U library. Unigene-length frequencies for each library are plotted in 100-bp bins.

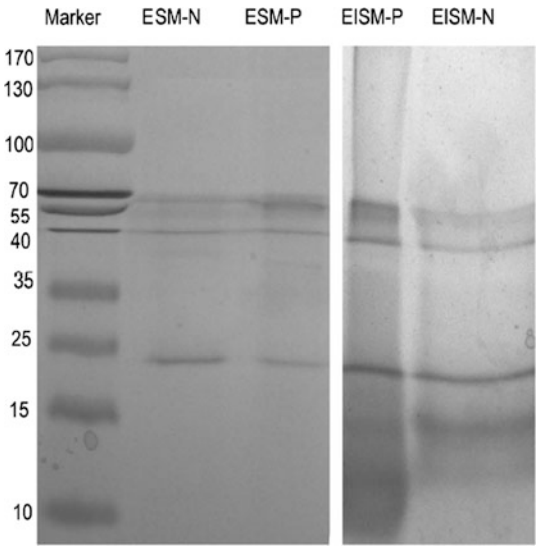

Fig. 1.9 SDS-PAGE of SMPs directly extracted from the shell for further LC-MS/MS analysis (*P* prismatic layer, *N* nacreous layer) [21]

epithelium. In other words, in addition to secreting SMPs for regulating shell formation, mantle is also indispensable to calcium absorption. Finally, large amounts of calcium ions are continuously deposited onto the framework under the precise regulation of matrix proteins.

After the injection of calcium isotope Ca^{45} into *Anodonta woodiana pacifica* (Heude), Ca^{45} was rapidly accumulated in gill, the radioactivity of which was the highest compared with other tissues or organs and lasted the longest. These results indicated that the gill exhibits the strong affinity and high metabolic rate to Ca^{2+}, which is quite crucial for the uptake, accumulation even storage of calcium. More to the point, the absorption of Ca^{2+} in mantle tissue is only second to gill. Furthermore, electrophysiologic [71] and isotopic studies [72] also showed similar effects that mantle epithelium did have high permeability to calcium. Calcium-positive reaction indicated numerous acidic mucous glands assembled in the inner mantle epithelium, suggesting the significant roles acid mucus is playing in Ca^{2+} interception, capture, and assurance. Due to the low concentration of Ca^{2+} in the surroundings, directly intake from environments is very critical for freshwater mollusks to accumulate calcium. It's difficult for Ca^{2+} to cross the mantle epithelium cell membrane in its original form, and this process needs ATP to provide energy. Therefore, there is a strong possibility that the carrier protein and the binding of Ca^{2+} induce a conformational (shape) change driving the ions to transport against the chemical gradient. But the calcium intake method may be different in mollusks living in seawater and hypersaline conditions, considering the similar ion concentration gradient between hemocytes and their living environments. Actually, the $CaCO_3$ solution is hypersaturated in both hemocytes and mantle cavity.

The digestive gland is the metabolic regulatory center in mollusks, besides participating in immune defense, detoxification, and elimination of xenobiotics;

