CHEMICAL REVIEWS

Review

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Jose# L. Arias, and Mari#a S. Ferna#ndez

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Polysaccharides and Proteoglycans in Calcium Carbonate-based Biomineralization

José L. Arias* and María S. Fernández

Faculty of Veterinary and Animal Sciences, and Center for Advanced Interdisciplinary Research in Materials (CIMAT), Universidad de Chile, Casilla 2 Correo 15, Santiago, Chile

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1. Introduction

Biomineralization is a widespread phenomenon in nature leading to the formation of a variety of solid inorganic structures by living organisms, such as intracellular crystals in prokaryotes, exoskeletons in protozoa, algae, and invertebrates, spicules and lenses, bone, teeth, statoliths, and otoliths, eggshells, plant mineral structures, and also pathological biominerals such as gall stones, kidney stones, and oyster pearls.[1–7] These biologically produced biominerals are inorganic−organic hybrid composites formed by self-assembled bottom up processes under mild conditions, showing interesting properties, controlled hierarchical structures, and remodeling or repair mechanisms which still remain to be developed into a practical engineering process.[8–10] Therefore, the formation of biominerals provides a unique guide for the design of materials, especially those that need to be fabricated at ambient temperatures. In biominerals, the small amount of organic component not only reinforces the mechanical properties of the resulting composite but also exerts a crucial control on the mineralization process, contributing to the determination of the size, crystal morphology, specific crystallographic orientation, and superb properties of the particles formed.[11–15] Therefore, biological routes of structuring biominerals are becoming valuable approaches for novel materials synthesis. Although several principles are applicable to the majority of the biominerals, herein we will focus on the role of polysaccharide polymers in calcium carbonate-based biominerals. As a general principle, the assembly of these biominerals consists of a four-stage process. It starts with the fabrication of a hydrophobic solid organic substrate or scaffolding onto which nucleation of the crystalline phase takes place closely associated with some polyanionic macromolecules. Crystal growth is then controlled by the addition of gel-structuring polyanionic macromolecules, and finally mineralization arrest is accompanied by the secretion of a new inert scaffolding of the same type or the deposition of other hydrophobic inhibitory macromolecules.[16]

Currently, a large number of proteins have been described which are involved in the control of biomineralization.[17–19] These proteins are usually highly negatively charged and contain carboxylate, sulfate, or phosphate as functional groups, which may bind Ca^{2+} ions and could control crystal nucleation and growth by lowering the interfacial energy between the crystal and the macromolecular substrate.[20–25] However, the precise mechanism involved in controlling crystal nucleation, growth, and morphology is far from being understood. Combinatorial biology techniques have been recently developed for testing the ability of randomly generated peptides to bind different substrates or ions, thus allowing a correlation between peptide structure and ion binding affinity.[26–29] However, the main focus is on the role of the backbone structure of the polymer due to the primary structure of the protein, because the synthetic technology does not allow the formation of post-translational modifications, such as sulfation and phosphorylation, which do occur in the eukaryotic cell. Even so, the occurrence of negatively charged groups in macromolecules involved in biomineralization, mainly derived from acidic amino acids, has inspired many researchers to produce synthetic polymers having such groups in order to control the size, orientation, phase, and morphology of inorganic crystals.[30–43] However, since Abolins-Krogis' work,[44] a slow but increasing interest has been developed to explore the role of polysaccharides in biomineralization, despite the fact that their involvement in biomineralization seems to appear very early in evolution.[45] There is no single type of polysaccharide associated with biominerals, but such polysaccharides are mainly hydroxylated, carboxylated, or sulfated or contain a mixture of these functional moieties.

2. Hydroxylated Polysaccharides

After cellulose, chitin is the second most abundant biopolymer in the biosphere. As chitin or its deacetylated form chitosan, it is widely distributed in the animal, plant, fungi, and protozoa kingdoms. Although its occurrence is not necessarily directly associated with biomineralization, its presence is crucial in biominerals such as crustacean shells, carapaces and gastroliths, and mollusks shells. Chitin is a linear polysaccharide of α- or β-(1−4)-2-acetamido-2-deoxy-D-glucopyranose where the monomeric residue is *N*-acetylglucosamine (Figure 1, upper structure).[46] It exists

* E-mail: jarias@uchile.cl.

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José L. Arias has a D.V.M degree (1973) from the University of Chile, Santiago, Chile. He did postgraduate research at the Institute of Chemistry and Biochemistry G. Rozoni, Milan, Italy, in glycosaminoglycans with Prof. B. Casu (1983−1984) and Case Western Reserve University, Cleveland, OH, where he worked with Prof. A. I. Caplan and A. H. Heuer on natural bioceramics (1989−1991). Presently, he is Professor of Cell Biology at the Faculty of Veterinary and Animal Sciences of the University of Chile and Principal Investigator of the Center for Advanced Interdisciplinary Research in Materials (CIMAT), Santiago, Chile. His current research interest is in biomineralization as a source for building biorelated materials. He has been a visiting scientist at different institutions of South and North America, Asia, and Europe.

