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Molluscan shell proteins

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Abstract

The shell secreted by molluscs is one of the most remarkable examples of a matrix-mediated mineralisation performed outside living tissues. The calcifying matrix is a mixture of proteins, glycoproteins, and polysaccharides that precisely self-assemble and control the $CaCO_3$ polymorph (calcite, aragonite), the size, the shapes of the crystallites, and finally, the texture of the shell. In spite of several biochemical studies, the molecular aspects of the shell building are far from being understood. The present article makes an overview of the most recent molecular data on the proteinaceous components of the shell matrix. These data put into question the classical models of molluscan mineralisation. Furthermore, they show that shell proteins are diverse and multifunctional and that they may have different origins. ***To cite this article: F. Marin, G. Luquet, C. R. Palevol 3 (2004)***.
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Résumé

Les protéines de coquille de mollusque. La calcification de la coquille chez les mollusques est l'un des plus remarquables exemples d'une minéralisation régulée par une trame organique extracellulaire. Cette trame est un mélange de protéines, glycoprotéines et polysaccharides, qui s'auto-assemblent et contrôlent le polymorphe du $CaCO_3$ (calcite ou aragonite), la taille, la forme des cristaux produits, mais aussi la texture de la coquille. Malgré de nombreuses caractérisations biochimiques, les aspects moléculaires de la fabrication de la coquille sont loin d'être compris. Le présent article fait le point sur les protéines coquillières connues chez les mollusques. Les données moléculaires remettent en question les modèles de biominéralisation de la coquille proposés jusqu'à présent et montrent que les protéines de coquille sont diversifiées et multifonctionnelles. Elles ont en outre plusieurs origines possibles. ***Pour citer cet article : F. Marin, G. Luquet, C. R. Palevol 3 (2004)***.
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Mots clés : Biominéralisation ; Mollusque ; Coquille ; Matrice calcifiante extracellulaire ; Protéines ; Gènes ; Microstructures ; Cambrien

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Parce qu'ils possèdent un corps mou, les mollusques ont développé des structures calcifiées externes de soutien, qui les protègent également des agressions environnementales et des prédateurs. Chez les mollusques les plus primitifs (solénogastres, monoplacophores), ce sont des spicules ou des plaques calcifiés. Cependant, chez l'immense majorité des mollusques, il s'agit d'une véritable coquille calcaire, élaborée à partir de deux des polymorphes du carbonate de calcium, la calcite et/ou l'aragonite (Fig. 1). Par-delà les aspects esthétiques, la coquille est un remarquable biomatériau composite, qui résulte d'un processus de minéralisation biologiquement contrôlé, et pour lequel la fraction minérale représente 95–99% du poids de la coquille (Fig. 1). Les 1 à 5% restants sont constitués d'une matrice organique, dont le rôle est, d'une part, de renforcer les propriétés mécaniques de la coquille et, d'autre part, de réguler de manière très précise la mise en place des cristaux d'aragonite ou de calcite [3,92,151,171]. Les constituants protéiques de cette matrice calcifiante font l'objet du présent article.

D'un point de vue ontogénique, la sécrétion de la coquille débute dès les premières phases de développement larvaire, puisque le stade trochophore voit la mise en place d'un groupe de cellules impliquées dans la sécrétion du périostracum, couche organique résultant d'un tannage quinonique, et qui constitue le premier support de la proto-coquille [78,140]. Ces cellules donneront par la suite le manteau calcifiant. Les analyses par diffraction des rayons X montrent que le premier minéral formé est du carbonate de calcium amorphe (ACC), qui se transforme en calcite ou en aragonite [178]. La mise en place de la coquille est caractérisée par une activité enzymatique intense [160]. Des travaux récents montrent que, chez les formes larvaires, le gène *engrailed* est activé dans une zone correspondant à la limite de la coquille [71,120,123,167]. Cependant, la contribution directe ou indirecte de ce gène à la formation de la coquille est encore mal évaluée.

Chez les mollusques en train de calcifier (Fig. 2), la minéralisation de la coquille a lieu dans l'espace extrapalléal, zone de très faibles dimensions délimitée par la coquille en croissance, le périostracum et le manteau calcifiant [92,140,151]. Cet espace contient un fluide précurseur de la minéralisation, le fluide extrapalléal, qui peut, sous l'action de pompes ioniques de l'épithélium calcifiant du manteau, atteindre les conditions de sursaturation du calcium et du bicarbonate (Fig. 2). Dans le même temps, ce même épithélium sécrète la matrice organique calcifiante, mélange complexe de protéines, glycoprotéines, polysaccharides acides et chitine. Il se produit alors un véritable processus d'auto-assemblage des ions minéraux et de la matrice calcifiante, aboutissant à des microstructures compactes et bien organisées [27,28,156,157].

La matrice coquillière, de nature hétérogène, est classiquement fractionnée en une phase insoluble, généralement hydrophobe (riche en glycine et alanine), et une phase soluble acide, riche en acide aspartique. Un modèle, élaboré au début des années 80, à partir de la nacre, indique que la matrice hydrophobe formerait une structure tridimensionnelle qui supporte la minéralisation, tandis que la fraction soluble polyanionique serait impliquée dans la nucléation cristalline, ainsi que dans l'arrêt de la croissance des cristaux [172,175]. Ce modèle a perduré deux décennies, jusqu'à ce qu'un modèle de nacre développé récemment montre que la fraction hydrophobe formerait un gel, plutôt qu'une structure rigide [89]. Une des faiblesses de ces modèles « topographiques », basés essentiellement sur des mécanismes de nucléation et d'inhibition, est qu'ils ne tiennent pas compte des données de séquences de protéines coquillières récemment acquises.

Pourtant, l'obtention des structures primaires des protéines matricielles a été envisagée depuis longtemps, et de nombreuses tentatives ont été faites, visant à fractionner proprement ces protéines par des techniques chromatographiques et électrophorétiques [26,170]. Cependant, dans la plupart des cas, les démarches biochimiques classiques ont trouvé ici leurs limites, du fait notamment du caractère polyanionique des matrices, de leur forte glycosylation et phosphorylation et de leur migration électrophorétique anormale sous forme de traînées. Ces difficultés expliquent pourquoi aucune séquence complète de protéine coquillière n'a été publiée jusqu'à fin 1996 (Table 1).

Avec l'utilisation des techniques de biologie moléculaire, des progrès notables ont été accomplis au cours de ces dernières années. Aujourd'hui, une quinzaine de protéines ont été identifiées (Table 2), dont douze par leur gène [80,100,115,117,118,141,

143,148,154,188] et trois par séquençage direct [97,111,176], à partir de quelques genres de mollusques : l'huître perlière *(Pinctada)*, l'ormeau *(Haliotis)*, la coquille Saint-Jacques japonaise *(Patinopecten)*, le jambonneau de mer *(Pinna)* ou encore deux genres de gastéropodes (*Turbo* et *Biomphalaria*). Cet essor des connaissances s'accompagne d'un renouvellement des concepts fondamentaux de la biominéralisation, puisqu'il remet largement en question les modèles de minéralisation de la coquille décrits cidessus. Presque toutes les protéines identifiées sont différentes. Certaines d'entre elles ont une organisation en modules, ce qui suggère qu'elles sont multifonctionnelles. Certaines d'entre elles (nacréine, N66) fonctionnent à la fois comme des enzymes et comme de possibles nucléateurs de minéralisation [80,117]. D'autres semblent avoir un rôle structurel. C'est le cas de la lustrine, de la MSI60 et de la MSI31 [148,154]. Parmi les domaines reconnus, on note la présence de ceux de type « anhydrase carbonique », « mucine », « lectine de type C », « IGF-BP » ou encore « dermatopontine ». La diversité des domaines laisse supposer que les protéines de la matrice calcifiante jouent beaucoup plus que les seuls rôles de support de minéralisation, de substrat nucléateur de cristaux ou encore d'inhibiteur de la croissance cristalline. En particulier, elles seraient impliquées dans les processus de signalisation cellulaire [179] et d'interaction avec des récepteurs membranaires, mais aussi dans des interactions avec d'autres constituants matriciels, en particulier les polysaccharides [97]. Un effet de rétro-contrôle de la matrice sur les cellules du manteau qui l'ont produite pourrait être envisagé lors des phases de redissolution partielle de la coquille (métabolisme anaérobie).

Les données de séquences permettent aussi de mieux comprendre l'origine de ces matrices. Les mollusques ont commencé à minéraliser leur coquille à l'aube des temps cambriens, à l'instar d'autres métazoaires [29]. Aujourd'hui, de nombreux indices montrent que les matrices calcifiantes ne sont pas apparues de novo à la base du Cambrien, mais qu'elles ont probablement été recrutées et orchestrées à partir de fonctions plus anciennes, par un processus d'exaptation [54,103]. De plus, la structure modulaire des protéines matricielles suggère que ces dernières auraient été produites par recombinaison d'exons (*exon shuffling*) à partir de gènes ancestraux dupliqués [127]. Un tel scénario permettrait d'expliquer la simultanéité de la minéralisation dans de nombreux embranchements de métazoaires déjà différenciés à la fin du Protérozoïque.