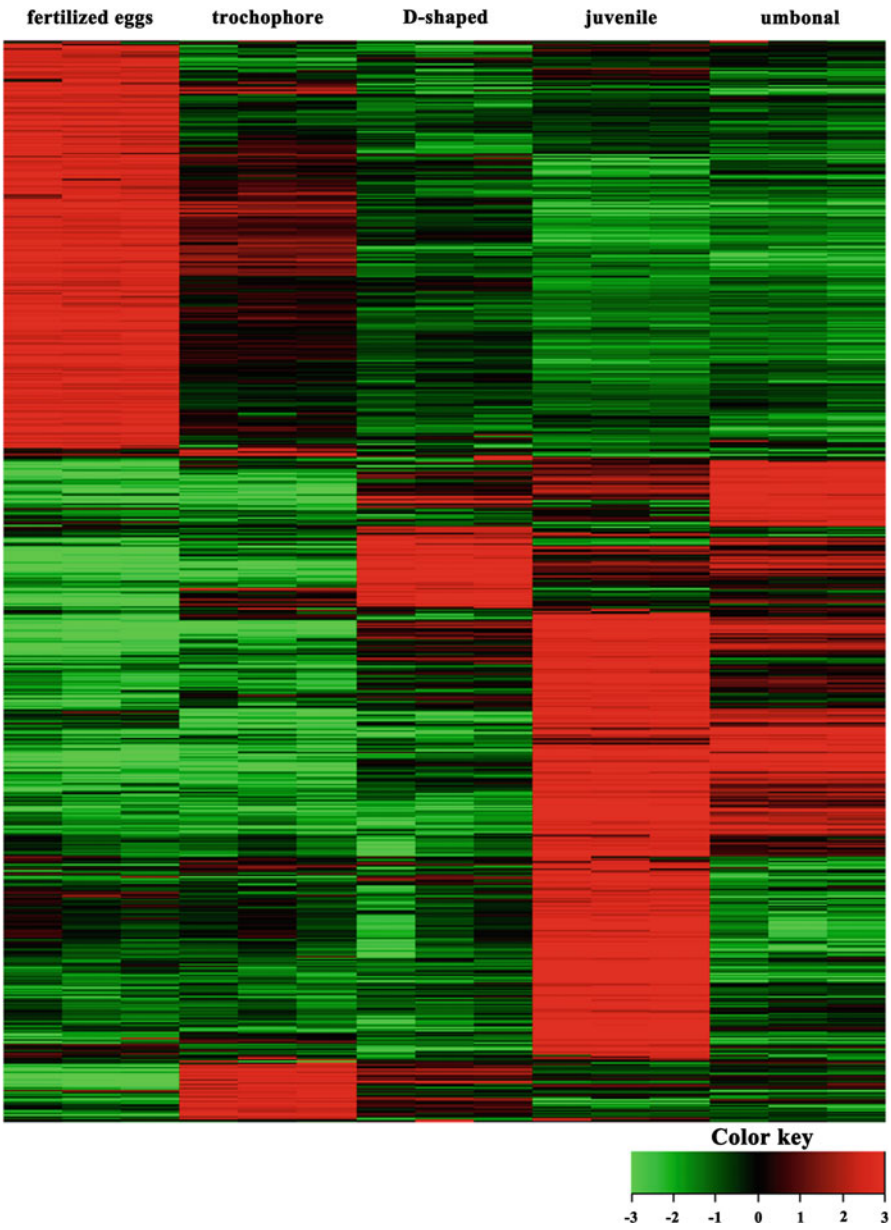

Fig. 1.10 Gene microarray analysis during the different larval development stages in *P. fucata* [36]

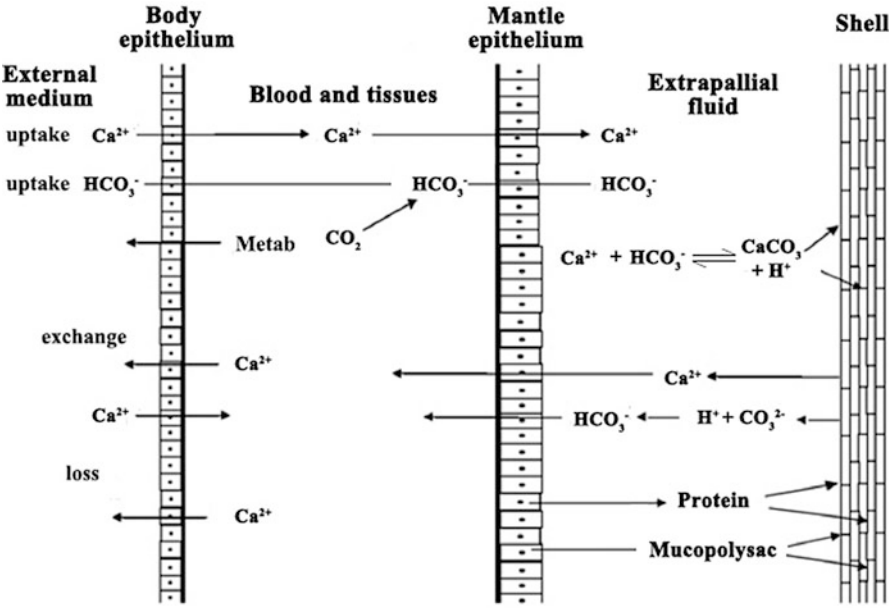

Fig. 1.11 The schematic diagram of calcium metabolism in mollusks [70]

it's also significant to homeostatic regulation of the internal medium (calcium balance, hemolymphatic pH, cell volume, etc.) [73]

Moreover, foot is involved in the calcium uptake process, too. Using the tracers like enzyme peroxidase and ionic lanthanum, paracellular uptake of Ca^{2+} was detected in the foot of the *Agriolimax reticulatus* [74]. On the other hand, electron probe microanalysis (EPMA) also detected the absorption of calcium and trace elements in byssus from mussels.

Thought with some progress, there are still lots of doubts and suspicion on the specific mechanism of calcium absorption waiting for exploration.

1.3.2 Calcium Transportation

There are two pathways in the transportation of Ca^{2+} between different epithelium cells: paracellular pathway [75–78] and transcellular pathway. The depolarization of epithelium cells indicated the apical barrier was permeable to Ca^{2+} when the concentration of calcium was raised from 1 mM to 6 mM the other side of the blood lymphocytes outside the mantle epithelium [79]. While with an exceeding of the equilibrium stage of 1–6 mM Ca^{2+}, calcium was then induced to transport to the other side shell surface [80]. These results together indicated at least part of the transepithelial calcium movements are transcellular. Bleher used lanthanum as an