María S. Fernández received an undergraduate degree in Medical Technology (1971) and a M.Sc. degree in Cell Biology (1995) from the University of Chile, Santiago, Chile. She spent three years at the Skeletal Research Center, Case Western Reserve University, Cleveland, OH, working with Prof. Arnold I. Caplan. Her main research interest is in morphogenesis and mineralization of hard tissues. Currently, she is Associated Professor of Developmental Biology at the Faculty of Veterinary and Animal Sciences of the University of Chile and Associated Researcher at CIMAT.

as either α-chitin or *β*-chitin, depending on whether the linkage between glucosamine units is α- or *β*-, respectively. While α-chitin occurs predominantly in the insect cuticle, in crustacean carapaces (e.g., lobster, crab, and shrimp), in the fungal cell wall, and, as recently described, in sponges,[47] *β*-chitin occurs in true mollusc shells or in vestigial ones such as in the squid pen, in diatoms, and in vestimentiferan sea worms.[48–50] However, the hard shell of the barnacle, in spite of its being a crustacean, contains *β*-chitin.[51] Adjacent α-chitin chains show an antiparallel configuration in the *c*-axis, thus having a highly ordered orthorhombic crystalline structure with extensive three-dimensional hydrogen bonding that gives rise to the rigid and insoluble properties of the polymer. *β*-Chitin has a monoclinic crystal structure with a parallel polymer chain arrangement which does not facilitate interchain hydrogen bonding between the C-6 hydroxyl groups along the *c*-axis but is favorable for the incorporation of water molecules between the weakly interacting chains.

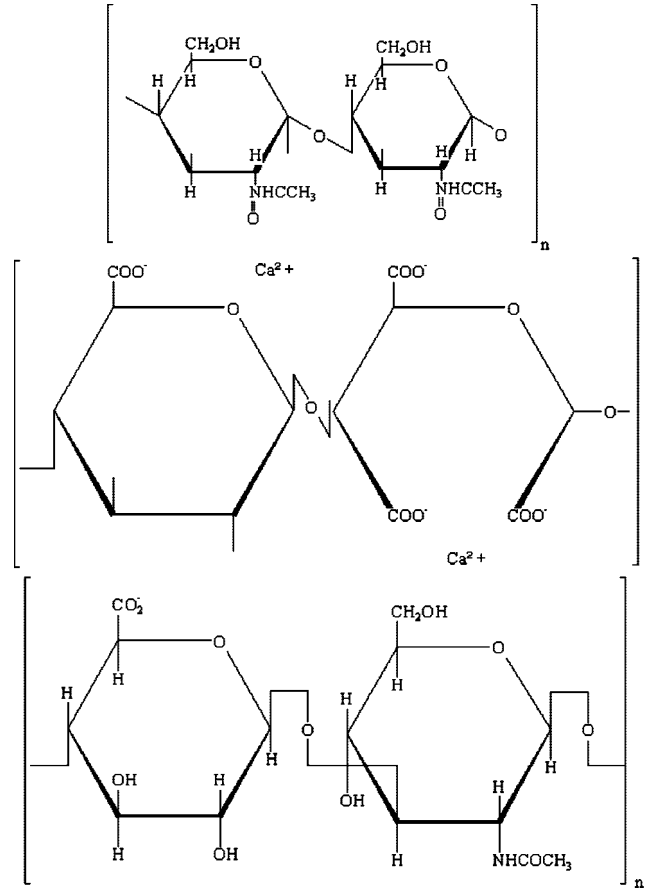

Figure 1. Structural formulas of polysaccharides: Upper structure, chitin or poly-*N*-acetyl-glucosamine; Middle structure, coccolith PS-2 with D-glucuronic, *meso*-tartaric, and glycoxylic residues; lower structure, hyaluronic acid disaccharide unit consisting of *β*-(1−4)-glucuronic acid and *β*-(1−3)-*N*-acetylglucosamine.