Pour conclure, les protéines des coquilles de mollusques offrent de tangibles perspectives appliquées. En particulier, le domaine des matériaux composites biomimétiques, la chirurgie osseuse réparatrice ou encore l'aquaculture et la perliculture pourront bénéficier des avancées réalisées ces dernières années. Cependant, de nombreuses questions demeurent, en particulier celles concernant la fonction exacte de ces protéines, leur multiples modifications post-traductionnelles, leur capacité à s'auto-assembler et à sélectionner le polymorphe (calcite ou aragonite), ou encore leur aptitude à contrôler la microstructure coquillière.

1. Introduction: the molluscan shell

Because molluscs are soft-bodied metazoans, they have developed external calcified structures to support their living tissues, and to protect themselves against predators. These protective biominerals exhibit a wide range of morphologies. They can be tiny spicules or scales, for the most primitive 'worm-like' solenogaster and caudofoveate molluscs or calcareous plates in the case of polyplacophorans, like the famous living fossil chiton [84,92]. However, in most of the cases, they are true rigid protections, shells. Shell-bearing molluscs, also named conchiferans, represent indeed the largest group, since they include the archaic monoplacophorans *(Neopilina)*, the bivalves (mussels, oysters, clams, scallops), the scaphopods (tusk-shells), the gastropods (snails), and finally the cephalopods *(Nautilus)* [84]. In total, more than 100 000 living mollusc species secrete a shell, in marine, lacustrine, or terrestrial environments. Molluscan shell secretion is probably one of the most common and abundant biomineralisation processes in the metazoan world, after coral mineralisation.

For aesthetic reasons, human beings have used molluscan shells for a long time. The children of Grimaldi cave, thirty thousand years ago, were buried with hundreds of pierced shells, originally sewed on their clothes [51]. The Mayas, several centuries ago, implanted pieces of nacre in jaws where teeth were missing [18]. In the 17th century, the nautilus shell, when finely set with gold and silver, was considered as a

goldsmith's masterpiece. Around the same period until recently, mother-of-pearl has been widely used for making buttons but also as inserts in wooden furniture. In the early twentieth century, the regular shape of nautilus shell was a source of inspiration for the famous naturalist D'Arcy Thompson [158]. Until now, shells exert an enduring fascination [164].

Beyond aesthetic considerations, the molluscan shell is a remarkable natural product: it is indeed the result of a fully controlled biomineralisation process [3,92,151,171]. The shell is a composite biomaterial, for which the mineral phase, calcium carbonate, accounts for 95 to 99% per weight. The remaining 1 to 5% represents an organic matrix. A shell, when observed in longitudinal section, exhibits different superimposed calcium carbonate layers, usually two or three, and an external organic layer, the periostracum [140]. The calcified layers, which are either made of aragonite or of calcite, correspond to different mineral textures, also called microstructures (Fig. 1). Among molluscs, the shell displays a large variety of microstructures [27,28,156,157]. One of the most known is the mother-of-pearl, or nacre, the iridescent aragonite layer that covers the inner surface of the shell of some popular molluscs: Pteriomorphid bivalves like the pearl oyster or the edible mussel, the abalone, a primitive gastropods or, the cephalopod nautilus (Fig. 1). Because nacre contains a little bit of an organic fraction, it exhibits high fracture toughness, three orders of magnitude higher than aragonite obtained from a chemical precipitation [32,70]. Because such a strong material is synthesized at "room temperature", nacre receives nowadays a great deal of attention from materials physicists [32,43,47,48,70,72,135], dentists [9,179], orthopaedists [8,10,90,91,134], and scientists involved in nanotechnologies [73,95,144,147,155].

The present paper deals with the molecular aspects of shell calcification. In particular, it presents the proteinaceous constituents of the shell matrix and their putative function in biomineralisation. Furthermore, it gives some insights on their origin and their evolution. At last, it reviews the ongoing and still-unanswered questions about their exact function and their ability to control shell mineralogy and textures.

2. The larval shell

Like all the exoskeletons of calcifying protostomians, the shell of molluscs has an ectodermic origin. Its synthesis starts at early stages of development. Contrarily to crustaceans, for which the calcified exoskeleton is replaced periodically by ecdysis [93], the molluscan shell grows continuously during the entire life of the animal. Two types of post-embryonic developmental processes have been observed among molluscs: a direct development, peculiar to the cephalopods – this mode of development implies that juveniles look like adults in reduction –, and a development with metamorphosis, seen in most of the other mollusc classes. Within these two types, there are several variations. It is therefore not our intention to cover this field, but to give only a schematic overview of the physiological events that accompany the development of the larval shell. To that end, we focus on the two most important shell-bearing molluscs, gastropods and bivalves, the developments of which present many similarities.

Soon after the fertilization, a bivalvian or gastropod egg undergoes a spiral cleavage, and becomes a morula, then a blastula. During the next stage, the gastrulation, the three germ layers are specified. The gastrula transforms into a ciliated transitory larva, the trochophore. The trochophore acquires a velum and become a swimming larva, the veliger. This last stage is typical of molluscan post-embryonic development. After a few days to a few weeks, the veliger looses its velum, settles down, and starts its complete metamorphosis to become a juvenile individual [19]. The shell is elaborated during these crucial early developmental stages. Curiously, only few reviews deal with the calcification of the early shell in connection with the ontogenic development [69,78,119]. The process itself has been monitored for a limited number of peculiar genera: the mussel *Mytilus* [66,77,87,112], the edible oyster [85,113], the pearl oyster [98], the giant clam *Tridacna* [82], the scallop [86], the water snails *Lymnea* and *Biomphalaria* [17,76], the marine gastropod *Nassarius* [64].

The first event precursor of the acquisition of a shell takes place at the end of the gastrulation stage, when a group of epithelial cells of the dorsal side increase in thickness [69,78]. This thickening defines the shell field. The transitory invagination of the central part of the shell field is currently called the shell gland during early trochophore stage [78]. In gastropods, the shell gland is a narrow pit with a circular opening. In bivalves, it forms a groove, which is thought to produce

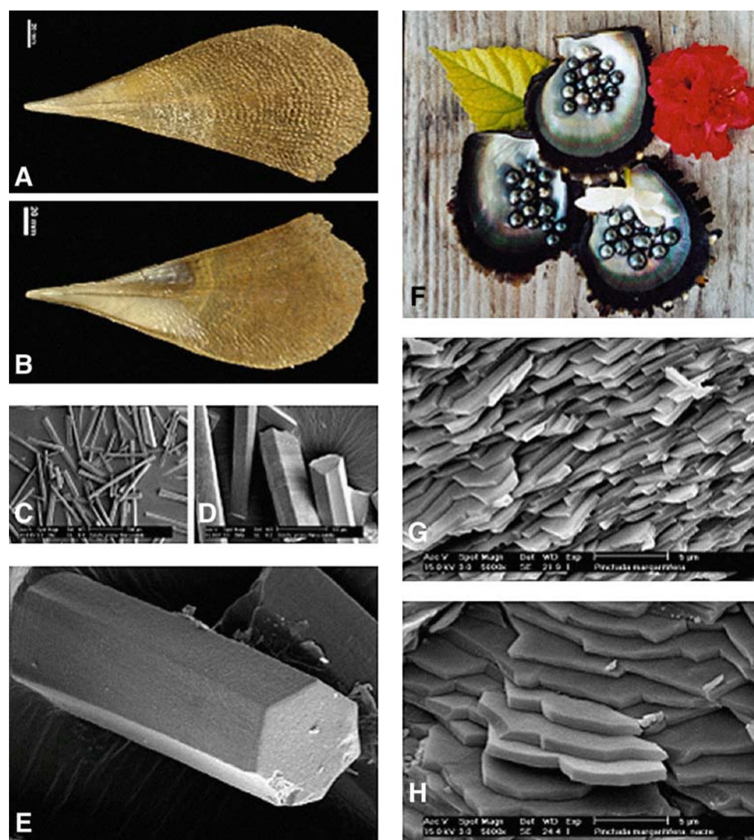

Fig. 1. The shell of two nacroprismatic bivalve genera and their corresponding shell microstructures, observed with ESEM. A, B : Shell of the Mediterranean fan mussel, *Pinna nobilis*, two-year-old specimen (length of the shell: 25 cm). **A**: Right valve, external surface, showing the prismatic layer. **B**: Right valve, internal surface. The internal nacreous layer is only present in the first half of the shell. **C**: When treated with sodium hypochlorite, the outer layer can be dissociated in calcitic prism units. **D**: Isolated prisms, higher magnification. **E**: Detail of one single prism (diameter : 40 μm, length 100 μm). **F**: Right and left shell valves and pearls of the 'black-lip' pearl oyster, *Pinctada margaritifera,* from French Polynesia (Manihi, Tuamotus). **G**: Nacreous layer of *Pinctada margaritifera*. **H**: Idem, higher magnification. The nacreous layer is made of superimposed aragonitic flat tablets, which are arranged in a 'brick-wall' microstructure.

