The biocalcification of mollusk shells and coral skeletons: Integrating molecular, proteomics and bioinformatics methods

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Chapter 1

Introduction
Chapter 1 Introduction

1.1 **General Concepts of Biomineralization**

Biomineralization refers to the process by which organisms produce minerals. It is a widespread phenomenon among living systems - a total of 55 phyla, across all 3 domains of life, produce mineralized structures, the so-called biominerals [1]. Among the most common forms of biominerals are calcium phosphates (in the form of hydroxyapatite, \( \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 \); e.g. in bones and teeth, Figure 1.1 A, and linguliform brachiopods), calcium carbonates (\( \text{CaCO}_3 \); e.g. in several protists, shells and coral skeletons, Figure 1.1 B, D and E) and silica (\( \text{SiO}_2 \); e.g. in skeletons of sponges and cell walls of diatoms, Figure 1.1 C and F). All these three main forms are broadly, yet irregularly, represented in the tree of life [2], letting numerous unsolved questions about the evolutionary origins of biomineralization.

![Figure 1.1: Scanning electron microscope (SEM) images showing the diversity of biomineral morphologies and microstructures.](image)

(A) human bone of calcium phosphate [3,4] (no scale attributed); (B) mineralized alveolar plates of calcium carbonate in the ciliate protist *Coleps hirtus* [5]; (C) siliceous skeleton of the sponge *Euplectella aspergillum* [6]; (D) the shell of the tropical abalone *Haliotis asinina* before (top) and after (lower) the mechanical removal of upper layers, and a closer view of the nacreous layer composed of superimposed tablets of \( \text{CaCO}_3 \) [7]; (E) the \( \text{CaCO}_3 \) skeleton microstructure (left) and morphology (right) of the scleractinian coral *Goniastrea favulus*.
Any living organism producing its mineralized tissue requires energy that incurs in a metabolic cost, which must be somehow compensated by the benefits given by the final product itself. In this matter biominerals provide a wide range of essential properties [10], which include – but are not limited to:

i. Support (e.g. skeleton produced by sessile organisms like corals and sponges);

ii. Mechanical strength (e.g. endoskeleton (bone) in vertebrates);

iii. Protection (e.g. molluscan shell protecting from damage and predators);

iv. Motion (e.g. endoskeleton (bone) in vertebrates);

v. Cutting and grinding (e.g. teeth);

vi. Optical, magnetic and gravity sensing (e.g. optical imaging in trilobites and gravity sense by fish otoliths in the inner ear and statoliths in jellyfishes);

vii. Detoxification (e.g. intracellular deposits to remove excess of Ca\(^{2+}\) or heavy metals in the cells);

viii. Storage (e.g. in amorphous CaCO\(_3\) concretions in plants and in some crustaceans).

While we can easily recognize the diversity of functions, forms and compositions of biominerals, among different taxa and environmental circumstances, understanding the molecular mechanisms underlying their formation remains much more challenging. Indeed, at the molecular level, biomineralization can be studied from chemical, physical and biological perspectives. In the present case, however, we will mostly focus on the biological aspects of the process.
All biominerals consist in organo-mineral assemblages where the mineral, the major component, is embedded in a minor organic matrix. This latter is often a complex mixture of macromolecules: proteins, glycoproteins, polysaccharides and lipids - which are produced by the organisms and directly involved in the formation of their skeletons, in a more or less controlled way. In most cases the macromolecules are embedded within the mineral during its growth, constituting the “organic fraction” of the biomineral or the more generally called organic matrix (OM). Despite this common feature among biominerals, the processes by which they are made are extremely diverse, in particular at the level of the control exerted by the organism, cells and ultimately by the OM. As a result biomineralization can be differentiated in two main types, first defined by Lowestam in 1981 [11]. The first type is the biologically induced, which refers to the unintentional precipitation of minerals as a consequence of ion deposits, resulting from the metabolic activities of the organism itself, or resulting from the environmental conditions. In this case, no special “molecular machinery” is generated for the purpose, the mineral deposition is mostly random, though the cell wall components (i.e. lipids, proteins and polysaccharides etc.) can influence the mineralization process by acting as generic surface for precipitation [10]. Several examples of this type of biomineralizations occur in bacteria. An interesting case is that of cyanobacteria participating in stromatolite constructions: they precipitate CaCO$_3$ from the supersaturated environment [12,13], contributing to the sediment accretion of these rocky structures occurring in shallow waters. Another example comes from several bacteria, which induce precipitation of a wide spectrum of salts (sulfates, carbonates, silicates) as a mechanism for extrusion of their metabolic products [14].

