Review

Atomic Details of Biomineralization Proteins Inspiring Protein Design and Reengineering for Functional Biominerals

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Abstract: Biominerals are extraordinary materials that provide organisms with a variety of functions to support life. The synthesis of biominerals and organization at the macroscopic level is a consequence of the interactions of these materials with proteins. The association of biominerals and proteins is very ancient and has sparked a wealth of research across biological, medical and material sciences. Calcium carbonate, hydroxyapatite, and silica represent widespread natural biominerals. The atomic details of the interface between macromolecules and these biominerals is very intriguing from a chemical perspective, considering the association of chemical entities that are structurally different. With this review I provide an overview of the available structural studies of biomineralization proteins, explored from the Protein Data Bank (wwPDB) archive and scientific literature, and of how these studies are inspiring the design and engineering of proteins able to synthesize novel biominerals. The progression of this review from classical template proteins to silica polymerization seeks to benefit researchers involved in various interdisciplinary aspects of a biomineralization project, who need background information and a quick update on advances in the field. Lessons learned from structural studies are exemplary and will guide new projects for the imaging of new hybrid biomineral/protein superstructures at the atomic level.

Keywords: biominerals; atomic details; interactions; calcium carbonate; silica; hydroxyapatite; 3D structures

1. Introduction

Biominerals are extraordinary materials with a variety of functions, including providing rigid support for soft tissue, breaking down food, protection from prey, sheltering, and more. Therefore, biominerals represent an attractive natural source of inspiration for novel bioengineered materials with properties adapted to vital functions [1–4]. From eggshells to pearls, bivalve mollusks to sponges, biominerals offer a variety of materials in nature, and like silica, often is produced on a scale than industrial amount [5–7]. Even more astonishing is the fact that these materials are made by organisms in very mild conditions.

Notoriously, chicken eggshells are made of more than >90 w/w% of a calcite mineral (calcium carbonate, CaCO₃) in association with ~0.03 w/w% uronic acid (carbohydrate) and small amounts of other amino acids [8]. These associations of biomineral and organic molecules/macromolecules make eggs resistant, but still fragile to allow etching [9–11]. Bones contain hydroxyapatite, consisting of a phosphate cluster anion counterbalanced by calcium and the addition of two hydroxy ions (Ca₁₀(PO₄)₆(OH)₂). This biomineral, together with collagen, the most abundant protein in our body, helps to confer our skeleton with strength and elasticity [12].

Organisms are equipped with biominerals, often interspersed with proteins (or other organic molecules), that help to glue tiny inorganic crystals to form biomineral aggregates. From a chemical perspective, these crystals are formed by salts of carbonate, citrate, iron oxide, oxalate, phosphate, silicate, sulfates, etc., often counterbalanced by cations of calcium, iron, magnesium, sodium, etc. Biomineral crystals in turn aggregate and assemble to form...
defined functional shapes ranging from beautiful spherical pearls and amazing spirals of snail shelters. Biominerals can even form tiny compasses assembled in strings that confer bacteria (and other organisms) sensing of the directional Earth’s magnetic field and to drive their motion towards nutrients and/or long-distance migration [9,13,14]. Notably, bacteria known as magnetotactic bacteria, a class of gram-negative bacteria, are able to biomineralize iron into magnetic particles of magnetite, a mixed oxo-iron compound (Fe(II)Fe(III)\textsubscript{2}O\textsubscript{4}), or greigite, the equivalent biomineral with sulphur atoms (Fe(II)Fe(III)\textsubscript{2}S\textsubscript{4}) [13,15,16]. Magnetite aggregates are grown in an enclosed lipid-bilayer membrane organelle known as magnetosomes [15,17–21]. In addition to the key role of crystal nucleation and mineral size regulation, proteins also confer properties such as elasticity and resilience to pressure.

Besides supporting mineral formation, proteins also provide other necessary tools (such as cell organelle membrane formation, etc.) or the cargo transport of inorganic ions for crystal formation. Proteins help to build cellular compartments to host the growing minerals or provide signalling factors for crystal growth. Proteins or designed peptides can stabilize an unusual crystal form aggregation that would otherwise be unstable and help to prepare mineralized mimicking bone structures [22,23]. Biomineral properties are not only derived from coexistence of different chemical entities, but also the synergy of several biophysical factors (e.g., pH, temperature, concentration, inhibitors concentration) can affect biochemical pathways synthesizing biominerals and can provide further insights into projects for artificial synthesis of biominerals [4,24–26]. From a chemical perspective, it is very intriguing to study the interactions of chemical entities that are so structurally different.

Considering the growing interest for biominerals and their potential applications as materials obtained from sustainable processes, biomineralization proteins involving all biochemical pathways of biomineral biosynthesis has grown [27]. Structural properties of biological/organic macromolecules affect biomineral properties on a macroscale level and through a common principle based on non-classical crystal growth [28,29]. In fact, distantly related organisms adapting to their environment generally use a common universal motif (aka given stretch of protein sequence) that in turn is used as a tool to assemble a material on to the macro scale level with shape and size as needed. In other words, the complex variety of shapes of biominerals on the macro scale seems to have a different and less organized level of complexity on the micro scale level [30,31].

