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Communication

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Distinct Effects of Avian Egg Derived Anionic Proteoglycans on the Early Stages of Calcium Carbonate Mineralization

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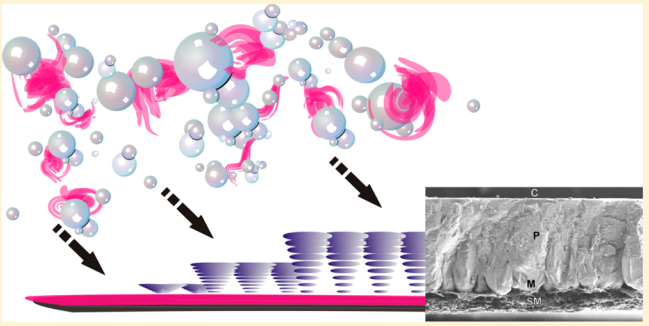
Supporting Information

ABSTRACT: This Communication addresses the effects of egg membrane- and shell-associated proteoglycans, namely, keratan sulfate and dermatan sulfate, respectively, on the nascent stages of $CaCO_3$ mineralization. Composed of calcitic columns intimately associated with a collagen membrane, the mechanisms underlying mineral growth are regulated by biomolecules. Of these, the role of proteoglycans is crucial because of their defined temporal and spatial distributions that direct mineral growth. The proteoglycans analyzed here induce dissimilar effects on the early stages of calcium carbonate mineralization. Egg-membrane associated keratan sulfate has a stabilizing effect toward soluble calcium carbonate prenucleation clusters and promotes formation of phases with lower solubility products after nucleation. In contrast, dermatan sulfate destabilizes prenucleation clusters and leads to more soluble phases of calcium carbonate postnucleation. The distinct effects of proteoglycans on calcium carbonate crystallization elucidate their unique spatiotemporal localization during egg mineralization.



The avian egg is a composite biomineral with material properties fine-tuned for protection, hatchability, and diffusion. The shell material is a bioceramic fabricated by combining specific extracellular matrix molecules with crystalline calcite.[1,2] Ultrastructurally, it is composed of shell membranes, mammillae, palisade, and cuticle (Figure 1). The shell membrane fibers are composed of a core, containing type X collagen, and surrounded by a fuzzy material referred to as a mantle.[3,4] Sparsely deposited on the outer side of the shell membranes are the mammillae (mammillary layer), which are the sites for crystal nucleation.[5] The calcified layer proper or palisade is formed by calcite columns. Closely associated with the calcite crystals of these columns is an organic material composed of vesicular structures interspersed with a fibrillar matrix material. Crystallographic and morphological studies of the palisade reveal three subzones (cone, central, and vertical crystal layers) differing in their crystal orientation.[1] This region contains many vesicles between the crystals.[1,4,6,7] The last formed layer, the cuticle, covers the entire shell and is made out of glycoproteins, the large part of pigments in colored eggs, and hydroxyapatite crystals.[6,8−10] It has been shown that, among other molecules described and reviewed elsewhere,[11−21] for which role in the calcification process has not yet been completely elucidated, the eggshell organic phase contains specific collagens and proteoglycans,[3,22−25] with regulated topographical and temporal distributions.[26]

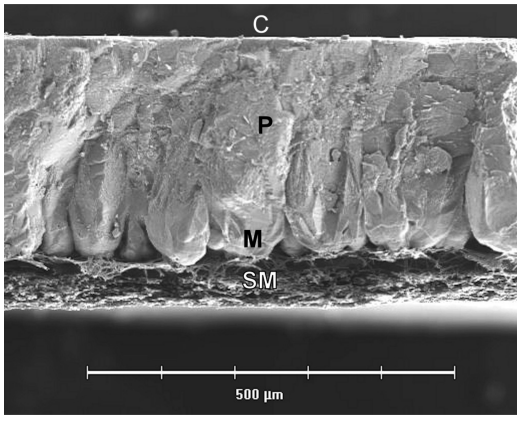


Figure 1. Scanning electron micrograph of an eggshell transversal section, showing its structure: C, cuticle; P, palisade; M, mammillary layer; SM, eggshell membranes.[1]