Fig. 1. Coquilles de deux bivalves nacroprismatiques et microstructures coquillières correspondantes, observées au MEB. A, B: Coquille de la grande nacre de Méditerranée, *Pinna nobilis,* spécimen âgé de deux ans environ (longueur de la coquille : 25 cm). **A** : Valve droite, vue externe montrant la couche prismatique. **B** : Valve gauche, surface interne. La couche nacrée interne chez *P. nobilis* n'est présente que dans la première moitié de la coquille. **C** : Après traitement à l'hypochlorite de sodium, la couche prismatique peut être entièrement dissociée en prismes calcitiques. **D** : Prismes isolés, plus fort grossissement. **E** : Vue agrandie d'un prisme (diamètre : 40 μm, longueur : 100 μm). **F** : valves droites et gauches et perles de *Pinctada margaritifera*, l'huître perlière de Polynésie française (Manihi, Tuamotus). **G** : Couche nacrée de *Pinctada margaritifera*. **H** : Idem, vue agrandie. La couche nacrée est constituée de tablettes de nacre arrangées en « mur de brique ».

the future ligament between the two valves [69]. The peripheral cells of the shell gland produce an extracellular lamella – the future periostracum, the function of which is to provide the early support for mineralisation. It is commonly accepted that the function of the shell field invagination is to bring together the periostracum-secreting cells, in order to prevent the formation of a hole in that layer [119]. Following the secretion of the periostracal lamella, the shell gland evaginates and the shell field spreads by flattening of the cells and by mitotic divisions, thus becoming the calcifying mantle. The evagination also provokes the lateral extension of the periostracum. Between the periostracum and the shell field, the primary mineralisation takes place. In bivalves, the early shell, called the prodissoconch I, exhibits a granular aspect and develops from the non-shelled trochophore [69]. It is followed by the prodissoconch II stage, formed during the

acquisition of the velum. The prodissoconch II shell is characterized by concentric growth lines, which mark a change in the calcifying regime [98]. At a later stage, the shell, called dissoconch, is produced after the metamorphosis of the veliger larva into a juvenile specimen. A sharp line in the shell outer surface marks the prodissoconch II–dissoconch transition. Among gastropods, the terminology is slightly different [69]: the protoconch I corresponds to the first shell developed in the late trochophore stage; the protoconch II is deposited during the veliger stage, and the post-metamorphosis shell is called the teleoconch.

The mineralogy of the larval shell has been studied in few cases with modern techniques: X-ray microdiffraction, X-ray absorption spectroscopy (EXAFS), IR spectroscopy, or Raman spectroscopy. In the freshwater snail *Biomphalaria glabrata,* the first deposited mineral is amorphous calcium carbonate (ACC) [62,109], immediately followed by aragonite. In *Mytilus edulis,* an amorphous phase has also been detected as a precursor [112]. In *Ostrea edulis,* the common edible oyster that exhibits a foliated calcite microstructure, calcite has been reported as the first mineral deposited, followed by aragonite in the prodissoconch-II stage. After settlement and metamorphosis, calcite is deposited [113]. In *Mercenaria mercenaria* and *Crassostrea gigas,* the combination of different analyses showed that ACC is present in the early shell (prodissoconch I) together with a poorly crystalline aragonite. Subsequently, the ACC partially transforms into aragonite [178]. These studies tend to show that ACC deposition in the early stage of shell formation has been widely underestimated until now. So far, no vaterite was found as a precursor. The question remains whether ACC deposition is a general mechanism common to all conchiferan molluscs and whether ACC is produced as a transient phase in adult shells.

The development of the larval shell is marked by the activation of homeobox-containing regulatory genes, in particular of *engrailed*. The basic function of *engrailed* in bilaterian metazoans is the patterning of the nervous system. In annelids and arthropods, *engrailed* specifies the body segmentation pattern (metamerism), whereas it is involved in the limb development in chordates [53,65]. Homologues of *engrailed* were found in bivalves, scaphopods, cephalopods, gastropods, and polyplacophores [184]. Four recent studies, performed on chitons [71], gastropods [120,123], clams [71] and tusk-shells [167], have shown that *engrailed* is specifically expressed in the cells at the borders of the embryonic shell. The exact role of *engrailed* in the formation of the larval shell is however unclear and controversial: according to Jacobs and co-workers [71], the expression of *engrailed* in ectoderms would play a direct role in skeletogenesis by marking the skeletal boundaries. They claim that the primitive role of *engrailed* would have been to delimit exoskeleton in calcifying metazoans, an assertion that suggests a single origin for the skeleton of all invertebrate bilaterians. On the other hand, Nederbragt et al. [123] suggest that the primary function of *engrailed* was to set up compartment boundaries during metazoan development, and that it was co-opted for delimiting the shell field in molluscs. If so, *engrailed* would have only an indirect contribution to the development of the larval shell.

At last, the development of the larval shell corresponds to an intense enzymatic activity [112,160,182] of the calcifying tissues. For example, carbonic anhydrase has been monitored during the whole developmental process. In *Mytilus* larvae, high expressions of carbonic anhydrase were found to precede the formation of the shell field in the gastrula stage, the formation of the shell gland and periostracum in the trochophore stage, and the mineral deposition in the prodissoconch-I and prodissoconch-II stages [112]. In the freshwater snail *Lymnea*, the expression of alkaline phosphatase, a marker of calcification, was the highest during the evagination process, while the expressions of DOPA-oxidase (tyrosinase) and peroxidase were maximal at the borders of the shell gland, after evagination [160].

3. The adult shell construction: physiological aspects

According to a classical terminology [92], the construction of the mollusc shell is a ‘biologically-controlled mineralisation’ process, by opposition to the ‘biologically-induced mineralisation’ performed by cyanobacteria, for example. This means that the process is entirely regulated by an extracellular organic matrix. This also means that the crystals synthesized by molluscs are different from ‘chemical’ calcium car-

bonate crystals [92,171]. Although the calcification is performed outside living tissues, it does not take place in contact with the ambient medium, seawater in most of the cases [151,182]. Fig. 2 describes the physiological process of shell mineralisation for a marine bivalve. Shell growth takes place on the distal border of the shell. It can be described as a growth by increments, where minute layers are added to subjacent layers. As a result, the shell grows more in length than in thickness. The kinetics of calcification is variable from species to species, and within a single species, it is condition-dependent [146].

Schematically, the shell growth requires three components: an enclosed compartment dedicated to calcification, membrane-bound ionic pumps, and an extracellular organic matrix, which shapes the forming crystals. We leave aside a fourth component, the up-stream regulation of calcification by hormonal signals. For this aspect, we refer to published works [35,36,138,139]. Fig. 2 details the three components cited above. Firstly, the shell calcification occurs in a tiny compartment, called the extrapallial space. This space is delimitated by the growing shell, the leathery periostracum and the calcifying mantle. The periostracum is secreted by the periostracal groove, which is situated between the outer and middle folds of the mantle edge among bivalves [25]. It is secreted as a soluble precursor, the periostracin [166], which becomes highly insoluble by a quinone-tanning process [165]. The periostracum has two main functions: it is the first support for calcium carbonate crystals, in the present example, prisms. Its additional role is to seal completely the extrapallial space, in such a way that supersaturation conditions – a pre-requisite for crystal formation – can be reached [140,151,182]. To this end, calcium and bicarbonate ions are taken up from the teguments (body surface, inner mantle epithelium), from the gills or from the gut [182]. They are transported via the haemolymph to the epithelial cells. They can be stored as amorphous intracellular or interstitial granules [45,68], which can be easily redissolved when needed. They are then actively pumped from the cytosol to the extrapallial space by putative calcium and bicarbonate channels, present in the membranes of calcifying epithelial cells. For molluscs, this mechanism is however poorly documented at the protein or gene levels. The result of the pumping activity is the formation of a supersaturated fluid, the extrapallial fluid, the composition of which is peculiar [116,121,131]. This fluid contains also minor and trace elements. Whether these elements are incorporated in the crystals in a controlled manner or not is not understood [182]. Because the crystallization of calcium carbonate releases protons, proton ATP-ases are also suspected to be present for re-absorbing these ions in the cytosol [151].