Although determination of the atomic details of proteins in complex with a biomineral is not straightforward, because of the different chemical entities involved, crystallography, NMR, and cryo-electron microscopy have been instrumental in understanding the nature of interactions of biomineralization proteins and biominerals. Despite the growing number of ordered biomineral protein structures in the Protein Data Bank (PDB), the archive of the macromolecular information, the structural biology of these well-ordered macromolecules represents a segment of larger protein sequence space [31]. In fact, a key role is played by biomineralization proteins that are intrinsically disordered (IDP proteins) or contain a sequence region that only becomes structurally ordered/disordered or are able to undergo variable post-translational modification in the presence of inorganic materials [32]. IDP proteins are more difficult to study by conventional structural biology techniques. Among IDP proteins, osteopontin, a protein expressed in our bones and tissues, is highly negatively charged and has multiple functions including bone structuring [33]. Even more insightful, is the role of biomineralization proteins with multifunctional capabilities such as deposit, nucleation, crystal formation and inhibition [34–36].

I have mined the RCSB PDB, a part of the wwPDB, to provide an overall view of the key atomic details of structurally ordered biomineralization proteins, an analysis of the type of nature of interactions between these macromolecules and biominerals [31]. Notably, a number of biomedical and other applications involving biomineralization proteins are rapidly growing [37–39]. Therefore, I have extended our search to find the 3D structural details of designed proteins or other known natural proteins, including enzymes, repurposed for the synthesis of novel biominerals. A list of unique experimentally determined
protein structures including functions, associated biominerals, bound ligands, and other key features (functional residues) is reported in Table 1. This review is organized in five different sections reflecting the available experimental structures, which include natural, designed or re-engineered biomineralization proteins. Finally, for each section indicated below, I have identified applications and inspiring lessons that can be learned.

**Table 1.** Natural, designed, and re-engineered proteins for the synthesis of functional biominerals. The unique entries are retrieved from the RCSB PDB (RCSB.org) [31,40]. The 3D structure for each indicated entry and other protein annotations can be explored by searching from the PDB code, indicated in the second column, and browsing the link below. Related entries can be retrieved from the structure summary page of each entry indicated in the column “PDB code ID”. The ligand name column refers to ligand of interest, if bound to the structure, and not any other solvent. Link: https://www.rcsb.org, accessed on 4 July 2022. The MamE structural model is available from the AlphaFold project [41].

| Source | PDB Code ID | Macromolecule | Crystal Material | Ligand of Interest | Function/Fold Type/Active Site Residues |
|--------|-------------|---------------|-----------------|--------------------|----------------------------------------|
| Porcine | 1q8h [42]  | Osteocalcin    | Hydroxyapatite  | Calcium ion        | Modified Glu residue at position 17, 21, and 24. A -S-S- linkage between two protein chains is present |
| Bovine | 1q3m [43]  | Osteocalcin    | Hydroxyapatite  | –                  | Dynamic binding study of calcium ions |
| Bovine | 4mzz [44]  | Osteocalcin (3 Glu form) | Hydroxyapatite  | –                  | Regulation of glucose metabolism. Helical propensity and Glu residues at positions 17, 21, and 24 are also of a focus |
| *Argyrosomus regius* /fish | 1vzm [45] | Osteocalcin | Hydroxyapatite | Magnesium ion | Modified Glu residue at position 17, 21, 24, and 25 |
| *Struthio camelus* | 4uww [46] | Struthiocalcin-1 | Calcite | – | α/β fold, C-type lectin. Intramineral protein. Glu63, Glu64, Glu65, Glu66 and Asp67, Asp93, Asp94, Asp95 and Asp96 |
| *Gallus gallus* | 1gz2 [47] | Ovocleidin-17 | Calcite | – | α/β fold, C-type lectin. Intramineral protein. Arg103, Lys106, Arg108, Arg109 and Arg117 |
| Flounder | 1wfa [48] | Antifreeze protein (AFP) | Ice | Water molecules | Inhibition growth of ice crystals. A simple alpha-helix |
| *Brachyopsis rostratus* | 2zib [49] | Antifreeze protein (AFP) | Ice | Water molecules | Inhibition growth of ice crystals. It is a C-type lectin protein |
| Pteria Penguin | 5yrf [50] | PPL3-A | Calcite/Pearl | Trehalose | Carbohydrates as mediators for biomineral recognition. Few -S-S- linkages are present. Asp32 and Glu86, interact with calcium ions, and positively charged residues, Lys83, Lys107, Lys118, Arg119, and Arg147 binding to carbonates anion. Aromatic residues are present |
| Sea urchin | 2jyp [35] | Aragonite protein AP7, C-terminal domain (36 residues) | Mollusk shell, nacre formation | – | Protein-protein interaction and other functions. Contains a Zn binding –Cys–(X)4–Cys– motif. Depending on biomineral type, binding function can be variable |
| Source | PDB Code ID | Macromolecule | Crystal Material | Ligand of Interest | Function/Fold Type/Active Site Residues |
|--------|-------------|----------------|------------------|-------------------|-----------------------------------------|
| Marine sponge <em>Tethya auriantium</em> | 6zq3 [51] | α-silicatein | Silica/sponges | – | Silic acid condensation. Hydrolyase. Functional residues S26, H165, Q20, N185. Silic acid is added to the crescent silica polymer |
| Human (chimeric construct) | 2vhs [52] | cathepsin L | Silica/sponges | Sulphate | Silic acid condensation. Hydrolyase. Catalytic mechanism is described [52]. |
| Magnetospirillum <em>griffiswaldense</em> | 3asf [53] | MamA, TPR like | Magnetite | – | Forms a homo-oligomeric scaffold for magnetosome associated proteins guidance |
| Magnetospira | 5ho1 [54] | MamB | Magnetite | Mg$^{2+}$ | Transport activity for protons and iron ions |
| Magnetosome protein | AF-QW8Q8-F1 [55] | MamE, HtrA protein | Magnetite | – | MamE functions as protease, and together with MamD and MamO, regulates crystal formation and size |
| Magnetospirillum <em>griffiswaldense</em> | 3w5x [56] | MamM | Magnetite | – | Transport activity for protons and iron ions |
| Magnetospirillum magneticum | 5jyg [57] | Mamk filament, Actin-like atpase | Magnetite | ADP cofactor, Mg$^{2+}$ | Mamk aligns magnetosomes. It functions as an actin homolog |
| Magnetospirillum magneticum | 5hm9 [58] | MamO | Magnetite | – | MamO supports metal transport inside the subcellular compartment. Presence of a surface di-His cluster (H148, H263). Promotes crystal nucleation. |
| Magnetospirillum magnetococcus | 4jj0 [59] | MamP | Magnetite | Heme C | Iron(II) oxidation and mineralization. It performs a control ratio Fe(II)/Fe(III) |
| Designed protein | 5chb [60] | nvPizza2-S16H58 | Nano crystal composed of 7 cadmium ions and 12 chloride ions | Tiny crystal composed of 7 cadmium ions and 12 chloride ions | Protein induce assembly of a small nanocrystal |
| <em>Stenotrophomonas maltophilia</em> | 6k1n [61] | γ-lyase smCSE | CdS, ZnS, AgS quantum dots | PLP cofactor | Function: lyase. Tyr 110 interacts with the aromatic group of the PLP cofactor contributing to its orientation and enzyme catalysis |
| <em>Escherichia coli</em> | 7mq6 [62] | Maltose binding protein (MBP) | Gold nanoparticle synthesis | Gold(I); Maltose | Repurposed enzyme. Peptide fused within protein sequence for binding of gold (functional residue Met 322). |
| Chicken | 3p64 [63] | Lysozyme C | Gold nanoparticle | 9 gold ions Au(I), Au(III) | Hydrolyase. Protein crystals form several gold ions aggregates |
| Reengineered human ferritin | 3es3 [64] | Ferritin heavy chain | Gold nanoparticle | Gold(I) ions | A total of 96 non-native cysteines added to the interior of the shell |
| <em>Pyrococcus furiosus</em> | 2x17 [65] | Ferritin homolog | Silver nanoparticle | Silver(I) ions | Natural protein repurposed for silver nanoparticle growth under reduction condition |
| Frog ferritin | 3ka3 [66] | Ferritin | Iron(II)/(III) biomineralization | Mg$^{2+}$; Cl$^-$ | “bucket brigade” pathway for the iron(II) movement towards the catalytic di-iron center |
2. Classical Protein Templates for the Nucleation and Growth of Biominerals