3. Polycarboxylated Polysaccharides

Distinctive types of polycarboxylated polysaccharides have been found in the calcium carbonate mineralized covering of some strains of unicellular marine algae referred to as coccoliths.[33,52] Although their composition varies among the species of algae studied, they can be classified into two groups: (a) polyalduronic acid polymers and (b) polymers of uronic, tartaric, and glyoxylic acids.[53] Polyalduronic polymers contain only a few uncharged glycosyl residues, have mainly alternating residues of D-glucuronic acid and D-galacturonic acid in a ratio of 1:3, and are referred to as PS-1. It is interesting to note that poly-D-galacturonic acid is the skeleton chain of pectins, a heterogeneous grouping of acidic structural polysaccharides found in fruit and vegetables, which are important in food manufacturing because of their gelling and thickening properties.[54] Polymers of uronic, tartaric, and glyoxylic acids are a unique kind of highly charged polyanion found in coccoliths and are referred to as PS-2.[33] They contain equimolar ratios of D-glucuronic acid, *meso*-tartaric acid, and glyoxylic acid as a repeating sequence of [→4) D-glucuronate (*β*1→2) *meso*-tartrate (3→1) glyoxylate (1-]$_n$ (Figure 1, middle structure). There are other acidic polysaccharides reported in coccoliths whose chemical structure still remains unknown.[55]

4. Sulfated Polysaccharides

Although there are several tyrosine-sulfated proteins in eukaryotes, whose functions are not well established,[56] the main occurrence of sulfate groups are in a particular kind of glycoconjugate called proteoglycans (formerly acid mucopolysaccharides). Proteoglycans consist of a protein core to which highly negatively charged linear polysaccharide side chains of variable length, called sulfated glycosaminoglycans, are covalently attached. Glycosaminoglycans (GAG) are alternating copolymers of a hexosamine and either a galactose or an alduronic acid. Individual GAGs differ from each other by the type of hexosamine or alduronic acid (or hexose), the position and configuration of the glycosidic linkages, and the degree and pattern of sulfation.[57–61] While hyaluronic acid or hyaluronan (HA) is a nonsulfated GAG (Figure 1, lower structure), the other types of GAGs are sulfated to a variable extent and form part of proteoglycans. GAGs are huge information-dense macromolecules due to the fact that modifications of sugar residues create an enormous molecular diversity.[62] If an octasaccharide unit allows more than 106 theoretical combinations, a 50−200-disaccharide unit represents almost infinite combinatorial possibilities.[63,64] The most common sites of sulfation are at O-4, O-6, or *N*- of the hexosamine or at O-2 of the alduronic acid. While the alduronic acid moiety can be D-uronic or L-iduronic acid, the hexosamine can be 2-amino-2-deoxy-D-glucose or 2-amino-2-deoxy-D-galactose, and the hexose is always galactose. The main differences among GAGs are as follows: (i) The uronic acid is D-glucuronic acid for HA and chondroitin 4- and 6-sulfate (C4S and C6S, respectively) (Figure 2, upper and middle structure), L-iduronic acid for dermatan sulfate (DS) (Figure 2, lower structure) and mainly for heparin (Hep) (with D-glucuronic acid as a minor constituent) (Figure 3, upper structure), and preponderantly D-glucuronic acid in heparan sulfate (HS) (with L-iduronic acid as a minor constituent) (Figure 3, middle structure); keratan sulfate (KS) is atypical, having D-galactose instead of alduronic acid residues (Figure 3, lower structure). (ii) The hexosamine is 2-amino-2-deoxy-D-glucose for HA, KS, HS, and Hep, and 2-amino-2-deoxy-D-galactose for C4S, C6S, and DS. (iii) D-Glucuronic acid is β-(1−3)-linked to the hexosamine in HA, C4S, and C6S and β-(1−4)-linked in HS. L-Iduronic acid is linked α-(1−3) in DS and α-(1−4) in Hep. (iv) The hexosamine residue is *N*-acetylated in HA, KS, DS, C4S, and C6S and (partially) in HS and *N*-sulfated in Hep and (partially) in HS. (v) L-Iduronic acid is 2-O-sulfated in Hep. Heparin is the most polyanionic polysaccharide known.

Biosynthesis of a proteoglycan starts with the formation of a polypeptide core composed of variable amino acids but always having serine residues. Through the action of the enzyme D-xylosyltransferase, D-xylosyl groups are *O*-linked to most of the hydroxyl groups of the L-serine of the core protein. Other glycosyltransferases then sequentially add two residues of D-galactose and one of D-glucuronic acid to each D-xylosyl group. This tetrasaccharide segment constitutes the protein core linkage region. Through the action of appropriate transferases, the GAG chains grow from these segments by alternate addition of 2-acetamido-2-deoxy-D-hexose and D-glucuronic acid or galactose. Depending on the GAG, these disaccharide units are then modified by a series of enzymes by sequentially performing *N*-deacetylation and *N*-sulfation of the hexose, C-5 epimerization of D-glucuronic acid, and sulfation.[57] However, KS-containing