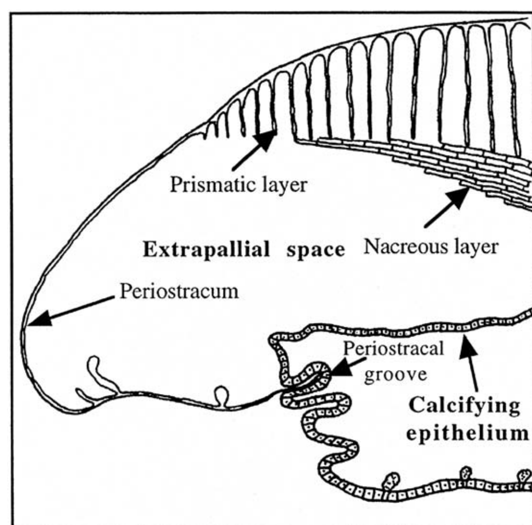

Fig. 2. Shell calcification process of a nacroprismatic bivalve. Redrawn from Saleuddin and Petit [140]. The calcification of the growing shell takes place at the distal border of the shell, in a minute compartment — the extrapallial space — enclosed by the calcifying epithelium, the periostracum and the shell itself. The cells responsible for the deposition of the nacreous layer (aragonite) and of the prismatic layer (calcite or aragonite) are not located in the same zone of the epithelium. The prisms and the nacreous tablets are not drawn to scale.

Fig. 2. Calcification de la coquille chez un bivalve nacroprismatique. Redessiné d'après Saleuddin et Petit [140]. La calcification de la coquille en croissance a lieu au niveau de la bordure distale de la coquille, dans un compartiment de très faible volume — l'espace extrapalléal — délimité par l'épithélium calcifiant, le périostracum et la coquille elle-même. Les cellules responsables de la sécrétion de la couche de nacre (aragonite) et de la couche prismatique (calcite ou aragonite) ne sont pas situées dans la même zone de l'épithélium. Les prismes et les tablettes de nacre ne sont pas dessinés à l'échelle.

The third component of the system is the calcifying matrix. This matrix is secreted in the extrapallial space by specialized cells of the calcifying mantle. It is a complex mixture of proteins, glycoproteins, proteoglycans, polysaccharides, and chitin [3,92,171,175]. The matrix interacts with the mineral ions. Together, they self-assemble in a precise manner to generate crystallites, which exhibit well-defined morphologies. The

calcifying matrix is suspected to play several roles: because the extrapallial fluid is supersaturated, the matrix in solution allows crystallization only where appropriate. Secondly, the matrix is the main calcium carbonate nucleating agent. Thirdly, it selects the calcium carbonate polymorph. Fourthly, it regulates the shapes and the orientation of the crystals. At last, it stops the crystal growth.

4. The shell matrix: a brief history of key-concepts

Since the shell organic matrix was suspected early to be the key-component for regulating the crystal growth, it was the subject of extensive characterizations [55,150]. Classically, the matrix is retrieved by dissolving the mineral phase with a weak acid or with a calcium-chelating agent like EDTA [26,104,150,181]. By doing so, one obtains two fractions, which can be separated by centrifugation: an insoluble fraction, and a soluble one. In the shell, the two fractions are intimately associated. Historically speaking, they were discovered at very different periods.

The insoluble shell matrix is known for more than one and half century. It was at first characterized by Frémy [46], who named it 'conchioline'. In his precursor article, he spoke about "*un résidu d'une matière organique fort remarquable, d'un aspect brillant et feutré, insoluble dans l'eau, l'alcool et l'éther, et qui résiste à l'action des acides étendus*". A century was needed before conchiolin was biochemically analysed by Grégoire and co-workers [56]. These authors evidenced that it was a heterogeneous mixture of proteinaceous substances. By using different solvents, they identified three fractions that they named nacrin, nacrosclerotin and nacroin, the last one being exceptionally rich in alanine and glycine residues. The numerous amino acid analyses performed subsequently on the bulk nacre insoluble matrix confirmed the predominance of these two amino acids [5,55,60]. Chitin was also detected as an optional component [187]. Amino acid analyses showed that the proteinaceous nacre matrix resembled silk-fibroin, the main protein of spiders and worm threads. This striking similarity in composition explained the peculiar elasticity of nacre material and its resistance to fracture. The numerous SEM observations performed on bivalvian nacre evidenced its brick-wall structure, with the nacre aragonitic tablets as the bricks and the hydrophobic matrix as the cement surrounding the bricks (Fig. 1H). From SEM observations emerged the idea that mantle cells secrete the framework hydrophobic protein and that crystallization of nacre tablets occurs more or less 'passively' into this pre-formed framework [15,16]. However, the 'compartment theory' did not take into account two mechanisms, which are essential for understanding crystal formation and control, namely, nucleation and growth inhibition.

Few after the formulation of this theory, the discovery of a shell soluble organic fraction brought a missing part to the puzzle. Simultaneously, Meenakshi et al. [114] and Crenshaw [30] detected the presence of an EDTA-soluble matrix in the molluscan shell. In particular, the work of Crenshaw underlined the importance of this matrix as the potential nucleator of calcium carbonate biominerals. Furthermore, Crenshaw could distinguish an intercrystalline matrix from an intracrystalline matrix, which was intimately associated with the mineral phase. Soon after, Weiner and Hood [172], in a milestone study, went one step further by reporting that the soluble matrix was polyanionic, i.e., that it was enriched in aspartic acid residues. Let us recall that aspartic acid (D in the one-letter code for amino acids) exhibits an anionic radical at physiological pH. They subsequently hypothesized that this polyanionic matrix acts as a template for crystal nucleation. Observing that aspartate residues of the matrix were easily released by mild acid hydrolysis, they proposed that ligation of calcium ions was performed via aspartate residues in a hypothetical $(D–Y)_n$ peptide. Six years after the finding of Weiner, Wheeler and co-workers [149,180] introduced another key-concept by observing that the soluble matrix, when added to a supersaturated solution, inhibited the precipitation of calcium carbonate. Inhibition and nucleation appeared as two antagonist mechanisms that explained how the growth of calcium carbonate crystals could be regulated. When adsorbed on an insoluble template, the polyanionic soluble matrix could act as a nucleating surface. The same matrix, when free in solution, could easily attach by electrostatic interactions on the cationic surfaces of growing crystals. By doing so, the soluble matrix would promote the growth of the crystals in privileged directions. The adsorption of the soluble matrix on all the sides of the crystals would

completely stop their growth. Numerous cycles of nucleation–maturation–inhibition would explain the regular and repetitive brick-wall structure of molluscan nacre.

The detailed studies performed by Weiner and co-workers [168–170,174] in the late seventies and early eighties led them to propose a 'topographic' and biochemical model of molluscan nacre [175]. In this model, the insoluble framework is a composite of chitin and hydrophobic (A/G-rich) proteins, which adopt a β-sheet conformation. This framework is coated by a soluble aspartic acid-rich matrix, which adopts the same conformation. Nucleation takes place on this polyanionic surface. Weiner and Traub [175], then Addadi and Weiner [2], suggested that the distance between consecutive negatively charged Asp radicals should allow the binding of one calcium ion. Repetitive sequences of the types $(D–X)_n$ or $(D–X–Y)_n$ would match the atomic distance between two consecutive calcium in the [001] crystallographic plan. Addadi and co-workers [1] completed and refined the model by pointing out the cooperative role of carboxylate groups of the D-rich proteins and the sulphate groups of polysaccharides, which were covalently attached to the protein core. The sulphate groups would participate in the concentration of calcium ions at the vicinity of the template. As described here, the nucleation and growth of nacre tablets from an underlying organic layer may be considered as epitaxy, sensu lato [175]. This view was put into question by the observation of minute pores in the insoluble interlamellar organic nacre matrix [145] of the abalone. The presence of such pores (15- to 40-nm diameter) suggests that each generation of nacre tablets may grow in continuity with the underlying tablets, through mineral bridges. The continuity from tablet to tablet ensures the perfect alignment of crystals axes along a stack in columnar nacre.

Recently, Levi-Kalisman et al. [89] proposed another model of nacre, on the basis of cryo-TEM observations. In this new model, the interlamellar framework is made of β-chitin, coated by soluble acidic macromolecules. The main innovation of this model comes from the state of the hydrophobic silk-like proteins, which would form a gel between the framework. This gel would also trap acidic macromolecules. This new model is consistent with the recent finding that a hydrophobic proteinaceous fraction may be obtained by an extraction with water [130].

While these models were developed, biochemical investigations were performed on the soluble matrix. A particular attention was drawn to fractionate it in discrete components, in order to obtain structural information. However, what is normally a routine procedure for standard proteins appeared soon to be considerably hampered by the non-standard behaviour of the soluble components extracted with EDTA or with acetic acid. Because of high polydispersity, multiple anionic charges, non-globular shape, post-translational modifications, these soluble matrix components never resolved in discrete components by gel permeation or ion exchange chromatography. Thus, the most relevant fractionation techniques were – and still are – a combination of HPLC and ion exchange [168–170] or polyacrylamide gel electrophoresis in denaturing conditions [12,13,22,26,74,75,104,105,108,118,173]. But even with these techniques, some obstacles still occurred: difficulty to stain gels, blocked N-terminus, ineffective internal cleavages. All these cumulated technical obstacles precluded for years the obtention of shell protein sequences.