Proteoglycans are heavily glycosylated proteins consisting of a peptidic core with one or more covalently attached sulfated glycosaminoglycan (GAG) chains. Mammillae are discrete aggregations of organic matter that intermix with the fibrillar material of the outer shell membrane fibers. Mammillae, specifically the 100−300 nm vesicles of the calcium reserve body, contain a calcium binding molecule (mammillan) which is a keratan oversulfated proteoglycan.[23,26] The shell matrix of the palisade contains hyaluronic acid[27] and ovoglycan, a unique

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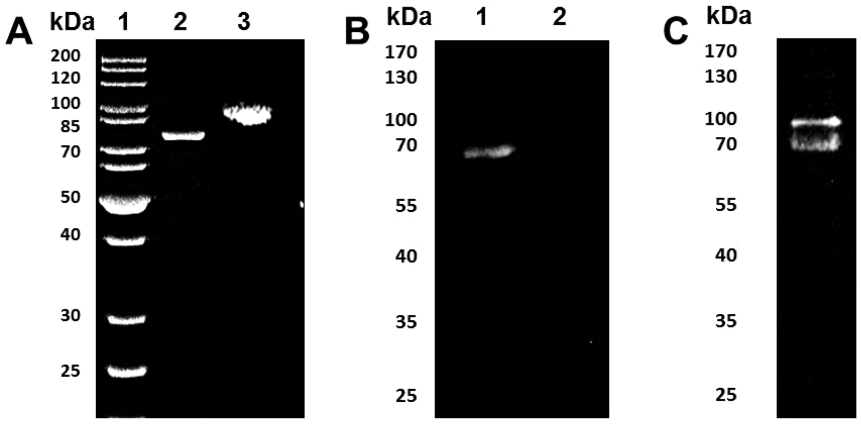

Figure 2. (A) SDS-PAGE of purified proteoglycans: Lane 1, standard molecular weight marker; Lane 2, dermatan sulfate; Lane 3, keratan sulfate. Western blots of (B) Lane 1, chondroitinase ABC, and Lane2, ACII treated dermatan sulfate, developed using 2B6 antibodies, as well as (C) keratan sulfate probed using 5D4 antibodies. Due to the specificity of the monoclonal antibody, the band roughly corresponding to 70 kDa should correspond to a degradation product.

200 kDa dermatan sulfate proteoglycan,[23,24,26] having a core protein (120 kDa) and glycosaminoglycan chains containing dermatan sulfate which average 22 kDa.[6,24,25] Known as ovocleidin-116, this protein core has been cloned and localized in the same shell matrix vesicles that are reactive to dermatan sulfate.[6,19,24,25,28] It appears that ovoglycan is a copolymeric proteoglycan containing unsulfated chondroitin, dermatan sulfate glycosaminoglycans,[24,27] and N-glycan structures.[29] Such proteoglycans have also been found in several other avian eggshells.[17]