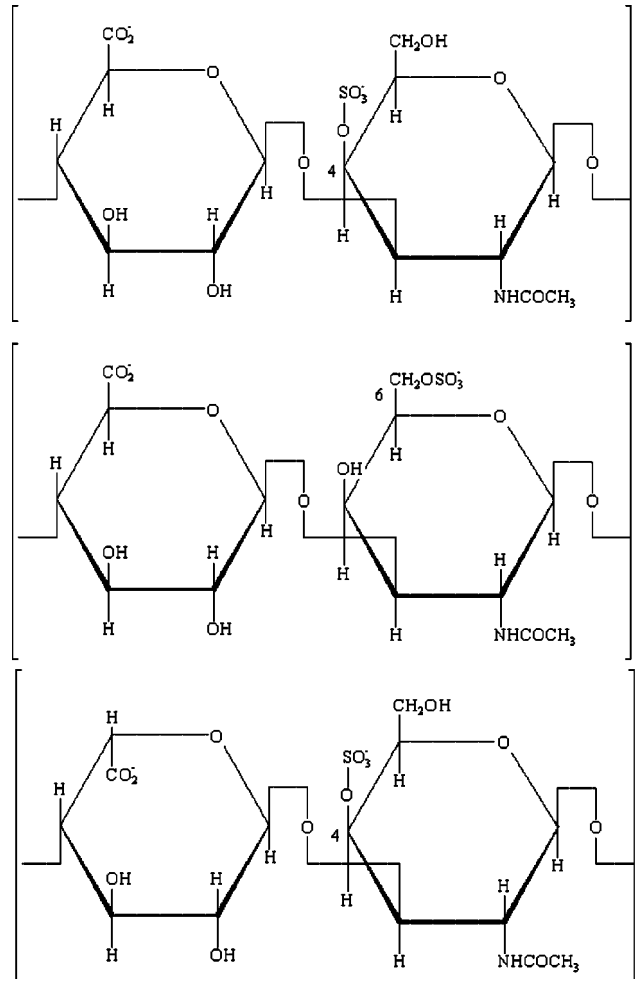

Figure 2. Structural formulas of the disaccharide unit of sulfated glycosaminoglycans: Upper structure, chondroitin 4-sulfate consisting of β-(1−4)-glucuronic acid and β-(1−3)-*N*-acetylgalactosamine-4-*O*-sulfate; middle structure, chondroitin 6-sulfate consisting of β-(1−4)-glucuronic acid β-(1−3)-*N*-acetylgalactosamine-6-*O*-sulfate; lower structure, dermatan sulfate consisting of β-(1−4)-iduronic acid and β-(1−3)-*N*-acetylgalactosamine-4-*O*-sulfate.

proteoglycans are typically attached to core proteins through either a mucin-type oligosaccharide to serine or threonine (O-linked KS) or a mannose-containing serum-type oligosaccharide to asparagine (N-linked KS).[60]

Depending on their GAG structure, core protein amino acid sequence, polymer conformation, and tissue distribution and function, proteoglycans have received different names, such as aggrecan, versican, and perlecan. Therefore, the modification of the polysaccharide residues is not the only source of diversity but also the primary structure of the core protein. Due to their diversity, grouping of proteoglycans has been a difficult task, but eventually these molecules have been classified into several families. One family contains the lecticans or hyalectans, of which the core protein has an N-terminal globular domain that interacts with HA and also has a C-terminal selectin domain. The GAG chains consist mostly of C4S, C6S, and KS. The characteristic cartilage proteoglycan, aggrecan, belongs to this group. Another family consists of those proteoglycans having leucine-rich repeat sequences in their core protein, thus referred to as SLRPs (small leucine-rich proteoglycans). Their GAG chains are mostly C4S, C6S, DS, and KS, and the main members include decorin, biglycan, fibromodulin, and keratocan. In general, SLRPs promote protein−protein interactions, for