The second type is the biologically controlled mineralization, which contrasts with the induced mineralization, in the sense that it is a highly regulated process at all stages of biomineral formation [1] requiring: (1) delineation of the mineralizing
space by cell membranes or polymers; (2) formation of an array of macromolecules, i.e. the OM, and its subsequent targeting to the site of mineralization; (3) pumping of ion precursors to set up a saturated environment; and finally (4), the control of the OM over crystal nucleation, growth and inhibition, providing a scaffold for mineral deposition. Although biologically controlled mineralization occurs in bacteria (magnetotactic [15]) and also in algae (diatoms [16] - Figure 1.1 F, coccolithophores [17]) or protozoa (ciliates - Figure 1.1 B), the process is undeniably more broadly represented in animals. Indeed, vertebrates produce mineralized endoskeletons made of bone and cartilage to provide support and other essential functions, while non-vertebrates can produce a wide-range of external biominerals such as shells (in mollusks – Figure 1.1 D, and in brachiopods), carapaces (in crustaceans) and skeletons (in corals – Figure 1.1 E, and in sponges) and calcified tubes (in serpulid annelids), but also internal ones like spicules (sponges and echinoderms). The focus of this dissertation is centered in this group of aquatic calcifying organisms - especially mollusks and scleractinian corals. In both organisms exoskeletons of CaCO₃ are produced through a process of biologically controlled calcification involving a specific OM in an extracellular environment, i.e. outside the cells.

1.2 Biocalcification in Scleractinian Corals and Mollusks

Biotic carbonate calcification typically involves the precipitation of at least two ions according to the following equation: Ca²⁺ + CO₃²⁻ ⇌ CaCO₃. This reaction is favored to the right, when concentrations of both ions attain the saturation state higher than 1 (or Ω > 1) at the site of calcification according to: Ω = [Ca²⁺] x [CO₃²⁻] / Kₛ_p, where Kₛ_p is the solubility of the calcium carbonate phase precipitated,
which usually corresponds to one of two forms: calcite and/or aragonite, by far the most thermodynamically stable polymorphs. It has been shown for mollusks [18] and corals [19] that the sources of carbon used for calcification come mainly from bicarbonate ions (HCO$_3^-$) or hydrated CO$_2$, according to the equations: Ca$^{2+}$ + HCO$_3^-$ ⇔ CaCO$_3$ + H$^+$ and CO$_2$ + H$_2$O + Ca$^{2+}$ ⇔ CaCO$_3$ + 2H$^+$. In both groups of organisms these reactions do not take place in direct contact with the aquatic environment but rather at specific sites, *i.e.* microenvironments specially delineated for calcification, as it will be detailed below.

When considering the physiology of shell calcification in adult specimens, there are at least four elements to take into account in the anatomy of mollusks [18] (Figure 1.2 B). The key element is the *mantle*, a thin tissue that coats the inner side of the shell and is divided in several regions including the outer and the inner epithelia. The outer epithelium produces the organic matrix and pumps the necessary ions (HCO$_3^-$, Ca$^{2+}$) to the *extrapallial space* where biocalcification takes place. The mantle, the periostracum and the shell enclose the latter. The periostracum is the outer layer covering the shell and consists of an organic sheet synthesized by the periostracal groove; it is formed along with the shell, providing support and delineation of the extrapallial space. It is worth noting that shells grow by increments, more in length than in thickness, and so does the periostracum [20] (for a review on the proposed mechanisms of shell mineralization see [21]). Shell microstructures from adult mollusks are very diverse (for a review see [22]). However, the physiological model described here generically represents that of a bivalve with two shell layers (Figure 1.2 B). The outer layer is made of calcitic prisms, while the inner one is the nacreous layer made of aragonitic nacre tablets. The outer epithelium specifically secretes the OM components to form each of these layers from distinct areas as drawn in Figure 1.2 B.
Calcium and bicarbonate ions are taken up from the body surface, inner epithelium of the mantle, from the gills or from the gut [18]. They are actively transported via the haemolymph to the outer epithelium, and then pumped to the extrapallial space, by calcium and bicarbonate channels located in the cells of the outer epithelium. This creates a supersaturated extrapallial fluid where precipitation occurs. Protons released from crystallization of CaCO$_3$ are absorbed by proton ATPases (Figure 1.2 C). The extrapallial space localizes the transition from the liquid state – the supersaturated extrapallial fluid – to the solid state, the biomineral. This classical view is commonly accepted without being firmly established from an experimental viewpoint.