Osteocalcin, one of the most studied biomineralization proteins, provides a reference point for the key interactions of biominal recognition, and its atomic details have been available since 2003. After collagen, osteocalcin is the second most abundant protein in our body/bones and acts as a hormone to promote bone growth [67]. In bones, it binds to the surface of hydroxyapatite crystals, a mineral composed of calcium and phosphate. The human osteocalcin is a small protein composed of only 49 amino acids with the presence in its primary structure of a modified amino acid, γ-carboxyglutamic acid residue (Gla, a glutamic residue with an extra carboxylic group), at positions 17, 21, and 24. Furthermore, a disulfide bond between two cysteine residues at positions 23 and 29 helps to stabilize the structure. The atomic details of the crystal structure of porcine osteocalcin, shown in Figure 1, reveals how this protein recognizes the inorganic mineral surface: an alpha-helix presents a set of negatively charged amino acids, spaced in a regular pattern with the ability to recognize calcium ions in the crystal mineral that are in turn structurally organized. The presence of doubly charged Gla residues further supports the importance of properly oriented side chains to coordinate calcium ions at the proper distance and geometry and plays a role in the helical propensity of this protein sequence and regulates its affinity for hydroxyapatite [44]. In fact, several calcium ions were seen in the crystal structure, showing a perfectly-matched spacing of amino acids to ions (Figure 1 showing PDB entry 1q8h) [42].

A more recent study based on mass spectrometry has revealed the presence of osteocalcin fragments presenting a fourth γ-carboxylation at residue 25 not previously detected in the structure of the porcine osteocalcin. A similar result emerged from a structural study from a fish osteocalcin [45,68]. In summary, from the wealth of the structural studies I have learned how calcium-hydroxyapatite is recognized by the protein and how the helical propensity/stability also play a role in mineral recognition.

Calcite, a calcium carbonate biomineral, is also synthesized by a protein similar to osteocalcin: struthiocalcin. Eggshells contain calcite, a mineral form of calcium carbonate, with a matrix of protein sandwiched in between. Naturally, CaCO$_3$ exists as different crystal polymorphs (i.e., calcite, aragonite, vaterite). Like osteocalcin, eggshell protein struthiocalcin, shown in Figure 1, (PDB entry 4uw) binds to the surface of the mineral crystals using an array of acidic amino acids that bind calcium, helping to direct the crystal growth of ostrich eggshell formation [46]. Struthiocalcin has a folding shape termed C-type lectin. Similarly, ovocleidin-17, the intramineral protein found in chicken eggs, offers a similar view of electrostatically charged surface protein residues interacting with inorganic material but with a subtle difference. In place of negatively charged acidic residues (glutamic and aspartic residues), it presents a stretch of positively charged residues (arginine and lysine residues, Table 1) [47,69].