In addition to the unique spatial localization of proteoglycans, their distribution also varies in a temporal manner. Shell membranes are formed in the isthmus region of the oviduct between 3:00 and 5:00 h post-oviposition (p.o.), that is the time elapsed after the last egg was laid.[26] Subsequently the inner shell membranes form, and coincidently the electron-dense vesicles of the isthmus tubular gland cells show a positive reaction to type X collagen.[19] Similar vesicles containing keratan sulfate are observed between 4:00 and 5:00 h p.o., when the outer shell membrane fibers are formed. The isthmus secretion of both molecules decreases later.[22] Mammillary formation occurs between 5:15 and 5:30 h p.o., when mammillan is detected in the vesicles of the calcium reserve body sac after being produced by the red isthmus tubular gland cells. Calcium crystals are formed in close association with the occurrence of mammillan in the mammillae together with the starting secretion of ovoglycan by the red isthmus lining epithelial cells.[19,26] These initial crystals are more randomly oriented than those of the palisade layer that forms later. Palisade forms between 6:00 and 18:00 p.o. when the egg is in the shell gland, and coincidently, large amounts of ovoglycan are secreted by the lining epithelial cells of this region. Ovoglycan is detected in 250−300 nm vesicles of the palisade shell matrix.[19,28] The secretion of ovoglycan has a crucial effect on crystal orientation, morphology, and growth. In fact, crude or semipurified extracts of eggshell matrix alter calcite crystal morphology deposited in vitro to closely approximate that seen naturally.[3,23,30−32] The dermatan sulfate chains of ovoglycan are polyanionic and acidic, having a high calcium affinity. After experimentally inhibiting sulfation in the laying hen, the palisade of laid eggs shows severe structural alterations.[19] Unsulfated dermatan sulfate does not modify calcite morphology. A keratan sulfate molecular species, not necessarily identical to mammillan, has been detected in the cuticle[33] and in the shell gland cells at 1 h p.o.[19] Although the role of keratan sulfate in the cuticle has not been fully established, involvement of this kind of molecule in calcium phosphate (hydroxyapatite) deposition and removal has been suggested. In fact, avian medullary bone, which acts as a reservoir of resorbable calcium in the laying hen, also contains keratan sulfate as its major glycosaminoglycan.[34]

Several macromolecules of the eggshell are deposited in a defined temporal−spatial order,[3,23,27,29,35] being shell membrane type X collagen responsible for the inhibition of mineralization of this fibrillar structure, and mammillan mainly involved in nucleation of the first randomly oriented and easily removable crystals of the mammillary layer,[36] while ovoglycan, a unique dermatan sulfate proteoglycan, relates to the growth of the crystalline palisade.[19,32] Despite their importance in mineralization processes, a precise role of specific glycosaminoglycans, as part of proteoglycans, on eggshell formation has not been described as yet. Moreover, recent studies emphasize the role of mineral precursors during mineralization and indicate that these inorganic species and their interaction with biomolecules are highly relevant for providing insights to biomineralization pathways.[37−39] Therefore, in the present study we describe the effects of keratan sulfate and dermatan sulfate isolated from egg membrane and shell, respectively, on the nucleation of calcium carbonate using potentiometric titration. This technique provides quantitative information on different aspects of mineral nucleation in bulk volumes.

■ ANIONIC PROTEOGLYCANS FROM THE EGGSHELL

SDS-PAGE profiles of proteoglycans isolated from egg shells and egg membranes indicate single bands roughly corresponding to 75 and 90 kDa for fractions purified from the shells and membranes, respectively (Figure 2A). Given the highly anionic nature of proteoglycans, PAGE is not an appropriate method to estimate molar mass information; nevertheless, the purification methods applied here are effective.[23] Egg-shell associated dermatan sulfate and membrane bound keratan sulfate, respectively, have been reported previously.[23,24] In order to ascertain the nature of the purified biomolecules, Western blots are performed using commercial antibodies for dermatan- and keratan-sulfate (Figure 2B,C). Using monoclonal antibodies that recognize specific proteoglycan epitopes,[23] the presence of

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dermatan- and keratan-sulfate in samples purified from egg shells and membranes, respectively, is validated. These anionic proteoglycans are further investigated with respect to their effect on calcium carbonate formation using potentiometric titration.

PROTEOGLYCANS MODULATE THE NUCLEATION REGIME

Potentiometric titration is the state of art technique to quantitatively investigate the early stages of mineralization in bulk solution.[40] This assay provides information about the interaction between the additives and ions/prenucleation clusters, inhibitory effects toward nucleation as well as the formation of particular mineralization precursors.[41,42] Systematic studies on the effects of carbohydrates and amino acids on the early stages of calcium carbonate mineralization show that molecular properties such as stereochemistry, polarity, presence of Ca^{2+} binding sites, as well as the nature of glycosidic linkages can influence mineralization.[43,44] To understand the role of the anionic proteoglycans isolated from egg-shells on the early stages of calcium carbonate mineralization, titration assays are conducted in the presence of such additives at different pH values. Plots representing development of free Ca^{2+} ions and solubility products are presented in the Supporting Information (Figures S1, S2). From these data, three aspects of the nucleation event, i.e., stability of prenucleation clusters, nucleation time, and solubility product of the initially formed phases are used for comparison purposes.