5. The molluscan shell proteins: a kit of multifunctional tools

The situation changed when it became possible to obtain N-terminal sequences from picomoles of proteins blotted onto nitrocellulose, and to develop degenerate oligonucleotide probes for fishing out the corresponding gene by RT-PCR or by screening a cDNA library. The use of molecular genetic techniques represents probably the major breakthrough in the field of molluscan biomineralisation, and, little by little, the molluscan shell proteins start to confide their secrets. We distinguish here the proteins, which have only been partly characterized by N-terminal and internal sequencing (Table 1), from those that have been fully sequenced or retrieved by their gene (Table 2).

From the partly sequenced proteins, RP-1 extracted and purified from the scallop shell is the best characterized [37,59,136]. It exhibits a yield of aspartic acid residues around 30%, which is in good agreement with the predictions of the model of molluscan shell matrix. However, cleavage of RP-1 with mild acid or with hydroxylamine yielded different acidic peptides, none of which exhibiting the D–Y or D–X–Y alternations,

Table 1
List of molluscan shell proteins, characterized only by their N-terminal sequence. In addition, the single extrapallial fluid protein known to date is indicated.
Liste des protéines de coquille de mollusques, connues partiellement par leur séquence N-terminale. En outre, la seule protéine de fluide extrapalléal connue à ce jour est indiquée.

	Species (Common name)	Microstructure (Mineralogy)	Names (Characteristics)	Ref.	SwissProt number
BIVALVIA	*Crassostrea virginica* (American oyster)	foliated *(calcite)*	fraction RP-1 (intern. D/S rich seq.)	[37]	_
	Adamussium colbecki (Antarctic scallop)	foliated *(calcite)*	fraction RP-1 (Idem)	[59]	_
	Pinctada maxima (Australian pearl oyster)	Nacre *(aragonite)*	p20 (4Y pattern seq.)	[12]	_
	Mytilus edulis (Edible mussel)	nacroprismatic *(aragonite + calcite)*	45–21–5-kDa prot. (hydrophobic)	[75]	Q9TWS3
	Pinna nobilis	Prisms	calprismin (acidic)	[99]	P83631
	Mytilus edulis (Edible mussel) (Fan mussel)	Extrapallial Fluid *(calcite)*	EP fluid glycoprot. (D- and H-rich seq) caspartin (acidic)	[63]	P83148
GASTROPODA	*Biomphalaria glabrata* (Freshwater snail)	cross-lamellar *(aragonite)*	19–61-kDa prot. (hydrophobic)	[107]	P83553

but rather polyaspartate domains. RP-1 is an effective inhibitor of calcium carbonate precipitation in vitro, but this effect is almost entirely conveyed by the phosphoserines [20,59], rather by the aspartic acid residues. Interestingly, RP-1 may exhibit homologies with phosphophoryns, highly acidic matrix phosphoproteins of the dentin [58,137].

The other partly sequenced proteins also include caspartin and calprismin, two acidic soluble proteins extracted from the calcitic prisms of the Mediterranean fan-mussel *Pinna nobilis* [99]. Calprismin was sequenced on 54 amino acids, but does not exhibit homologies with known proteins. Caspartin is striking since it seems to be composed of a long poly-Asp domain. Together, Asx and Gly represent 77% of all the amino acid residues. Caspartin binds calcium with a low affinity, but strongly interacts with the in vitro precipitation of calcium carbonate. It is located within and around the prisms.

Other N-terminal sequences of shell proteins have been published in the nineties, until recently (Table 1). Particularly interesting is the protein purified from the extrapallial fluid of the edible mussel [63]. This protein is acidic, glycosylated and enriched in histidine residues. It dimerizes and binds calcium. Furthermore, it seems to self-assemble in multimeric complexes. Whether this protein is incorporated into the shell or not is not known.

The few other sequences, especially those obtained from the mussel or the pearl oyster, are too short to be truly informative [12,75,107]. In addition, they do not exhibit any homologies with known proteins. They represent however a starting point to develop degenerate DNA probes for fishing out the corresponding gene.

With the intrusion of molecular genetic techniques in mollusc studies, an increasing flux of information became available in the last few years. Table 2 summarizes our present knowledge on the primary structure of molluscan shell proteins. About 16 proteins have been characterized so far. Few were obtained by a direct sequencing, all the others, by RT-PCR or library screening. For obvious reasons related to the pearl industry and to the remarkable mechanical properties of the nacre – by far, the most studied molluscan microstructure –, a particular attention was put on the genes encoding proteins that are associated with nacre matrix. The first gene discovered to date was that of nacrein, an EDTA-soluble protein from the mother-of-pearl of *Pinctada fucata,* the Japanese pearl oyster [117]. Nacrein, a 50-kDa protein, exhibits 26 G–X–N motifs, where X is frequently D or N, and more rarely E or Y residues. The acidic repeats are suspected to be

Table 2
List of the 16 known molluscan shell proteins. Their primary structure was obtained by a direct sequencing [97,111,176], or by fishing out the corresponding gene [80,100,115,117,118,143,148,154,188]. The main characteristics of each protein are given: molecular weight (MW), theoretical isoelectric point (Theor. IP), solubility, main amino acid residues (Dominant AA). In this latter case, the letter followed by a number corresponds to the one-letter code for amino acids and the percentage of this amino acid in the protein. The functions of the protein domains were deduced from sequence analysis, and are consequently hypothetical.
Liste des 16 protéines de coquille de mollusque connues à ce jour. La structure primaire de chacune d'elle a été obtenue, soit par un séquençage direct [97,111,176], soit par le gène correspondant [80,100,115,117,118,143,148,154,188]. Les caractéristiques principales de chaque protéine sont indiquées : masse moléculaire (MW), point isoélectrique théorique (Theor. IP), solubilité, acides aminés prédominants (Dominant AA). Dans ce dernier cas, les lettres suivies de nombres désignent chaque acide aminé, selon la nomenclature internationale ainsi que le pourcentage de cet acide aminé dans la protéine. Les fonctions des domaines protéiques, déduites de l'analyse des séquences, demeurent par conséquent hypothétiques.

	Genus	*Sp*	Protein name	Microstruct. *(mineral)*	Accession number	Ref.	MW (kDa) *(aa)*	Theor IP	Solubility	Dominant AA	Domains	Putative functions
BIVALVIA	*Pinctada*	*maxima*	N66	nacre *(aragonite)*	JC7210 *(PIR)*	[80]	62,3 *568*	8,68	soluble	N21, G16	2 carb. anhydrase GN domain	HCO_3^- synthesis
			N14	nacre *(aragonite)*	JC7211 *(pir)*	[80]	16,4 *140*	5,4	soluble	G13, Y11, N11	Short acidic domains GN domain	Ca-binding ?
		fucata	nacrein	nacre *(aragonite)*	Q27908 *(trEMBL)*	[117]	50,1 *447*	6,8	soluble	N13, G13	2 carb. anhydrase 27 GXY (X=D, E, N)	HCO_3^- synthesis Ca-binding ?
			N16 or pearlin	nacre *(aragonite)*	O97048 *(trEMBL)*	[141] [118]	15,4 *129*	6,1	soluble	G11, Y10	Short acidic domains GN repeats	Ca-binding ?
			MSI 60	nacre *(aragonite)*	O02402 *(trEMBL)*	[154]	61,7 *738*	4,9	insoluble	G37, A26	11 poly-A blocks 2 A-rich domains 39 poly-G blocks short acidic domains	structural id. id. Ca-binding ?
			MSI 31	prisms *(calcite)*	O02401 *(trEMBL)*	[154]	32,8 *334*	3,8	insoluble	G24, S11, V10	10 poly-G blocks 6 ESEEDX	structural (ß-sheet) Ca-binding ?
			MSI 7	prisms *(calcite)*	Q7YWA5 *(trEMBL)*	[188]	9,3 *95*	6,7	insoluble	G28, L12	G-rich domain	structural (ß-sheet)
	Pinna	*nobilis*	mucoperlin	nacre *(aragonite)*	AF145215 *(GenBank)*	[100]	66,7 *636*	4,9	soluble	S15, P13, L9	13 SP-rich modules short acidic motifs	$CaCO_3$ inhibition ? Ca-binding ?
	Patinopecten	*yessoensis*	MSP-1	foliated *(calcite)*	Q95yf6 *(trEMBL)*	[142] [143]	76,4 *840*	3,4	soluble	S31, G24, D20	4 GS domains 4 D-rich domains 1 basic domain 3 G-rich domains	loop Ca-binding ? anchorage ?