Besides the interest in understanding how calcite material is deposited in eggs, these proteins are also studied for material applications and goose eggshell ansocalcin, an ovocleidin-17 protein, is capable of inducing crystallization of calcite crystals in vitro [71,72]. Studies of ostrich egg fossils show that the interaction between the mineral and the protein is so strong that fragments of the protein-mineral complex can last for millions of years [73,74]. Struthiocalcin is a C-type lectin protein, and though it binds to a mineral entity, the shape is similar to antifreeze proteins that bind ice crystals to their surface (Figure 2, PDB entry 2zib), and it is thought to have evolved from a common ancestor [49,75].
Figure 1. Atomic details of biomineralization proteins binding a calcite biomineral. The biomineral represented as a light blue color shape is interfaced with the rich acidic segment of osteocalcin (PDB accession code 1q8h [42]) and struthiocalcin-1 (PDB accession code 4uww [46]). Both proteins are represented as spheres. Red colors indicate acidic residues (Glu, Gla, and Asp residues). Calcium ions are indicated in cyan colored spheres. This figure is obtained from the Molecule of the Month column “Proteins and Biominerals” (Di Costanzo, L; Goodsell, D. 10.2210/rcsb_pdb/mom_2019_4) [70].

Figure 2. Superposition of biomineralization proteins reveals similarities with the ice binding protein (PDB entry 2zib [49], green color). Superposition of osteocalcin (brown, PDB entry 1q8h [42]) and ice binding protein (left side, rmsd 2.3 Å, 21 Ca atoms). Superposition of struthiocalcin (brown, PDB entry 4uww [46]) and ice binding protein (right side, rmsd 1.5 Å, 119 Ca atoms). Protein structures are represented as ribbon plot. Superpositions were calculated using the Pairwise Structure Alignment tool of the RCSB PDB server.

Even though more than 95% of bivalve shells are composed of calcium carbonate, a quantity less than 5% of an organic matrix reinforces shells mechanical fracture 3000 times as compared to monolithic CaCO₃ by allowing the formation of layers with a distinctive pattern to form complex biomineral microstructures [37]. From a biosynthetic perspective, the proteome of oysters’ shell proteins offers insights on how these resilient intertidal zone organisms need to adapt and quickly (even short time) build shells that withstand environmental and climate changes [76]. During the early stage of larva development, typical protein expression includes fibronectin-like protein and chitin synthase, events that are associated with template formation on which crystals of calcium carbonate can form [76]. Generally, the small amount of the organic matrix does influence the mechanical properties...
of the biomineral and has a key role in controlling the mineralization process, size, crystal morphology, specific crystallographic orientation, polymorph or amorphous phase stabilization, and/or crystal growth inhibition. The studies of protein templates and calcium carbonate is shedding new light on bioceramics and other engineered applications [77,78].

2.1. Pearls

Among known 3D structures of biomineralization, proteins templating for biominal growth of the mother pearl formation are of great interest not only to understand how these beautiful structures, which are used for jewelry, are made. Similar to eggshells, oysters’ pearls are made with ~95% calcium carbonate and ~5% of an organic/biological macromolecule such as chitosan and other proteins. Beyond jewelry, mother pearls’ powders are studied for a variety of applications, particularly with regard to health care products, considering their relatively low or no cytotoxicity and osteogenesis stimulation [79].

Upon a stimulus, oysters produce a substance, within a small pearl bag, known as nacre that is made of crystalline layers of calcium carbonate and other organics and biomolecules. The final product is the well-recognized mother pearl. Ongoing research is endeavoring to clarify the mechanism of pearl formation beginning with the nucleation and crystallization of calcium carbonate. This mineral growing into the pearl gem is supported by lectin proteins, a class of biomolecules able to bind sugars. Unlike eggshells, pearl’s calcium carbonate crystal growth is mediated by the binding of sugars, and these in turn could either inhibit or support crystal formation. In vitro crystallization experiments performed with the isoforms of lectin known as PPLs (PPL3A-C and PPL4) have shown a significant effect on the number of calcium carbonate crystals and their size in the presence of sugars. Smaller crystals were increased in a dependent manner of sugar concentration, indicating that its binding might suppress and control crystal growth of calcite [50]. From a structural perspective, as shown in Figure 3, PPL3B 3D-structure to calcite crystals clearly suggest that its interaction on the calcite crystal surface is comprised of negatively charged residues, Asp32 and Glu86, interacting with calcium ions, and positively charged residues, Lys83, Lys107, Lys118, Arg119, and Arg147, binding to carbonates on the crystal faces [5]. Similar conclusions were drawn from the structural analysis of the other PPL3s. The morphology, size, and number of crystals that formed PPL4 are regulated by sugars, resulting in considerably different biomineralization functions, which can either suppress or enhance the crystal growth. Therefore, PPL3 and PPL4 supports calcium carbonate formation or inhibition of mineral formation through carbohydrate binding. Therefore, the mineral recognition mechanism is different with respect to the osteocalcin mechanism (see section above).
Chemistry 2022, 4, FOR PEER REVIEW 8

(-RLPCPNCSKLP) that confers a helical shape similar to amelogenin, a binding mineral produced on the scale of gigatons per annum, an amount higher than the whole chemical protein (14 KDa) is conserved among a variety of plants and contains 16% calcium-binding present that could facilitate protein orientation for binding to the mineral surface. The insets provide zoom views of the bound carbohydrates (purple color) interacting with the represented side chains as found in the experimental structure.