During the prenucleation regime, the slope of the increase in free Ca^{2+} provides information on the interactions between additives, free Ca^{2+} ions, and solute ion associates.[41] Destabilization of prenucleation clusters is reflected by a higher slope of the linear prenucleation regime because the equilibrium moves toward free Ca^{2+} ions. In contrast, a lower slope indicates stabilization of clusters mediated by additive molecules. With respect to the stability of prenucleation clusters, distinct effects are observed for the two proteoglycan additives assayed here (Figure 3A). At pH 9.0, dermatan sulfate leads to a 20% increase in the prenucleation slope, whereas keratan sulfate promotes a decrease in the slope by about 13%. Similar trends are observed at lower additive concentrations at the pH 9.0. At pH 9.75, both proteoglycans induce stabilization of prenucleation clusters with respect to reference experiments. However, similar to the observations for experiments conducted at pH 9.0, keratan sulfate induces about 50% greater cluster stabilization in comparison to dermatan sulfate (Figure S3). Recently the effect of the prenucleation cluster stability on the thermodynamic stability of initially nucleated products, i.e., polymorph/polyamorph selection has been proposed.[45] Considering the opposite effects of these anionic proteoglycans (isolated from the same biomineral) on the prenucleation slope (Figure 4), it seems that biomolecules modulating the stability of prenucleation clusters can potentially modulate the early stages of biomineral growth in local environments. Such distinct effects can be attributed to differences in sugar residue conformations, acidic residues, and glycosidic linkages, as well as proteoglycan associated proteins.[43]

Significant inhibitory effects toward the nucleation of a solid phase of calcium carbonate are exhibited by the anionic proteoglycans assayed here (Figure 3B). These effects are dependent on the proteoglycan concentration as well as the pH of the carbonate buffer. A scaling factor F is applied, which is the quotient of the nucleation time in the presence of an additive and that of the corresponding reference. At pH 9.0, shell-associated dermatan sulfate exhibits F values of 1.1 and 1.8