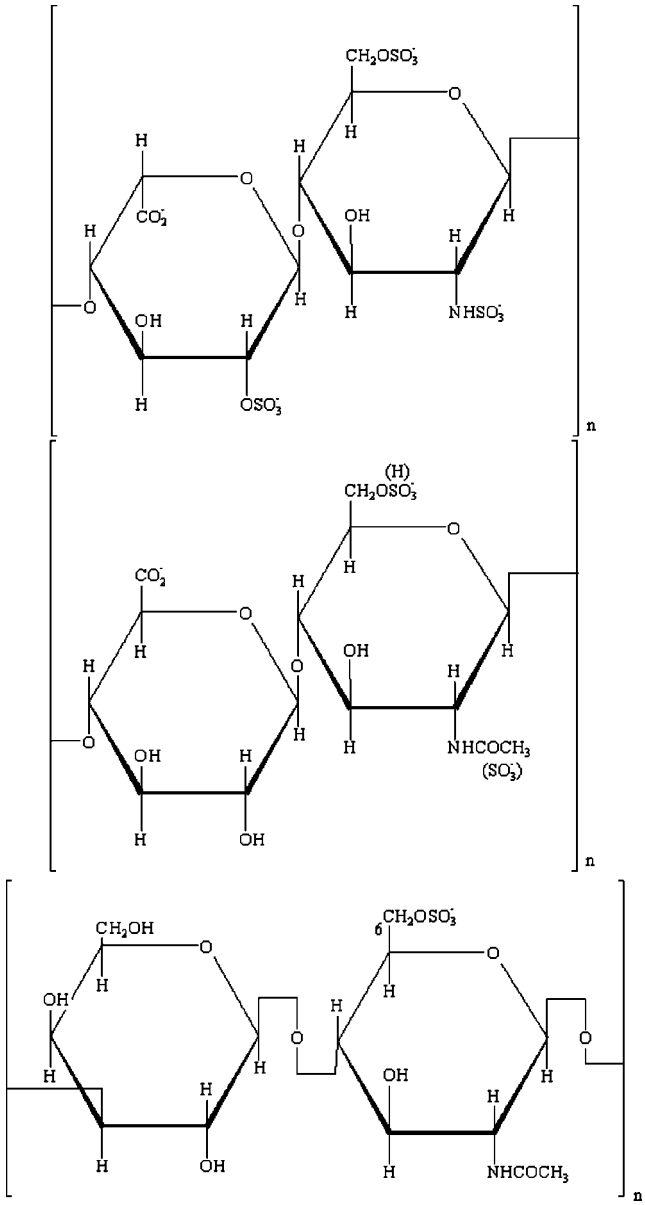

Figure 3. Structural formulas of the disaccharide unit of sulfated glycosaminoglycans: upper structure, heparin or β-(1−4)-iduronic acid-2-*O*-sulfate and β-(1−4)-*N*-sulfonyl-glucosamine-6-*O*-sulfate; middle structure, heparan sulfate or β-(1−4)-iduronic acid and partially β-(1−4)-*N*-sulfonylglucosamine-6-*O*-sulfate; lower structure, keratan sulfate or β-(1−3)-D-galactose and β-(1−3)-*N*-acetylglucosamine-6-*O*-sulfate.

example organizing collagen networks. A third group is the family of HS-rich proteoglycans, such as perlecan, syndecan, and agrin, which are generally associated with or form part of cellular membranes and not only function in cell adhesion to the extracellular matrix proteins but also as receptors of signaling for cell migration, proliferation, and differentiation. Other groups include the so-called "part-time proteoglycans", whose core proteins do not always contain attached GAG chains, and also the spongicans. These proteoglycans function in signal transduction processes and self−nonself recognition and in primitive tissue formation in sponges.[64–66] Because of their long polyanionic GAG chains, proteoglycans provide highly hydrated spaces outside of cells, forming gels of varying pore size and charge density, and serving as selective sieves to regulate ions, molecules, and cell traffic. Due to their carboxylate and sulfate groups, they show a high cation affinity. In spite of that, not all proteoglycans are involved in biomineralization, as described below.

Unusual sulfated polysaccharides have been found in coccoliths.[33,55,67] One of these is a sulfated polysaccharide composed of galacturonic, glucuronic, and iduronic acids in a molar ratio of 2.4:1.1:1.0, respectively.[67] A second one is predominantly *O*-sulfated poly-D-galacturonic acid.[67] A third one, referred to as APS-A92, consists of (1→3)-linked D-mannosyl residues with sulfate groups and has abundant side chains, in which two or three D-galacturonic acid residues occur.[68] The last one is PS-3, a galacturonomannan with high levels of rhamnose and xylose residues and a significant amount of sulfate ester groups.[33] There is no information concerning the linearity or branching structure of these polymers. Although these are sulfated polysaccharides, they are not glycosaminoglycans and have not been reported to be attached to a protein core.

5. Polysaccharides in Biomineralization

Although chitin occurs in many calcium carbonate-based biominerals, its role is almost passive. In fact, is has been demonstrated that neither chitin nor chitosan *per se* modify *in vitro* calcium carbonate nucleation and growth.[69,70] However, in biominerals chitin occurs not by itself but constitutes an insoluble two-dimensional scaffolding or three-dimensional compartment wherein chitin-associated polyanionic polysaccharides or proteins control crystallization in a confined space.[16,71,72]