2.2. Protein Templates and Biominerals in Plants

Biomineralization is also related to higher plants and biominerals play a variety of functions [80]. In fact, the precipitation of calcium oxalate within specialized plant cells has been associated with biochemically controlled processes and other cellular mechanisms [81]. L. Wang and co-workers have shown the presence of a nanofiber template protein that assists the formation of hundreds of aligned needle-shaped crystals called raphides (e.g., in banana) with a typical dimension of 150 µm in length and 4 µm in width [82]. This small protein (14 KDa) is conserved among a variety of plants and contains 16% calcium-binding amino acids of the total protein sequence, several of which include glutamate, aspartate, and cysteine. This protein has a C-terminal 11-mer proline rich peptide with a sequence (-RLPCPNCSKLP) that confers a helical shape similar to amelogenin, a binding mineral found in enamel [82,83]. In summary, the study of structural protein templates for biomineralization is not only limited to the formation of calcium carbonate or phosphate and the advancement of three-dimensional electron microscopy technique will allow imaging of new hybrid biomineral/protein superstructures at the atomic level [51].

3. Polycondensation and Depolymerization of Silicates

In addition to calcium carbonate and phosphate, biosilica (SiO₂) is a natural widespread biomineral found in organisms such as sponges and diatoms, and some evidence also suggests the presence of organosilicon adducts in plant cell walls [84,85]. From a chemical point of view, silica forms several 3D polymeric arrangements with a common repeating tetrahedral unit with a central silicon atom covalently bonded to four oxygen atoms [86]. Although silica appears as an amorphous material, it forms multiple symmetrical architectures known as spicules found in marine sponges and diatoms [31]. Natural silica is produced on the scale of gigatons per annum, an amount higher than the whole chemical industry production of silica, estimated to be in the megatons [87]. Silica is a very ancient biomineral that is already present in the spicules of marine demosponges, earlier multicellular organisms that emerged from the Marinoan glaciation period (~635 Myr ago) [88,89]. Besides the formation of beautiful geometric structures and patterns, spicules often range in size from a few microns to centimetres, have a structural support function, help the organism by keeping prays away, and, like antennas, capture sunlight at lower temperature,
even at the bottom levels of the oceans [90]. The study of silica formation is important because of the many technologies relying on silica, including cellulose mineralization [91]. While the natural silica polymer is produced in mild conditions and neutral pH condition by self-assembly of the enzyme silicatein, other proteins participate in the 3D structure on the macroscopic level of organization [92].

For the biosynthesis of silica, proteomics studies and phylogenetic tree analysis have revealed the role of the enzyme silicatein [93–95]. This enzyme is present in nature as two isoforms that share similar sequence identity (>50%) to a cysteine protease cathepsin L, a serine protease (or hydrolase), and contains the catalytic triad composed of Ser, His, and Asn. In cathepsin L, the catalytic Ser is mutated in Cys, and both enzymes are thought to have derived from the same plant cysteine protease from *A. thaliana* [96]. In humans, cathepsin L has the function of processing multiple proteins, providing their activation. Silicatein operates by the condensation of acyclic acid to the crescent polymeric silica via nucleophilic attack of an -OH atom group to the Si atom group of the incoming acid and with a departure of a water molecule (Table 1). The silicatein shown in Figure 4 (from pdb entry 6zq3) is strikingly similar to human cathepsin L [51]. A recent study by I. Zlotnikov and coworkers, based on high-resolution transmission electron microscopy (TEM) and serial electron crystallography, has revealed the atomic details of the 3D view of the hybrid silica/protein forming a highly symmetric skeleton in demospongiae [51]. This study is also exemplary since it reveals the utility of these structural techniques in imaging, at sub-nanometer scale, the intimate association between biominerals and proteins. As shown in Figure 4, the TEM images highlight the structure of hybrid silica/protein material consisting of a honeycomb structure corresponding to the hexagonal superstructure made by the packing of silicatein with the hexagons filled with amorphous silica material. In other words, the symmetric structure corresponds to the crystal packing directions of the c and a-axes of the silicatein asymmetric unit [51].

As discussed above, organisms use silica to support their skeletons. A question could be raised as to how these large polymeric structures can be degraded [97]. W.E.G. Müller et al., in the course of elucidating the metabolism of silica in Demospongiae spicules, have identified an enzyme known as silicase, which is able to depolymerize silica in its building blocks [98]. They were able to isolate silicase from the marine sponge *S. domuncula* and related it to carbonic anhydrase from a sequence alignment analysis. It would be interesting to explore the structure and mechanism of this enzyme [99].
shown in Figure 4, the TEM images highlight the structure of hybrid sub-nanometer scale, the intimate association between biominerals and proteins. As serial electron crystallography, has revealed the atomic details of the 3D view of the hybrid.

In other words, the symmetric structure corresponds to the crystal packing directions of the c and a-axes of the silicatein asymmetric unit [51]. Asymmetric unit crystal packing of α-silicatein reveals the regions available for filling with amorphous silica in a spicule specimen. This packing was also observed from transmission electron microscopy imaging of the real spicule specimen. (Lower panel, Left side) (A) Schematic representation of the studied slice of spicule sample by Focused Ion Beam (FIB) milling. This method allows for the preparation of thin slices of a specimen to be imaged through cryo-electron tomography. (B,C) Images obtained by studying the cross-section (along the [001] zone axis) (B) and the longitudinal section (along the [100] zone axis) (C) of the axial filament as depicted in A. The scheme and photographs were reprinted with permission from Ref. [51].