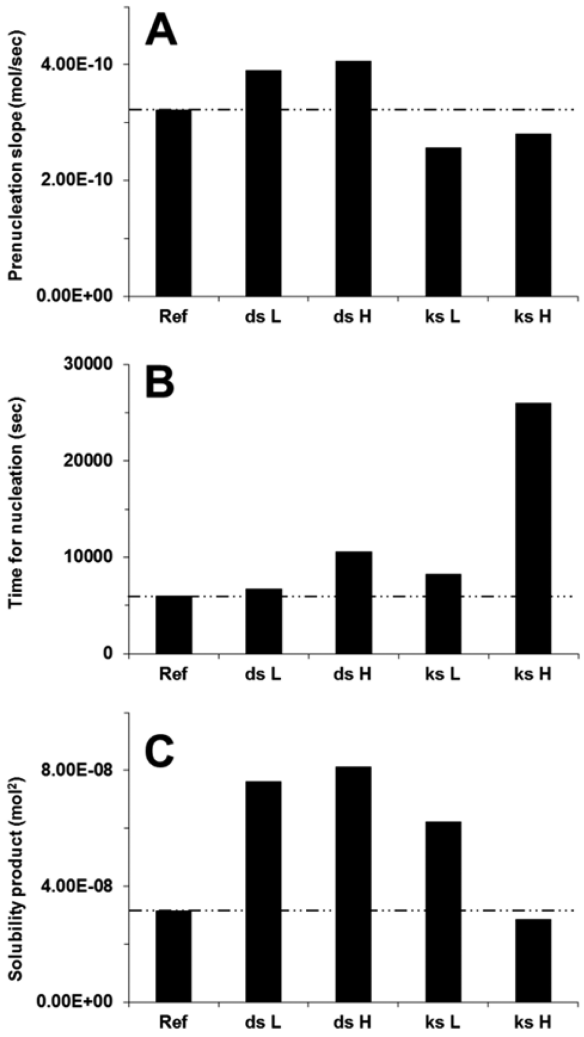

Figure 3. Bar plots representing the effects of proteoglycans dermatan sulfate (ds) and keratan sulfate (ks) on calcium carbonate formation at pH 9.0 in terms of (A) prenucleation slope, (B) nucleation time, and (C) solubility of the initially precipitated phase at 10 (L) and 100 (H) μg/mL.

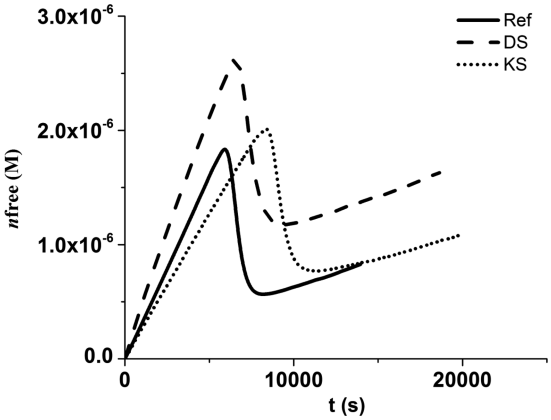

Figure 4. Time development of the amount of free Ca^{2+} ions in the presence of 10 μg/mL dermatan sulfate (DS, dashed line) and keratan sulfate (KS, dotted line) at pH 9.0. Reference experiment is shown with a continuous curve.

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at 10 and 100 μg/mL, respectively. This inhibition is, however, diminished at pH 9.75 wherein corresponding F values of 1.04 and 1.4 are observed (Figure S3). The inhibition of calcium carbonate nucleation by keratan sulfate derived from egg membranes is more pronounced. The induced inhibition of calcium carbonate nucleation is evident from F values of 1.4 and 4.3 at 10 and 100 μg/mL at pH 9.0, respectively. For keratan sulfate, the time required for nucleation also is pH dependent. This is reflected by F values of 1.6 and 1.7 at the corresponding low and high additive concentrations. Thus, both anionic proteoglycans are inhibitors of calcium carbonate nucleation of which keratan sulfate is more potent.

The solubility products corresponding to the initially precipitated phases are derived from titration experiments (Figure 3C). These values refer to the thermodynamic solubilities based on the ion products of free species.[46] Reference experiments performed in the absence of additives at pH 9.0 and 9.75 yield solubility products for initially formed phase of about 3.1×10^{-8} and 3.8×10^{-8} M^2, respectively. These are in good agreement with previous literature.[40,47] At pH 9.0, the initial phases nucleated in the presence of dermatan sulfate exhibit values of solubility products between 8×10^{-8} and 7.5×10^{-8} M^2. These are higher than those observed for reference experiments. The solubility products of polymer induced liquid-like precursors synthesized in the presence of acidic polymers such as are in the range of 40×10^{-8} and 20×10^{-8} M^2.[42] In the present study, the intermediate solubility product values might reflect the presence of a transient precursor to amorphous calcium carbonate or a highly hydrated form of amorphous calcium carbonate. In the presence of 10 μg/mL keratan sulfate, the solubility products of the initially nucleated phase also indicate similar amorphous calcium carbonate that gradually transforms to the crystalline phase. However, at higher concentrations of keratan sulfate, vaterite is the initially nucleated product. Toward the end of the titration experiments, the final values of solubility product correspond to vaterite (2.4×10^{-8} M^2) and calcite (1.2×10^{-9} M^2) at low and high keratan sulfate contents, respectively. As solubility products from titration assays correspond to the most soluble of all phases present, keratan sulfate appears to promote the transformation of amorphous phases to crystalline forms at relatively high concentrations. This is supported by a secondary nucleation event observed in the presence of 100 μg/mL keratan sulfate at higher pH (Figure S1). At higher pH (9.75), both proteoglycans lead to formation of crystalline calcium carbonate immediately after nucleation (Figure S3). The postnucleation solubility products are in the range of 2×10^{-8} and 2.7×10^{-8} M^2 which reflects the formation of vaterite. Thus, under identical mineralization conditions, dermatan sulfate leads to the formation of more soluble phases, whereas keratan sulfate induces crystalline phases after nucleation. Moreover, the formation of distinct phases relates to the stability of prenucleation clusters, i.e., keratan stabilizes calcium carbonate prenucleation clusters and leads to less soluble phase postnucleation, whereas dermatan sulfate induces opposite effects.