This process is done in a controlled way through self-assembling of the organic matrix components (mainly (glyco)proteins (Figure 1.2 E) and polysaccharides) with the mineral phase.
Figure 1.2: **Overview of the multi-scale shell calcification process in a bivalve producing two distinct shell layers (nacre and prisms), highlighting the main compartments and scales involved in shell-formation.** (A) An opened pearl oyster (left) and an oyster removed of a visceral mass (right). Adapted from Awaji & Machii [23]. (B) Tissue morphology in a bivalve (e.g. pearl oyster). Calcification takes place in the extrapallial space. In the outer epithelium, the cells responsible for the deposition of nacre are in a different area from those responsible for the production of prisms. The prisms and nacre tablets are not drawn to scale. Extracted from Marin et al. [21]. (C) Schematic physiological model of calcification for bivalve shells with ion fluxes of Ca\(^{2+}\), H\(^+\) and HCO\(_3^-\). Scheme adapted from Hippler et al. [24]. (D) and (E) Schematic representation of mollusk (eukaryotic) cells and molecules. Adapted from [25,26].
As for the physiological model of calcification in corals, there are also several versions but our focus is mainly on scleractinian corals (i.e. corals that generate a hard exoskeleton) living in a mutualistic relationship with zooxanthellae - photosynthetic algae that also contribute to the calcification process [27,28]. Symbiotic corals live in colony and are very common in warm shallow waters, being the major contributors of tropical reefs. Therefore they are also called hermatypic (or reef-building) corals.

Figure 1.3: Overview of the multi-scale calcification process in the hermatypic coral. (A) Schematic view of two polyps in a colony linked by the coenosarc. Adapted from Tambutté et al. [29], (B) Diagram of the calcium pathway through the calicoblastic epithelium during calcification. Adapted from Allemand et al. [30], (C) Diagram of the bicarbonate pathways from the seawater through the different layers of the coenosarcs, reaching the mineral deposition front. ECM = extracellular calcifying medium. Adapted from Allemand et al. [30], (D) and (E) Schematic representation of coral (eukaryotic) cells and molecules. Adapted from [25,26].
Scleractinian corals produce an external skeleton that provides support and is covered by the colony soft tissue. The individual units of the colony are the polyps, which are linked together by the coenosarc (Figure 1.3 A). The polyps and coenosarcs are organized in aboral and oral tissues (Figure 1.3 C) separated by the body cavity (the coelenteron). The oral tissue is divided in oral ectoderm (in contact with the seawater) and oral endoderm (containing the zooxanthellae) while the aboral tissue is divided into aboral endoderm and aboral ectoderm (facing the skeleton). The latter is relatively thin (0.5 to 3 µm) [30] and contains the calicoblastic cells, which are responsible for the formation of the aragonitic skeleton, by controlling the transport of ions and the secretion of the organic matrix to the mineralizing space - the extracellular-calcifying medium (ECM). Although this space is present, it is not always visible and has varied thickness (< 1 µm). Moreover the ECM does not extend through the entire gap between the skeleton and the soft tissues since both remain attached by specialized anchoring cells, the desmocytes [31,32].