For some polycarboxylated polysaccharides in coccoliths, the mechanism of calcium−polymer interaction could be in some sense similar to that acting in pectin. That is, carboxylate groups cooperate together in sequestering the bound water away from the calcium ions to form the salt links and thereby increase the gel strength.[73] It has been demonstrated that polyanionic polysaccharides accompany calcite crystal formation from nucleation to growth and also regulate crystal morphology by enhancing precipitation of calcium carbonate ions on the acute crystal face.[74–76] While PS-2 facilitates crystal nucleation, since mutants which do not produce this polyanion produce fewer crystals,[77] the sulfated polysaccharide PS-3 seems to control calcite growth and morphology.[78]

The first indication about the possible role of proteoglycans in calcium carbonate-based biominerals came from the occurrence of sulfated mucopolysaccharides (presently named GAGS) in the calcifying granules formed on glass coverslips inserted either between the shell and the shell-forming cells of the mantle or at the initial site of shell regeneration.[79,80] Additional evidence comes from studies in which mollusk shells were decalcified in solutions which fix glycoproteins. In these studies, nucleation sites showed calcium-binding material containing sulfur, acid mucopolysaccharides, or GAGs.[81–92] In fact, it has been demonstrated that sulfate is an important component of the intracrystalline organic matrix. [93] Although it has been shown that mantle cells produce proteoglycans, their involvement in shell formation has not been well established.[94] An immunoultrastructural localization of keratan sulfate in the abalone shell is shown in Figure 4.

Additional evidence comes from eggshells. These are natural biocomposites containing organic (5%) and inorganic (calcite) components (95%).[14,95,96] Eggshell mineralization starts with the nucleation of calcite crystals on randomly deposited discrete organic aggregations (mammillae), which

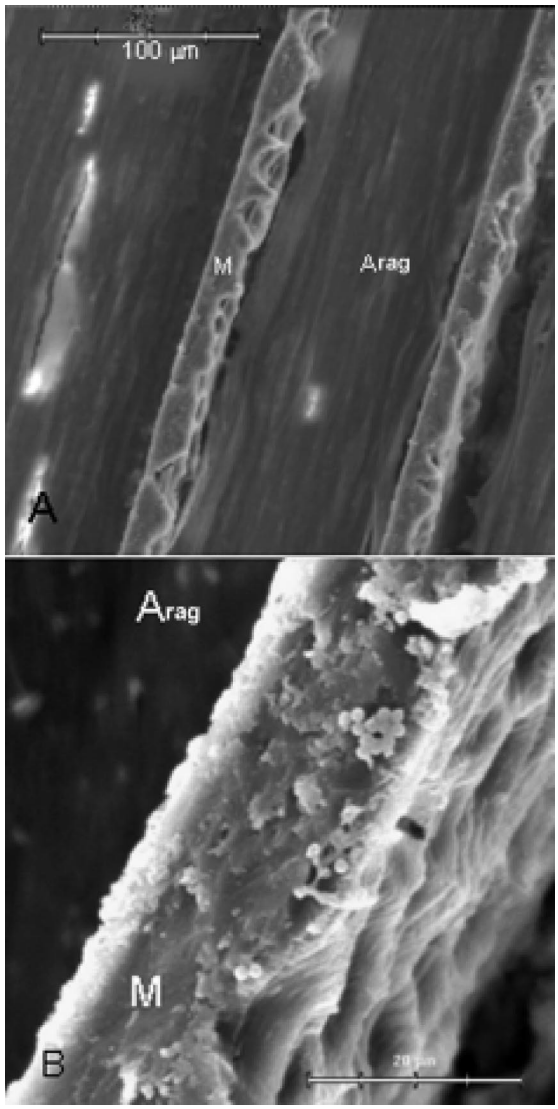

Figure 4. SEM image of red abalone shell (*Halliotis rufescens*). (A) Shown are the chitin-rich mesolayers (M) and the aragonitic region (Arag) between them. (B) Polystyrene microspheres (1 μm in diameter) indicate a keratan sulfate immunopositive reaction mainly located on chitin mesolayers (M) and significantly less in the aragonitic region (Arag).

contain mammillan, a proteoglycan that has oversulfated KS GAGs.[97–100] Columns of calcite grow on the top of these mammillary knobs, and their crystal orientation and morphology are affected by ovoglycan (also known as ovocleidin-116), a unique DS proteoglycan.[99–101] When sulfation of these macromolecules is experimentally affected, the eggshell crystalline calcite columns show severe structural alterations.[102] These types of sulfated macromolecules have also been found in eggshells other than chicken.[103] In addition, a KS proteoglycan has been reported to be closely associated with calcite crystals (statoconia) in the chicken ear, and noncharacterized proteoglycans have also been found to be involved in the calcification process of fish otoliths.[104–106]