(continued on next page)

Table 2
(continued)

	Genus	*Sp*	Protein name	Microstruct. *(mineral)*	Accession number	Ref.	MW (kDa) *(aa)*	Theor IP	Solubility	Dominant AA	Domains	Putative functions
GASTROPODA	*Haliotis*	*rufescens*	lustrin A	nacre *(aragonite)*	AF023459 *(GenBank)*	[148]	142,2 *1428*	8,1	insoluble	S16, P14, G13	10 C-rich modules 8 P-rich modules GS domain basic protease inhib.	structural id. loop anchorage? protease inhib.?
			AP7	nacre *(aragonite)*	AF225916 *(GenBank)*	[115]	9,9 *88/66*	5,2	soluble	L9, C8, S8, Y8	altern. hydrophob/ hydrophil. motifs	calcite-binding
			AP24	nacre *(aragonite)*	AF225915 *(GenBank)*	[115]	19,6 *171/147*	5,3	soluble	N8, T8, A7	short acidic motifs 2 N-glycosylations	calcite-binding
		laevigata	Perlustrin	nacre *(aragonite)*	P82595 *(SwissProt)*	[176]	9,3 *84*	8	soluble	C14, P9, L8	IGF-BP domain	IGF-binding cell interaction
			Perlucin	nacre *(aragonite)*	P82596 *(SwissProt)*	[97]	18,2 *155*	7,2	soluble	L10, R8	C-type lectin domain 2 adhesin-like repeats	sugar-binding
	Biomphalaria	*glabrata*	Dermato-pontin	Cr-lamellar *(aragonite)*	P83553 *(trEMBL)*	[111]	16,6 *148*	6,3	Soluble	S8, V8	dermatopontin N-glycosylation	ECM-binding cell interaction
	Turbo	*marmoratus*	Nacrein	nacre *(aragonite)*	AB073680 *(Genbank)*	[185]	57,6 *538*	5,8	soluble	G20, N17	2 carb. anhydrase GN domain	HCO_3^- synthesis

involved in calcium binding, a function coherent with the staining of nacrein in blue by Stains-All. In addition to these central repeats, nacrein exhibits a rather unexpected feature, the presence of two carbonic anhydrase-like sub-domains. Homology search shows that these domains are 28% similar to human carbonic anhydrase II. Carbonic anhydrases are a family of well-characterized zinc-containing cytoplasmic enzymes, which catalyse the hydration of carbon dioxide into carbonic acid [152]. In vitro enzymatic tests confirm that nacrein has a significant carbonic anhydrase activity, although reduced in comparison to that of a true carbonic anhydrase. Nacrein would be a polyvalent protein able to work as an enzyme as well as a template for binding calcium. Northern-blot analysis indicates that nacrein may be expressed at high level in the mantle zone responsible for nacre secretion. A homologous of nacrein, N66 [80], was isolated from *P. maxima,* a species very close to *P. fucata.* The two carbonic anhydrase-like sub-domains of N66 are almost identical to those of nacrein. N66 differs from nacrein by a longer central repeats domain made of 46 G-X-N motifs (where X is mostly N and rarely D, Y or G) interspersed by 12 G-N motifs. Since this domain is far less acidic than the one of nacrein, a calcium-binding function seems less likely. N66 may be expressed in both the dorsal region and the edge of the mantle, and, consequently, may be present in the nacre and the prismatic layer. N66 inhibits crystallization in solution. Interestingly, a third nacrein was obtained from the nacreous gastropod *Turbo marmoratus*, the great green turban [185]. The *Turbo* nacrein exhibits a high homology with N66, since 29% of the residues are conserved. This percentage increases to 59% in the GN domain. In the future, it will be fascinating to find homologues of nacrein in the other mollusc classes, namely, in cephalopods and scaphopods, and to see whether nacrein is strictly correlated to the presence of nacreous microstructures.

The two following genes to be isolated encode insoluble framework proteins of the shell of *P. fucata* [154]. They were isolated with a DNA probe encoding a hydrophobic peptide, which was obtained by dissolving the nacre powder with concentrated methanoic acid and treating the matrix with cyanogene bromide. MSI60 is a nacre protein, which exhibits 11 polyalanine blocks and 39 polyglycine blocks dispersed throughout the sequence. Consequently, MSI60 has a putative β-sheet conformation. The polyalanine blocks confer to MSI60 a high homology to spider-silk fibroins. The N and C-termini of MSI60 contain two and one aspartic acid-rich domains, and four and one cysteine residues respectively. MSI31, the second insoluble framework protein from the prismatic calcitic layer exhibits short (3 to 5 residues) polyglycine blocks, mainly in its N-terminus half. MSI31 has only a limited homology with a family of glycine-rich cell wall plant proteins. In addition, six XSEEDY motifs (where X is D or E, and Y is M or T) in the C-terminus confer to that protein an acidic isoelectric point. These motifs would bind calcium. Interestingly, MSI60 and MSI31 are expressed in different locations of the mantle. In situ hybridisation shows that MSI60 is secreted by the outer epithelium of the mantle in a nacre-secreting zone, whereas MSI31 would be secreted as a prism matrix component. Recently was discovered a third protein, MSI7, which exhibits a high homology (70%) with the G-rich N-terminal domain of MSI31 [188]. MSI7 might be a truncated hydrophobic variant form of MSI31. However, it would be involved in the formation of both the prismatic and the nacreous layers. A recombinant MSI7 was found to accelerate in vitro the nucleation of calcium carbonate, and to modify the morphology of the crystals. MSI7 is suspected to form supramolecular complexes.

Chronologically speaking, the fourth gene encodes a modular protein of the nacreous layer of the abalone, *Haliotis rufescens.* With 1428 amino acid residues and a theoretical molecular weight of 142 kDa, lustrin A is the longest protein identified to date [148]. Its complex primary structure is constituted by nine cysteine-rich modules interspersed by eight proline-rich modules, followed by a long GS domain, a cysteine-rich module, a short basic domain and a protease inhibitor-like C-terminus. The presence of this last domain suggests that lustrin is self-protected against degradation in the nacre. The first proline-rich domain is 53% homologous to a fragment of collagen I-alpha 1 chain, whereas the GS-loop domain exhibits a high homology (up to 66%) with GS domains deduced from ORFs of the slime mold *Dictyostelium discoideum* and of the fungi *Neurospora crassa.* Lustrin A is clearly an insoluble multifunctional protein. It would stick nacre tablets together [153], by behaving like a chain of springs, which would elongate one after each other when an increasing stretching force is applied. In other

terms, lustrin A would act as energy-absorbing filler. The mechanical behaviour of Lustrin A would be similar to that of titin, a protein involved in muscle contraction/relaxing [163]. Lustrin A may be a member of a multigene family, because northern-blot performed with lustrin A-derived probes indicate the presence of two messengers with markedly different lengths in the mantle tissues of *Haliotis.*

The fifth gene, MSP-1, was found in the scallop *Patinopecten yessoensis* [142,143]. Interestingly, MSP-1 was the first protein published which does not belong to the 'nacro-prismatic type'. The scallop exhibits indeed a foliated calcitic shell microstructure. MSP-1 is a very acidic soluble molluscan shell protein, with a theoritical isoelectric point around 3. MSP-1 is enriched in serine, aspartic acid, and glycine residues. Furthermore, it exhibits a modular structure, with a short-basic domain, close to the N-terminus, and two GS domains that alternate with D-rich domains. The GS-domains are similar to the GS loop of lustrin A, and, subsequently, may be involved in the flexibility of the protein. The two aspartic acid-rich domains are 89% similar. They fit with the model of Weiner and co-workers, since they exhibit DGS and DS motifs. They also present numerous DD repeats. All these motifs are suspected to bind calcium ions. Furthermore, MSP-1 is enriched in serine residues. Thus, it is likely that this protein is phosphorylated and /or glycosylated and that the post-translational modifications also participate in the binding of calcium ions. Like RP-1, MSP-1 exhibits homologies with dentin phosphophoryns. RP-1 and MSP-1 may be homologous.

A family of low molecular weight proteins, with 129, 131 and 140 amino acids respectively, was also genetically characterized and identified as proteins of the nacre of *Pinctada sp.* These proteins are referred as pearlin [118] (primarily called conchiolin 15) or N16 in *P. fucata* [141], or N14 [80] in *P. maxima*. They differ only by few amino acids and exhibit a moderately acidic to moderately basic isoelectric point. The members of this family are enriched in glycine and tyrosine. They exhibit NG repeat sequences in addition to four short acidic domains (3 to 12 residues). Ten cysteine residues are present in the first two thirds, at conserved positions. N14 may be specific of the nacre layer, as suggested by northern-blots. Furthermore, it may dimerize, since a 28-kDa protein is also visible as a minor component in SDS-PAGE. When adsorbed on an insoluble matrix, N14, together with N66, seem to be responsible for the nucleation of 'platy nacreous' tablets. In solution, these two proteins inhibit crystallization [80].

Another nacre protein was characterized from a cDNA library constructed from mantle tissues of the nacro-prismatic bivalve *Pinna nobilis,* the Mediterranean fan mussel. This expression library was screened with antibody probes [101]. The corresponding protein, which was named mucoperlin [100], is acidic and exhibits three regions: a short N-terminus (probably incomplete), followed by a long set of 13 almost identical tandem repeats, which are enriched in serine and proline residues. The C-terminus contains short acidic motifs, and three cysteine residues, putatively involved in intermolecular bond, and two potentially sulfated tyrosine ones. Mucoperlin is a glycoprotein, with potentially 27 sites for O-glycosylation, in the tandem repeat domain. It exhibits some homologies with PGM, a pig gastric mucin. More generally, because mucoperlin possesses P–S-rich tandem repeats and because these repeats are likely to be O-glycosylated through S residues, it can be classified in the mucins group. Mucins represent a growing family of heavily glycosylated proteins involved in the protection of mucosa in many different biological systems [52]. They are often associated with systems, which are supersaturated with respect to calcium: the buccal cavity [124], the gallbladder [132], or the urine bladder [4]. A polyclonal antibody raised against the recombinant non-glycosylated mucoperlin clearly shows that this protein is located around polygonal elements of the nacreous layer, but is totally absent from the calcitic prismatic layer. Although putative, the function of mucoperlin may be to control the lateral extension of nacre polygons. To date, this is the first protein, which has been directly localized in the shell.