Similarly to mollusks, there is transport of Ca\textsuperscript{2+} and HCO\textsubscript{3}\textsuperscript{-} from the seawater through the epithelial layers in order to reach the ECM (Figure 1.3 B), which is the thin space delineated by the skeleton and the calicoblastic epithelium and where calcification takes place. Ion transport may occur by diffusion/seawater flow or by active/facilitated transport. At the level of the calicoblastic epithelium however there are evidences suggesting that a combination of both ways is involved in the transport of calcium: by diffusion in the septate junctions between calicoblastic cells and by active transport through pumps [33] (Figure 1.3 B). Concerning inorganic carbon, a large part of it comes from metabolic CO\textsubscript{2} [34] and the remaining part should come from inorganic carbon in the seawater (Figure 1.3 C). Altogether these mechanisms and recent studies at the level of the ECM [35]
confirm that the ionic composition in the calcifying medium is different from the seawater.

While there is control in the supply of ions to the mineralizing space in both mollusks and coral examples, the extent of the control exerted by the organic matrix over the morphology and microstructure of coral skeletons is still a matter of debate. Barnes (1970) and Constantz (1986) stated that coral skeleton is formed by a physicochemical dominated process, and that only competitive crystal growth determines the morphology of the skeleton [36,37]. From another perspective, Johnston (1980) sustained the idea of a biologically controlled process [38] and, later on, Lowenstam and Weiner (1989) [1] and, for example the work of Gladfelder (1983) [39], considered corals as an intermediate case, were both physicochemical and biologically-driven mechanisms control the mineral deposition. More recently, Veis (2005) also referred corals has an example of biologically induced mineralization, arguing that the biominerals “adopt crystal shapes similar to those formed by inorganic processes and have essentially random crystal orientations” [40]. Nevertheless there are many evidences in support of a biologically controlled skeletogenesis in corals. Works on the microstructure of scleractinian corals have shown a great variation among coral species [41]. Moreover structural units differ in composition [41–45], mostly due to the presence of the organic matrix, suggesting a strong biological control of skeletogenesis. In view of these results, new biomineralization models have been proposed [33,45–47], that take into account the organic matrix in the control of skeleton formation.

In both corals and mollusks, biocalcification is a physiological process that can be approached in many scales, from the final macromorphology to the underlying genetics of calcification (Figure 1.2 and 1.3). Although there is still much to study about the exact mechanisms by which the components of the organic matrix, in particular the proteins, control the formation of their mineralized tissues, some key
features of these proteins are recognized as functionally important, since they are generally common to corals and mollusks. The first common trait is the high content in aspartic acid and, in less degree, glutamic acid. This feature indicates that the most abundant proteins in shells and skeletons are acidic. Moreover, they are recognized to have strong inhibitory function on the crystal growth and control on the morphology, due to their polyanionic character [48–50]. Another interesting feature of the OM proteins are the post-translational modifications, in particular, glycosylations and phosphorylations, that can greatly contribute to the polyanionicity of the proteins [51,52]. Besides glycoproteins, the OM contains polysaccharides which are important at providing structure [32] to the organic-mineral framework but also at interacting directly with the crystals [52]. The enzyme carbonic anhydrase has also been identified often in organic matrices. The function of this metalloenzyme is to catalyze the reversible hydration of CO₂, forming one bicarbonate ion and one proton, according to the following reaction:

\[ H_2O + CO_2 \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H^+ \]

A famous example is Nacrein, a modular protein with two carbonic anhydrase domains intercalated by an acidic domain [53] which was first identified in the shell of the pearl oyster *Pinctada fucata*. Modularity is indeed another common feature of OM proteins, pleading for the idea of multifunctional molecules. Among these modules were recognized other enzymatic domains in mollusks [54] and regions of biased compositions in a few amino acids (i.e. low complexity regions) - or tandem repeats [55,56].