The occurrence of a mucopolysaccharide layer at the outer surface of the mineralizing ectoderm of the polyp suggested its role in coral mineralization.[107] More recently, there is additional evidence confirming this role.[108] A high content of probable proteoglycan-originated sulfur has been found in the skeletal soluble matrices and within the centers of calcification of corals.[109–112] Additional evidence comes from the reported adsorption ability of GAGs on coral surfaces,

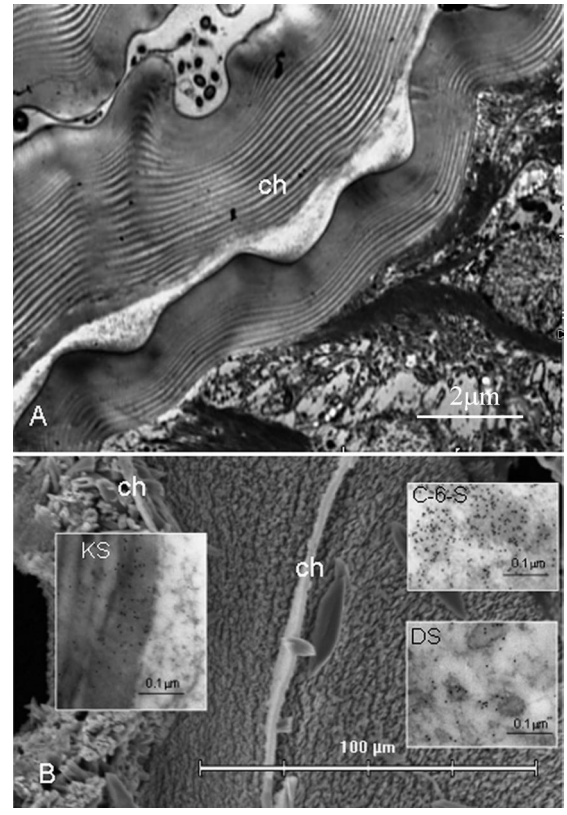

Figure 5. Electron microscopic images of giant barnacle shell (*Austromegabalanus psittacus*). (A) TEM image of decalcified shell showing chitin layers (ch); (B) SEM image of partially decalcified shell showing the organic matrix between chitin layers (ch). Insets show keratan sulfate (KS) immunogold positive reaction at the borders of the chitin layers, while chondroitin 6-sulfate (C6S) and dermatan sulfate (DS) positive reactions are detected in the organic matrix located between chitin layers.

which depends on the charge density due to sulfate groups.[113,114]

The occurrence of proteoglycans in crustacean carapaces has not been fully documented. However, we have shown that the barnacle shell does show a precise distribution of specific proteoglycans.[16,51] The barnacle shell is built as concentric mineralized layers where a KS proteoglycan is located in close association with an inert chitin lamina (Figure 5). Growing of calcite crystals occurs in a gel phase,[115] where C6S and DS proteoglycans are found. Preliminary results show that a similar pattern is found in crustacean gastroliths, disk-shaped calcified structures built by organic−inorganic alternate layers.[116] In some of these systems (caparaces and gastroliths), the main mineral is amorphous calcium carbonate.[117] The role of proteoglycans in the stabilization of this mineral form remains unexplored.

6. Calcium Carbonate Growth in the Presence of Sulfated Polymers

It has been shown that the glycosaminoglycans C4S, C6S, and DS in solution stabilize the spontaneous precipitation of vaterite and influence the crystal size distribution.[118] However, in order to understand the precise role of these sulfated macromolecules in calcium carbonate nucleation and growth, not only their structure but also their supramolecular assembly must be considered. In fact, the occurrence of proteoglycans as a fixed substrate, as a gel, or in solution should lead to different effects when calcium carbonate