4. Protein Tools for the Synthesis of Magnetotactic Sensing Biominerals

Amazingly, many organisms, including bacteria, fish, and birds, grow tiny crystals of magnetite that can act as compasses to sense the Earth’s magnetic field. Birds need these crystals to guide their continent-spanning migrations. Similarly, anaerobic bacteria away from the equator can use magnetite crystals like a GPS tool for the sensing of oxygen.
directionality. Magnetite clusters are also found in Atlantic salmon heads used for long-distance guidance [100–102]. Crystals of magnetite are grown within magnetosomes, which are subcellular compartments delineated by the cellular membrane. Biomineralization proteins have been found in all domains of life and, therefore, magnetite sensing (as well as greigite) is now considered to be a universal process relying on a handful of distant magnetosome genes [101–103]. For instance, eleven biomineralization proteins have been identified in fish, although the precise function for some is still an ongoing process [101].

From a structural point of view, among known 3D structures of magnetosome proteins in bacteria, seven key macromolecules participating in magnetosome formation are known to atomic details but not all of them are involved in nucleation and mineral growth [104].

In Figure 5 and Table 1 are shown the available structures (including a homology model) of known biomineralization proteins and a summary of their functions. Biomineralization proteins are known as the acronym of magnetosome-associated proteins (Mams) and completely regulate the biomineralization process. As mentioned, magnetite crystals are grown within enclosed membrane organelles and aligned forming a line of crystals along the cellular body that can enhance magnetic field sensing.

The available structural studies of Mams proteins have been reviewed [104]. The latest studies of magnetite biomineralization, included in Figure 5, have revealed new functions for the Mam protein known as MamE. Besides metal binding and cellular translocation of metals, MamE is a dependent proteolysis of biomineralization factors. MamE is a structural homologue of the high temperature requirement A (HtrA), within the family of trypsin-like serine proteases (Figure 5), and in turn is mutually regulated by two other biomineralization factors: MamD and MamO [55,105,106]. MamD is a protein tightly bound to the forming mineral crystals, inside the magnetosome cellular membrane, and is proteolytically processed by MamE. Therefore, MamE can inhibit crystal growing by processing MamO, which in turn is required for the upstream activation of MamE protease activity. MamO also has a metal binding activity. In summary, the interplay of MamD, MamE, and MamO regulates crystal formation and growth. Among other interesting magnetite biomineralization proteins, magnetochrome or MamP, (shown in Figure 5 from the PDB entry 4jj0) is crucial for the building of perfect crystals of iron oxide [59]. MamP is a modular protein composed of a central domain linked to two consecutive magnetochrome domains. The central domain brings together acidic glutamate amino acids to form a pocket for iron binding, and nucleation is possibly initiated. The hemes in magnetochrome domains, which are similar to cytochrome c, shuttle electrons to switch the oxidation state of the iron atoms as they are combined with oxygen, especially during the exponential growth phase of magnetite crystal [107].
From a structural point of view, among known 3D structures of magnetosome proteins are represented as ribbon plot and correspond to PDB codes reported in Table 1. MamA function as a protein scaffold for biomineralization proteins assembly. MamB and MamM have similar shapes and represent transport proteins of iron ions and protons through the cellular membrane and in an opposite flow direction. MamK functions as an anchoring string for the alignment of magnetosomes and to enhance the overall magnetosensing function. MamE and MamO are two proteases mutually regulated, and together with MamD, a protein tightly bound to the mineral, regulate crystal formation and growth. In addition, MamO has a metal binding activity [38]. The MamE model is derived from the AlphaFold project [41]. Figure made with BioRender (https://biorender.com/; accessed on 1 June 2022). (Right panel) MamP is a modular protein composed of a central domain linked to two consecutive magnetochrome domains [59]. The central domain brings together acidic glutamate amino acids to form a pocket where iron nucleation is initiated. The hemes in magnetochrome domains (purple color), are similar to cytochrome c, and shuttle electrons to switch the oxidation state of the iron atoms as they are combined with oxygen in the growing magnetite crystal. Negatively charged residues are represented in red. This figure is obtained from the Molecule of the Month column “Proteins and Biominerals” (Di Costanzo, L; Goodsell, D. 10.2210/rcsb_pdb/mom_2019_4) [70].

5. Design of Peptides and Proteins for the Synthesis of Artificial Biominerals

Structural studies of natural biomineralization proteins have inspired scientists to design short peptides or even proteins that are able to synthesize biominerals. Typically, designed peptides for biomineralization purposes mimic the osteocalcin mineral-binding surface and consist of a simple short alpha helix that contains in their sequence a regular arrangement of acidic amino acids (see Section 1). Following this simple strategy, researchers have engineered two decapeptides containing Glu residues spaced by one (-ELELELELEL-) or two Leu residues (-ELLELLELLEL-). These two simple sequences are able to stabilize a beta-strand (50% Glu residues) or an alpha-helix (33% Glu residues) [108]. Experimentally, both peptides can form vaterite, although the beta-strand peptide, with higher secondary structure propensity, forms a more stable and a unique crystal form biomineral [108].