### 1.3 Why to Study Biomineralization Proteins of Aquatic Calcifiers

Biocalcification is the most common form of biomineralization in the freshwater and marine environments. Mollusks and scleractinian corals do biologically controlled calcifications to produce their external shells and skeletons, the primary
function of which is to provide support and protection of their soft tissues. Moreover, as biomaterials, these composites exhibit complex microstructures and are source of particular interest in the material science and medical fields due to their exceptional mechanical and osteogenic properties. For example nacre, also known as mother of pearl, has been the subject of multiple biomimetic studies [57]. This calcified layer is present in several mollusk shells, including bivalves (pearl oyster, freshwater mussel) and gastropods (abalone, top and turban snails). As an inorganic-organic nanocomposite material, nacre has excellent strength, toughness and stability, appealing many material scientists in the creation of nacre-inspired materials. Although material scientists have been successful in obtaining a layered nanostructure, they do not achieve the same impressive mechanical properties as the original nacre [58,59], which can show an ultimate strength ten times higher than pure mineral [60]. Nacre is not only considered an inspiring biomaterial, for example for bone and dental support [61], but also as an osteoinductor with capacity for cell differentiation [62] and bone regeneration [63]. Altogether, nacre properties are related to its hierarchical microstructural organization where superimposed flat aragonitic tablets with 0.5-1.0 μm of thickness are surrounded by an organic matrix with approximately 5% of the total mineral weight [21].

As for coral skeletons, they were reported to have lower values of strength (stress and fracture) than most other carbonate skeletal materials [64]. Still scleractinian skeletons are considered biocompatible, osteoconductive and biodegradable. They have been used with contrasted success as implants, e.g., bone grafts substitutes [65].

Coral skeletons and molluskan shells are also useful source of fossil records [66] and are frequently used to study the impact of environmental changes in the marine environment [67]. Indeed, a significant decrease in the biocalcification rates of some mollusks and reef-building corals has been observed [68–71], among other
marine calcifiers. The decline of calcification is a direct effect of ocean acidification, \textit{i.e.} the decrease of pH in the seawater due to the higher diffusion of CO$_2$ that is released in excess into the atmosphere.

Thus, a full identification of the organic matrix components and a complete elucidation of their interactions with minerals and cells will contribute to many \textbf{open questions} on:

- \textbf{the mechanical, biocompatible and inductive properties of mineralized structures}, that are of interest for biotechnology applications;
- \textbf{the calcifying mechanisms of marine organisms}, that ultimately help to predict the impacts on calcification in a global changing environment;
- \textbf{the evolutionary paths of calcifying systems}.

The study of the organic matrix, in particular the proteins composing it, is the main subject of this thesis. The protein repertoire is also considered as the “biomineralization toolkit”, \textit{i.e.} the key element of organic matrix-mediated processes, which bares the genetic background involved in the process (Figure 1.2 and 1.3 D-E).

With the research described here, we aimed at characterizing several biomineral-occluded proteins by integrating molecular biology, biochemistry, proteomics and bioinformatics’ methods and focusing mainly in two species: the first one belongs to the phylum Mollusca – the freshwater mussel \textit{Unio pictorum} (Bivalvia class), and the second belongs to the Cnidaria - the reef-building coral \textit{Acropora millepora} (Scleractinia class).
1.4 Outline of the Thesis

The research described in this thesis was supported by the EU Seventh Framework Programme (FP7) within the Marie Curie Initial Training Network BIOMINTEC entitled: “Biomineralization: Understanding of basic mechanisms for the design of novel strategies in nanobiotechnology”, in the period 2009-2012. The BIOMINTEC project gathered together a consortium of 10 teams (8 universities and 2 private companies) from Germany, France, Italy, UK, Greece, The Netherlands, Austria and China, and was coordinated by Prof. Dr. Dr. Heinz C. Schroder from the University Medical Center of the Johannes Gutenberg University Mainz (UMC), Germany. The goal was to build a multidisciplinary and international network of researchers and biotechnology companies in the EU, dedicated to the study of basic mechanisms of biomineralization (biocalcification and biosilification) and ultimately translate this knowledge into novel strategies to apply in bio- and nanotechnology. This project also offered the opportunity to young researchers to receive their PhD training in the field of biomineralization, and conduct their research activities in more than one host institution.