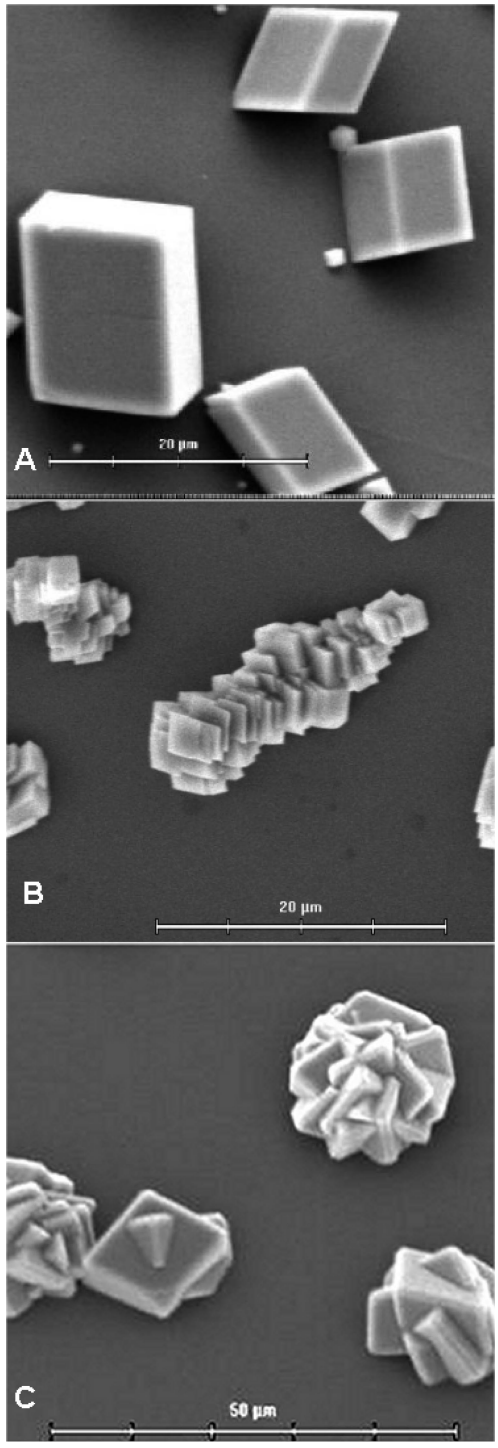

Figure 6. *In vitro* calcium−carbonate crystallization. (A) In the absence of polymers showing typical rhombohedral calcite crystals; (B) Piles of calcite crystals grown in the presence of hyaluronic acid; (C) Rosette-like aggregates of calcite crystals grown in the presence of heparin.

precipitation is induced. When calcium carbonate precipitation is assayed in solutions of GAGs, a clear effect of the sulfate groups has been observed.[119] When HA, a nonsulfated but carboxylated GAG, was added, 20-μm long piles of unmodified calcite crystals were observed as compared with calcite crystallization in the absence of the polymer (Figure 6A,B). The edges of the crystals in the pile are parallel to each other, probably indicating that HA induced a monocrystalline aggregate. In the presence of desulfated DS, which is an epimeric form of HA but a shorter polymer, which has the carboxylate groups in an inverted configuration, isolated rhombohedral {104} calcite crystals showing rounded corners with planes oriented parallel to the *c* axis were observed.[120] When DS, itself, was added, isolated calcite crystals were formed in a columnar morphology as a {*hk*0} cylinder with three {104} faces forming a cap at both ends. The addition of Hep, a highly sulfated helicoidal polymer, induces the formation of large rosette-like aggregates of clockwise helicoidally disposed calcite crystals, where the majority of the {104} faces appear not to be lost (Figure 6C). In the presence of KS, cuboctahedral calcite crystal aggregates are formed.[121] Although the described cooperative role of the carboxylate and sulfate groups[29] could also beting in GAGs, the pattern of sulfation appears to be crucial for the interactions established between the GAG and the mineral.

It has also been shown that functionalized self-assembled monolayers with sulfate groups are more active than other negatively charged groups in inducing calcium carbonate nucleation, and the sulfate groups induce a face-selective nucleation.[32,122,123] Calcium carbonate crystallization on solid functionalized substrates depends on the spacing, ordering, and orientation of the terminal group.

7. Concluding Remarks

During the past decade, there has been remarkable progress in the development of bioinspired procedures for controlling inorganic crystal nucleation and growth. Although many macromolecules of various types have been described to be involved in biomineralization, keratan sulfate is a molecule that has been described at the nucleation sites of different models of calcium carbonate-based biomineralization where a spherulitic growth occurs. Similarly, a common feature to all calcium carbonate-based models of mineralization studied is the occurrence of confined spaces delimited by a water insoluble material (e.g., chitin, type X collagen, or cell membrane phospholipids), inside which crystal nucleation and growth occur. However, there are many questions waiting to be addressed. Due to the fact that a great variety of proteins and polysaccharides have been shown to be involved in controlling biomineralization, the precise role of sulfate groups and their interaction with other chemically active groups is still unknown. Three approaches have being followed to understand processes of biomineralization: (i) characterization of biomineralized structures, (ii) characterization of the assembly process during biological development of such structures, and (iii) characterization of the assembly process occurring during wound healing and repair of biomineralized systems. A promising approach should be the use of synthetic polymers functionalized with specific groups in precise locations relative to the backbone in order to correlate the influence of these groups with their ability to affect the nucleation, growth, and morphology of inorganic crystals. Questions such as how mineralization is controlled in a micro- or nanosized confined space or how is it possible to regulate the spatiotemporal deposition of particular solid interfaces or macromolecules in solution to produce self-assembled mineralized structures will require new interdisciplinary approaches to be answered.

8. Acknowledgments

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