The anionic proteoglycans display behaviors typical of type II, III, and V additives, influencing cluster formation and equilibria, inhibiting mineral nucleation and affecting initially formed phases.[41] The distinct behavior of keratan and dermatan-sulfate on calcium carbonate crystallization associates with their actual localization in the eggshell. Keratan sulfate is located in vesicles of the calcium reserve bodies of the mammillary crystalline layer where mineralization initializes during eggshell formation and where calcium carbonate dissolution occurs as a calcium ion source during bone formation in chick embryo formation. Thus, an additive such as keratan sulfate that stabilizes prenucleation clusters during transport, prevents premature nucleation, as well as modulates the phase formed postnucleation is ideal. On the other hand, dermatan sulfate, located in the highly mineralized and insoluble palisade, is involved in regulation of mineral growth and crystal morphology. A destabilization effect toward prenucleation clusters and formation of soluble amorphous phases indicates that this proteoglycan is possibly involved in the transport of mineral precursors. A highly soluble nucleation product might also promote the dissemination of mineralization precursors in confined environments and interactions with other additives. This is also supported by reprecipitation experiments using quail egg shell components that indicate mineralization takes place through the amorphous calcium carbonate precursor phase.[48] Our results indicate that, in addition to concentration and pH dependence effects, each proteoglycan leads to distinct schemes of calcium carbonate mineralization (Figure 4). To better simulate in vivo environments, optimization of titration experiments at physiological pH is in progress.

ONE ADDITIVE: SEVERAL SPATIOTEMPORAL ROLES?

In conclusion, biomineralization studies generally associate the morphological and crystallographical features to address formation mechanisms and emergent properties of sophisticated biominerals. Such investigations provide important information on Nature's design strategies and suggest routes of mineral nucleation and growth. However, at the submicron scale, it becomes important to address the interactions of biomolecules with mineral precursors that eventually lead to manifestation of the biomineral structure. With the recent emergence of mineral precursors species such prenucleation clusters, liquid condensed phases, and polymer induced liquid precursors,[37,38,40,49,50] it is crucial to investigate interactions of biomolecules and transient precursors to gain a better understanding of biomineralization processes. By using sensitive techniques that provide quantitative information on nucleation events in bulk solutions, one can monitor the presence and properties of transient mineral phases in the presence of additives. In the present study, titration assays show that dermatan- and keratan-sulfate distinctly affect early stages of mineralization by modulating cluster stability, nucleation events, and polymorphs nucleated. Since these molecules are also involved in the regulation of mineral morphology and mineral storage, a single additive can potentially affect nascent mineralization processes as well as later events including crystal morphology and assembly. Given the unique spatiotemporal distribution of egg-associated anionic proteoglycans and the composite structure of egg shells, the regulation of mineral formation emerges to be dependent on local in vivo variations and distributions of certain biomolecules. This hints toward a complex scenario of biomineralization processes that are typically regulated by diverse biomolecules where each biomolecule regulates different stages of mineralization in a temporal manner.

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■ ASSOCIATED CONTENT

Supporting Information

Purification procedures and experimental details are provided. Supplementary data include potentiometric titration data and plots. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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