Three other proteins were partly, then fully characterized by direct sequencing. Interestingly, their respective sequences do not fit in the simplistic nucleation/inhibition model. The two firsts, called perlucin and perlustrin, were obtained from the nacreous layer of the abalone *Haliotis laevigata* [97,176,177]. Perlucin is a non-acidic 18-kDa protein, for which leucine is the dominant amino acid. In its C-terminus, perlucin exhibits two short identical repeats, similar to those of the P32 adhesin. However, the most remarkable feature is the presence of a functional

mannose/galactose-binding C-type lectin domain [97]. By definition, C-type lectin domains are calcium-dependent carbohydrate-recognition domains [38]. Such domains have been found in different calcifying systems: lithostathin also called pancreatic stone protein [33], diverse sea urchin spicule matrix proteins [183], the two eggshell matrix proteins ovocleidin and ansocalcin [83,96], or the cartilage tetranectin and aggrecans [23,50,122]. In solution, perlucin accelerates the precipitation of calcium carbonate and modifies the shape of the produced crystals. Perlustrin, the second small protein, has an even more striking primary structure, since it exhibits many similarities with N-terminal sequences of vertebrate IGF-BP (Insulin-like Growth Factor Binding Protein) [176]. In vitro measurements showed that perlustrin is able to bind different IGFs. Perlustrin would interact with other components of the matrix, or with the epithelial cells of the calcifying mantle. The third protein was obtained from the aragonitic crossed-lamellar shell of a freshwater snail, *Biomphalaria glabrata* [107,111]. It exhibits remarkable sequence homologies with vertebrate and invertebrate dermatopontins, a group of proteins also called TRAMP (Tyrosine-Rich Acidic Matrix Protein). Dermatopontins are extracellular matrix proteins present in skin, skeletal muscles, kidney, cartilage, and bone [44]. They bind to decorins [125] and to Transforming Growth Factor-β (TGF-β) [126]. In addition, they promote cell attachment, cell aggregation and the self-assembling of collagen into fibrils. These functions suggest that the shell dermatopontin may be involved in the supramolecular organization of the calcifying matrix.

At last, two major proteins have been identified from their gene as matrix components of the abalone shell [115]. Named AP7 and AP24 (for Aragonite Protein), they are small soluble and moderately acidic and seem associated with the nacre microstructure. They do not exhibit homologies with known proteins. AP24 is putatively N-glycosylated, whereas AP7 presents four cysteine residues that may be involved in disulphide bond formation. Their N-terminus interacts with calcite crystallization. Very recently, another pair of minor proteins, AP8α and AP8ß has been identified [49] from the same shell matrix. They have not been sequenced yet.

Three other proteins have been characterized from the abalone nacre [162]. These proteins are called perlinhibin, perlwapin and perlbikunin. No sequence data are available yet.

6. Molluscan shell proteins and the model of shell mineralisation

Which properties do all these shell proteins share? Can they be grouped in one family? With the exception of the two nacreins and N66, which are homologous in three different species, all the other proteins appear, a priori, as a heterogeneous group of exotic proteins, which do not present clear relationships. However, a careful examination of the sequences permits to underline some important characteristics. Firstly, these shell matrix proteins are characterized by the predominance of few amino acids, usually two to four. This feature is often observed among proteins of the extracellular matrix of vertebrates [161]. As a consequence, their amino acid composition deviates – at different degrees – from that of 'standard' proteins [94]. Among the most commonly used amino acids, one finds glycine, aspartic acid, serine, and, to a lesser extent, proline, cysteine, tyrosine, leucine and asparagine. Alanine is only abundant in MSI60.

The second important feature is that the primary structure of molluscan shell proteins is modular, namely that it is organized in different functional domains. This property is also known in extracellular matrix proteins of vertebrates [39,40]. Usually, one domain corresponds to a unique function. Some domains like the carbonic anhydrase domains of nacrein and N66 are clearly identified. Some domains are composed of tandem-arranged repeat units. In simple cases, the repeat can be two amino acids, like GN [80]. In other examples, the repeats are longer: six residues for the C-terminal domain of MSI31, repeated six times [154], or 31 residues repeated 13 times in the case of mucoperlin [100]. In the most complex case – lustrin A –, the elastic domain is composed of a tandem of two different modules, one Cys-rich, one Pro-rich, which are repeated eight times [148]. All the domains can be classified into four groups: the first group comprises enzymatic domains, the second one, all the 'structural' (sensu lato) domains, the third one, the domains that interact with calcium carbonate, and, at last, the receptor or binding domains. The modular construction of molluscan shell proteins with different

combinations of these domains strongly suggests that each shell protein is able to perform different functions. It also explains why homologies with known proteins are usually low, when the whole sequence is considered.

The multifunctionality of molluscan shell proteins puts into question one common idea on molluscan-shell mineralisation, based on the interaction of two sets of proteins: the insoluble silk-fibroin-like hydrophobic proteins, and the soluble acidic proteins [175]. As shown in § 4, the dichotomy between insoluble/hydrophobic and soluble/acidic is essentially technical and comes from bulk amino-acid analyses of both matrices. The sequence data makes this dichotomy less and less obvious: MSP-1 is a soluble acidic protein with a structural motif, the GS loop, found also in the insoluble lustrin A [143,148]. MSI31, a framework protein, exhibits both structural hydrophobic (G-rich) and acidic (E/D-rich C-terminus) domains [154]. In addition, we do not exclude the possibility that two states of the same protein may coexist in the shells: a soluble state, when the protein forms monomers, an insoluble state, when this protein polymerises. Our recent data on caspartin suggest such a phenomenon [99].

A second common idea, which can be discussed briefly, is the central role played by aspartic acid residues in the shell matrix. For more than two decades, several amino acid analyses performed on different shell textures have shown that aspartate residues were preponderant in the bulk soluble fraction. In some cases, because of the conversion of asparagine residues into aspartic acid during hydrolysis, the possibility that the amounts of aspartic acid were overestimated cannot be excluded. To our knowledge, only once was demonstrated the 'aspartic-acid origin' of an aspartate-rich hydrolysate [168]. Nowadays, the belief that aspartic acid-rich proteins are widespread among molluscan shell textures may have to be revised. It is striking to observe that all aspartic acid-rich shell proteins characterized so far are associated to calcitic microstructures: foliated calcite (RP-1, MSP-1) or calcitic prisms (caspartin). The molecular data published at mid-2004 (see Addendum) confirm this tendency. By opposition, none of the proteins characterized from nacre is rich in aspartic acid residues. For example, N66 exhibits more than 20% asparagine residues, but only 5.5% aspartic acid, and its theoretical isoelectric point is rather basic [80]. Similarly, the proteins of the N14/N16 family are soluble but not acidic. The observation that Asp-rich proteins are associated with calcite is, however, restricted to the mollusc (and maybe to the octocoral) biomineralisation, since the matrix proteins extracted from the (high magnesium) calcite of echinoderms are moderately acidic and do not belong to the aspartic acid-rich family [183].

The diversity of shell proteins, as seen from Table 2, drastically modifies our view of the molluscan shell as a calcifying system. Until now, the mainstream was that molluscan shell proteins provide a framework for crystals, allow crystal nucleation, and regulate crystal growth by inhibition [92]. Obviously, shell proteins do more than these three roles. In particular, they may be involved in two additional functions: interactions with other macromolecular components of the matrix, and cell signalling. For illustrating the first function, the C-type lectin domain of perlucin specifically binds galactose/mannose present in the sugar moieties of the matrix, or of the cell surfaces of the mantle epithelium [97]. Because it exhibits some homologies with vertebrate dermatopontins, the dermatopontin of the snail *Biomphalaria* shell matrix is probably involved in the supramolecular organization of the matrix, by interacting with other matrix components [111,125,126].

The second putative function, cell signalling, calls for few developments. Perlustrin seems to be a receptor for growth factors, IGF-like. The reason for the presence of such a receptor in shell matrix is still not understood. It suggests however that the shell is not a dead terminal product of calcification (as it is often considered!) and that subtle feedbacks occur between the shell and the calcifying epithelium, which synthesizes it. One possibility would be that perlustrin first captures and concentrates IGF-like growth factors, present in the extrapallial fluid during calcification, and that these factors are released when the shell is slightly re-dissolved during the anaerobic phases [31]. The recognition of IGF-like factors by membrane receptors would trigger the epithelial cells to re-calcify the shell by synthesizing the shell matrix proteins. Of course, we do not exclude the possibility that the IGF-BPlike domain of perlustrin was co-opted for a completely different function.