Using a computational approach, J.J. Gray and co-workers were able to engineer six variants of 16-mer peptides starting from a poly-Ala sequence peptide ending in a Tyr, used to simply allow peptide concentration measurement. The peptides were designed with the software Rosetta using two different calcite crystal planes as a template, namely...
were able to biomineralize amorphous calcium phosphate nanocomposites (ACP-NCs). The approach of controlling mineral growth by inhibiting crystal formation has inspired the use of other chemical entities with regular secondary structures such as a small DNA fragments able to inhibit and control crystal growth and represent a reference point for the engineering of novel biominalization proteins. The approach of controlling mineral growth by inhibiting crystal formation has inspired the use of other chemical entities with regular secondary structures such as a small DNA fragments able to inhibit and control crystal growth and represent a reference point for the engineering of novel biominalization proteins.

Natural biomineralization proteins have inspired the design of proteins for the synthesis of nanocrystals with tailored magnetic, optical, for use in engineered materials. The designed protein npizza2-S16H58 sandwiches a precise 3D lattice nanocrystal consisting of seven Cd(II) ions and twelve chloride ions. This protein was designed placing with a perfect symmetry β-propellers (aka blades) consisting of a 42 residues subunit. The term “Pizza” indicates the canonical symmetry as shown from the 3D structure shown in Figure 6 (Table 1). The hybrid protein/nanocrystal material shows the nanocrystal coordinated by a set of symmetrically positioned histidines extending inward from the propeller-shaped protein complex.

Figure 6. Designed protein for the synthesis of nanocrystals. Two orthogonal views of the designed protein npizza2-S16H58 (PDB entry 5chb) [60]. The protein sandwiches a small nanocrystal, a precise 3D lattice consisting of seven cadmium ions and twelve chloride ions. In the designed protein, the cadmium-chloride nanocrystal is coordinated by a set of symmetrically positioned histidine residues extending inward from the propeller-shaped protein complex. A sulphate anion is also present (red and yellow spheres).

6. Re-Purposed Enzymes for the Synthesis of Biominerals

Few natural enzymes have been re-purposed to regulate nucleation, crystallization, size, and the morphology/orientation of biominerals (see Table 1). M.B. Gu and co-workers were able to biominalize amorphous calcium phosphate nanocomposites (ACP-NCs) using the enzyme carboxyl esterase, a hydrolase that cleaves an ester bond into an acid and an alcohol [112]. With its structural features, the enzyme functions as an entrapping
material for the growth of the composites. In presence of the enzyme, calcium chloride, and disodium hydrogen phosphate (main ingredients), two separate phases are mixed: a hydrophobic phase, for micelle formation (obtained using cyclohexane), and a gel-water system, obtained using non-ionic emulsifier for non-polar solvents [113]. The resulting ACP-NCs, in the range size 100–200 nm, are interspersed with enzyme, identified within small capsules, still partially active, and stable over a period of 10 days [60,112]. The current use of ACP-NCs and CaCO₃ nanocomposites are used for a variety of biomedical purposes including skin (e.g., sunscreens formulation) and oral care, in addition to deodorants as absorbing materials [114].

The tetrameric enzyme cystathionine gamma-lyase has been used to prepare aqueous metal quantum dots (QDs) in the presence of the enzyme prosthetic group pyridoxal phosphate (PLP) [61]. This enzyme cleaves cystathionine, a molecule derived by side chain condensation of a serine and cysteine into α-ketobutyrate, ammonia, and cysteine. B.W. Berger et al. were able to prepare an optical responsive nanocrystal of cadmium sulphide by using cystathionine gamma-lyase and its substrates L-cysteine and cadmium acetate [115]. In addition, the use of glutathione as stabilizing capping agent for the resulting CdS nanocrystals. These nanocrystals show a nice photoluminescence under UV light, which undergoes to redshift of absorbance and fluorescence maxima, indicating an increase in the size of the CdS nanocrystals with incubation time period (Figure 7) [115]. One key lesson from this study is that although bulk CdS nanocrystals can be formed, it is only in the presence of the enzyme that a controlled growth is obtained [116].

Similarly, Chen et al. were able to prepare QDs of CdS, ZnS and PbS using the same enzyme to break of cysteine into pyruvate, ammonia, and release of hydrogen sulphide, which in presence of acetate salts of cadmium, or zinc, or lead cause precipitation as QDs. Typical QDs produced in aqueous media, where the enzyme is active, have sizes of ~3.5 nm. An X-ray structural study (Table 1) has revealed the role of three key enzyme residues PLP cofactor: Tyr-108, Arg-370 and Lys206 [61]. The aromatic residue and the guanidinium properly orient the aromatic ring of the PLP cofactor which in turn is covalently bound to the side chain of lysine. The addition of an intermolecular stabilizing loop region, consisting of a dodecapeptide at the N-terminal domain of the enzyme, resulted in an engineered variant that facilitated QD formation as compared to the wild-type enzyme. The authors were also able to modulate the QDs size by stopping the enzyme reaction with inhibitors
or EDTA [61]. In a similar process, aqueous phase biomineralization of CdSe and mixed CdSe-CdS core-shell nanocrystals were obtained by S. McIntosh et al. [116].