Concerning the research training that contributed to this thesis, it was conducted first at the University of Burgundy (France) and supervised by Dr. Frédéric Marin over a period of 18 months between 2009-2010. Subsequently, the project continued at the University of Amsterdam (The Netherlands) under the supervision of Dr. Jaap Kaandorp, for an equal period between 2010-2012, which included some short-term stays back in France to perform experiments. Finally the BIOMINTEC program was followed by a 1-year extension supported by the EU FP7 Knowledge Based Bio- Economy project BioPreDyn.
In this thesis, we aim to present a comprehensive understanding of the molecular processes of biocalcification in mollusks and corals, by identifying new proteins directly involved in biocalcification, and by obtaining, whenever possible, the complete repertoire of skeletal proteins. To this end, we made use of high-throughput technologies, such as proteomics, combined with other molecular and bioinformatics approaches. This goal was largely achieved. Our work has contributed to a deepened knowledge of organic matrix–mediated mechanisms underlying the process of skeletogenesis (in corals) and shell formation (in mollusks), as well as to shed light on the macroevolution of metazoan biocalcification.

Following the introductory chapter, Chapter 2 describes the identification and molecular characterization of Upsalin, a novel protein associated to the nacreous layer of the freshwater bivalve *Unio pictorum*. Upsalin is a small protein of unknown function that does not exhibit the classical features of other shell-associated proteins. The discovery of Upsalin highlights the diversity of proteins associated to calcified tissues and the need to invest on new experimental methods to unveil their functions.

Chapter 3 describes the proteomic study applied to the skeleton of the scleractinian coral *Acropora millepora*. After extraction of the organic matrix, the proteome was analyzed by liquid chromatography-tandem mass spectrometry, which, combined with the coral transcriptome, enabled the identification of 36 skeletal matrix proteins. This dataset comprises proteins exhibiting important and recognized domains in biomineralization: acidic, extracellular matrix and enzymatic, but also novel signatures. In addition, we show that some proteins have signal peptides being directed to the extracellular calcifying space, whereas others may act in the calcifying space while attached to the cell membranes. The 36 skeletal proteins were then compared with three cnidarian genomes and other
organic matrix proteomes from calcifying metazoans owing to bioinformatics tools. The results suggest a two-sided scenario for the evolution of coral calcification: by mechanisms of cooption and domain shuffling.

**Chapter 4** is a small report that deals with the challenges faced by us and other scientists when using proteomics to determine organic matrix proteins directly involved in biomineralization. Since part of these proteins are embedded in the mineralized tissues, they can easily be identified by liquid chromatography-tandem mass spectrometry combined with the nucleic acid dataset of the corresponding species, after decalcification and purification of the organic fraction as described in Chapters 2 and 3. However, the diversity of protein hits that has been resulting from some of the works applying these methods is worrying. Many protein datasets published in the recent years include intracellular proteins and other contaminants that hamper the interpretation of the results and the conception of new models, in particular those of biocalcification occurring in an extracellular medium. Therefore, we discuss the importance of a thorough cleaning of biomineral samples prior to highly sensitive coverage by proteomics.

**Chapter 5** is a structural description of the skeleton of *A. millepora*, together with an overall biochemical, compositional and functional characterization of the skeleton organic matrix. The skeleton of *A. millepora* is fully aragonitic but incorporates some minor elements such as sodium, strontium, sulfur and magnesium. The organic matrix is not only composed of the proteins described in Chapter 3, but also by a large amount of sugars, among which arabinose is by far the most abundant monosaccharide. This unusual biochemical signature has not been found in other biominerals besides Acroporid skeletons and mucus. Additionally, new evidences are provided to strengthen the hypothesis developed in Chapter 3, in such a way that some proteins of the organic matrix are transmembrane, and their extracellular parts are the ones acting in the mineralizing space, being subsequently occluded in the skeleton. At last, the OM was
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characterized for its CaCO$_3$-mineralization activity \textit{in vitro} showing strong control on the morphology of the crystals.

**Chapter 6** summarizes the thesis, with a general discussion and conclusion of the previous chapters, and brings new perspectives for the study of biomineralization proteins.

In brief, each chapter has been adapted from the published [72–75] and submitted work. Chapters 2, 3 and 5 are organized in the form: Introduction, Background, Materials and Methods, Results, Discussion and Conclusions (the latter only in Chapter 3). Chapter 4 was adjusted to include the work described in one editorial letter and viewpoint articles and is only divided in two sections. All figures are numbered according to the number of the corresponding chapter and all references are numbered in the order they are found along the dissertation.