The analogy with vertebrate mineralised tissues can be pushed one step further. Bones and teeth mineralising matrices are known to contain low amounts of

bioactive factors [133,186], among which BMPs (Bone Morphogenetic Proteins) are the most known [57]. BMPs trigger osteoblasts to mineralise by a mechanism similar to what described above. We cannot exclude the possibility that homologous factors are disseminated in the shell of molluscs. Their presence may explain why the shell matrix activates in vitro different cell lines to produce mineralised tissues [8,134], and why nacre, when implanted in vivo, is able to promote new bone formation [9,10,90,91,179]. In the coming future, the finding of such bioactive factors and their potential use in bone repair represents a promising challenge.

7. On the origin of molluscan shell proteins

When did molluscan shell proteins appear? The fossil record indicates that molluscs, like several metazoan groups including sponges, cnidarians, ecdysozoans, lophotrochozoans and deuterostomes started to calcify at the dawn of the Cambrian times, about 540 million years ago [29]. Calcification was indeed one of the most visible aspects of a spectacular biological event, the 'Cambrian explosion', which was marked by a prodigious diversification of most of the known phyla. Molluscs were a part of this diversification, and shell-bearing representatives of gastropods, bivalves, and monoplacophorans have been found in the Lower Cambrian [29,81]. Before this event, molluscs were already in existence since the Proterozoic fauna – 'Ediacara-type' – contains soft-bodied metazoans, which are usually considered as molluscs: *Kimberella* is the most famous example [42]. More generally, phylogenetic reconstructions based on molecular data showed that the main steps of metazoan radiation occurred before the Precambrian/Cambrian boundary [11,24], namely, before they started to calcify.

For explaining how calcification emerged from ancestral soft-bodied molluscs, two scenarios are possible. On the one hand, molluscan shell proteins were true innovations, which took place somewhere in the Late Proterozoic or Early Cambrian. On the other hand, molluscan shell proteins were recruited and orchestrated from pre-existing Precambrian functions, which were not related at all with calcification [103]. This process called exaptation [54] is supported by the following arguments.

At first, serological comparisons performed with polyclonal antibodies raised against acetic acid-soluble shell matrices evidenced unexpected cross-reactivities with the matrices of different extra-groups, like brachiopods for example [102]. Although we do no exclude the possibility of false positive signals obtained by serological techniques, and of true positive signals due to common but unrelated epitopes, these findings may also suggest deep similarities, at the protein level, between the tested matrices across phyla. If so, it is likely that the similarities reflect a common origin rather than evolutionary convergences.

Furthermore, in a paper published some years ago, we observed that mucus substances and acetic acid-soluble shell matrices extracted from molluscs also exhibited immunological similarities. Furthermore, we showed that mucus substances were able to inhibit in vitro the precipitation of calcium carbonate, in a manner similar to that of soluble shell matrices [106]. We proposed the 'anti-calcification hypothesis', where proteins of the molluscan shell matrix may have been recruited from ancestral anti-calcifying mucus, to keep crystallization in check. 'Anti-calcifying mucus' (mucus that inhibits the precipitation of $CaCO_3$) may have represented the first adaptative response of naked metazoans for preventing themselves of being encrusted by calcium carbonate nuclei, in the heavily supersaturated seawater of the Late Proterozoic [79]. The fact that mucoperlin exhibits mucin-like properties gives further support to this idea [100].

The strongest argument in favour of a Precambrian origin of molluscan shell proteins comes from the analysis of their primary structure. As shown in the previous paragraph and in Table 2, molluscan shell proteins are made of different modules, which represent functional domains [21,61]. This 'mosaic' structure suggests that the genes encoding shell proteins were constructed by exon shuffling [21,127,128]. Exon shuffling is a powerful and parsimonious gene tinkering mechanism invented by metazoan to create new genes (and new functions) from old ones [67,129]. It involves the duplication of ancestral genes, and the subsequent swapping and rearrangements of the exons of the duplicated genes. In the particular case of nacrein and N66, exon shuffling was performed from very old modules, carbonic anhydrase domains that were inherited from bacteria. In other cases, shuffled modules might have been more 'recent' and character-

istic of metazoans. Particularly relevant is the fact that three modules are homologous to extracellular matrix modules of vertebrates. The C-type lectin domain of perlucin, the dermatopontin domain of the *Biomphalaria* shell protein, the IGF-BP domain of perlustrin are the best illustration that these functions were existing at least before the protostome/deuterostome split, and that they were co-opted for calcification somewhere in the Late Proterozoic. In the case of C-type lectin domains, it is striking to notice their broad repartition, in association with phylogenetically distant calcifying systems [23,33,50,83,96,122,183]. Exaptation for mineralisation is certainly a general process, which may have occurred in other mineralised tissues. Recent data [34] have shown that the appearance of the exon 2 of the amelogenin gene predates by more than 100 million years the Cambrian emergence of teeth among vertebrates.

8. Several unsolved questions

To conclude this review, the study of molluscan shell proteins has made important advances in the last decade, but the field is still in its infancy. Molluscan shell proteins open new vistas in the domain of biomimetic materials and in orthopaedics. The most promising applications are the synthesis at room temperature of organic-mineral composite materials of high mechanical properties, the use of bioactive matrix components (growth factors, growth factor receptors) for tissue repair, the use of shell proteins as biodegradable anti-fouling agents, and finally, the improvement of pearl culture and production. However, several questions remain unanswered.

The first one concerns the exact function of molluscan shell proteins. The primary structures of 16 proteins have been elucidated, but the function of most of them was deduced from sequence analysis and homology search. Some of the proteins or protein mixtures could be tested in vitro [6,88,99,115,188] for their ability to modify the shape of $CaCO_3$ crystals. However, it is very unlikely that the assays mimic the real conditions that take place in the extrapallial fluid environment. Furthermore, the proteins were tested one per one, but in reality, they work all together. An approach, which would allow a better understanding of the functions, would be to knockout genes in larvae. So far, this technique has not been tried on molluscs. Another improvement will consist in continuing to develop conceptual tools for better understanding the supramolecular chemistry of the shell proteins and the self-assembling processes.

The post-translational modifications are another point in case. Sugar moieties represent an important part of the shell matrix, but this fraction is usually neglected. Computer programs, which detect post-translational modifications, indicate that phosphorylation [59,136], O and N-glycosylations [100,111] and tyrosine-sulfation are commonly found in shell proteins. Only in few cases, the role of these modifications in shell mineralisation was emphasized [7,110].

One open question, which has puzzled geologists for more than four decades, is the calcite/aragonite problem. Molluscs can modulate precisely the precipitation of the two polymorphs in the same shell, the stable calcite, and the metastable aragonite. In Pteriomorphid bivalves (mussels, pearl oysters), calcite forms the outer prismatic layer, whereas the inner nacreous layer, mother-of-pearl, is made of aragonite. Several biochemical analyses have shown that the matrices associated with these two microstructures are different [104,170] and some of the proteins described in Table 2 are layer-specific, in particular MSI60, MSI31 and mucoperlin [100,154]. Key-experiments performed independently by two teams few years ago showed that soluble components of the shell matrix play a role in determining the polymorph [14,41,159]. It is however not clear whether they were sufficient alone to select calcite or aragonite, as the experiment of Belcher et al. tends to show [14]. On the other hand, the experiment performed by Falini and co-workers demonstrated that an appropriate microenvironment is also required, in particular, a template made of β-chitin and silk fibroin-like proteins [41]. In both cases, a mixture of soluble proteins was tested. Thus, the key-components responsible for the deposition of aragonite – if they exist – have not been identified so far.

The last question tackled in this review concerns shell microstructures. As briefly mentioned in the introduction of this review, the shell of molluscs, in particular of bivalves, displays a large variety of microstructures [27,28,156,157]. However, our knowledge of the shell proteins is almost entirely limited to the nacreous layer and, to a lesser extent, to the calcite prisms. We virtually know anything about the compos-

ite prismatic, the crossed-lamellar, the complex crossed-lamellar, the foliated, the granular, or the homogeneous textures. Although it is likely that the shell matrix controls the microstructure, we do not have the slightest idea on how this control is performed and what the key-regulators are. Shell microstructures are probably an emerging property, controlled at supramolecular level by a limited number of attractors. If so, we are probably still very far from being able to synthesize shell textures in a test tube.

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Addendum

While this paper was submitted, three new molluscan shell proteins were discovered: these proteins, named respectively prismalin, aspein and asprich, were retrieved from cDNA sequences. All three are present in calcitic prisms: of *Pinctada fucata* (prismalin, aspein) and of *Atrina rigida* (asprich). Aspein and asprich exhibit an unusual composition dominated by aspartic-acid residues. These proteins give consistency to the idea that highly acidic proteins are associated with calcite among molluscs.

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