Another approach to obtain stable and functional biomineral particles is represented by the repurposing of natural proteins’ primary function as a template for metal aggregation. Among hollow proteins, cage-shaped ferritin allows the formation of regular nano aggregates of biominerals within their confining space. Ferritin represents the iron reservoir for our living tissues and up to 4500 iron ions can be present within the hollow shape assembly of 12- or 24-ferritin subunits composed of a 4-α-helix bundle macromolecule. Each subunit contains a classical di-iron center where the oxygen dependent oxidation of iron occurs before entrance into the assembly interior space [117]. Crystallography studies of frog ferritin revealed a movement of iron (II) from the entry point, around the three-fold symmetry center of three adjacent subunits, to the di-iron catalytic site within the core of a single subunit four-helix bundle where the iron is oxidized to iron (III), before entering the hollow space for storage [66]. The ~27-Å path is characterized by an arrangement of Asp and Glu residues and a helix kink that help Fe(II) ions move towards the di-iron oxidation center [66]. In addition to this channel, the current view for ferritin iron recognition begins at its surface, since coordination of specific residues and electrostatic properties are able to drive iron directionality into the protein interior upon iron oxidation [118]. Taken together, these studies underline a rather complex mechanism for iron recognition and accumulation, but also provide insights for protein engineering for biotech applications through the deposition of a variety of metals including silver and other nanoparticles [119]. Despite the opportunity represented by these hybrid materials, safety issues represent a great limitation to their practical use [119].

For biomineralization purposes of metals other than iron, Dmochowski, I.J. et al. elucidated how the human heavy chain ferritin (HuHF), could be redesigned to facilitate noble metal ion (Au\(^{3+}\) or Ag\(^{+}\)) binding, reduction, and nanoparticle formation within its interior [64]. In fact, HuHF forms a ~500 kDa protein macromolecular cage forming a that assembles 24 four-helix bundles to form an internal cavity of ~8 nm. A variant with a total of 96 cysteines and histidines, removed from the exterior surface, and 96 non-native cysteines added to the interior surface retained wild-type stability and structure and promoted the formation of silver or gold nanoparticles within the protein cavity, as shown in Figure 8 [64]. Crystalllographic studies with HuHF variants provide insight into how ferritins (Table 1) control the access of metal ions to interior residues that perform chemistry. The metal ion deposition from these studies involves the coordination of the chemistry properties of ferritin. However, the formation of silver or gold nanoparticles within the protein has been obtained by the reduction in ferritin crystals of silver and gold ions owing to harsh reduction conditions [65]. This strategy has allowed nanoparticle formation independent of the coordination chemistry.

The protein quaternary structure as “nanoreactor” for biomineral synthesis is not the only chemical space that induces crystal formation. The whole protein crystal formed on average by ~50% of water and, often, the presence of large cavities can also be re-purposed for the growth of ordered 3D nano-biominers [120,121]. In fact, Y. Lu, I.M. Robertson, J. Xuo, and their coworkers synthesized gold nanoparticles within single crystals of lysozyme, an enzyme found in saliva and other bodily secretions with the function of cleaving a peptidoglycan bond (glycoside hydrolase) that perforate the cell wall leading to bacteria death [63,122]. Crystal structure analysis has revealed the interaction of four or more Au(I) and Au(III) ions coordinated to side chains of His or Tyr (Table 1). Interestingly, this crystal, while growing over time, shows the presence of additional gold ions and a dynamic coordination sphere. Crystals of lysozyme were used as a whole architectural framework for the synthesis of quantum dots (QDs), inorganic nanomaterials widely studied for their optical properties and applications for solar cells and microscopy imaging [123]. QDs of CdS, synthesized through stabilized lysozyme crystals by glutaraldehyde cross-linking, show an average size of ~10 nm [123]. Similar QDs were synthesized for ZnS and Ag\(_2\)S. QDs of CdS within protein crystals show red fluorescence emission that can be enhanced...
or quenched by adding Ag(I) or Hg(II) ions, respectively, gaining the formation of a hybrid mixture. The structural study of these materials has revealed the location within the crystals of nine Cd(II) ions coordinated to -N or -O atom groups of several protein residues (Table 1).

Figure 8. Re-engineered human ferritin for the synthesis of gold nanoparticles shows the presence of a significant amount of bound gold ions (PDB entry ID 3es3 [64]). Protein represented as a ribbon plot of half of the symmetrical sphere to visualize the protein shell interior. Gold and chloride ions are represented as golden and green-colored spheres, respectively.

7. Conclusions

The study of biomineralization proteins involves a variety of research areas ranging from chemical, material, and biomedical sciences. Calcium carbonate, hydroxyapatite and silica represent widespread natural biominerals. The atomic details of the interface between macromolecules and biominerals is very intriguing from a chemical perspective, considering the association of chemical entities that are structurally different. With this review, I provide an overview of structural studies of biomineralization proteins and how these studies are inspiring the design and engineering of novel proteins able to synthesize biominerals in mild conditions, and often in aqueous solution. The study of biomineralization proteins through structural biology is advancing owing the recent progress of cryo-electron microscopy and its related techniques [51]. These allow the imaging of biomineral layers and reveal interactions between proteins and biominerals at atomic level, including amorphous material [124]. I have mined the PDB to find the three-dimensional structures, signaling mechanisms for pH sensing and applications from a chemical point-of-view up to in vivo biochemical applications. The progression of this review from classical template proteins to silica polymerization seeks to benefit researchers involved in various interdisciplinary aspects of a biomineralization project who need background information and a quick update on advances in the field. Lessons learned from structural studies are exemplary and will guide new projects for imaging of new hybrid biomineral/protein superstructures at the atomic level.

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