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MORPHOLOGICAL EFFECTS OF GLYCOSAMINOGLYCANS ON CALCIUM OXALATE MONOHYDRATE CRYSTALS

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Abstract

The effects of individual glycosaminoglycan (GAG) species on calcium oxalate monohydrate (COM) crystal growth were studied *in vitro* by the observation of crystal morphology grown in a supersaturated calcium oxalate solution in the presence of GAGs using optical and scanning electron microscopes. GAGs affected the morphology of COM crystals differently depending on the species. The growth rates of the crystals formed in the presence of chondroitin-6-sulfate (ChS-C) were higher in length and lower in width and thickness than those of control crystals. The incorporation of dermatan sulfate or heparin into the crystals formed in the presence of these GAGs was revealed by X-ray microanalysis, whereas ChS-C was not detected in the crystals grown with it. The experiment using dicarboxylates, as a simple model of GAG molecules, showed that a distance between the side groups was important for their morphological effects. These findings suggested that the different effects of GAGs on the crystal morphology resulted from the differences in their interaction modes with COM crystal faces, that is, the differences in their binding behavior, their inhibition modes of crystal growth, and other roles played after binding to the crystals.

Key Words: Calcium oxalate monohydrate, crystal morphology, crystal growth, crystallization process, glycosaminoglycans, inhibitor, scanning electron microscopy, X-ray microanalysis.

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Introduction

Glycosaminoglycans (GAGs) are known to be inhibitors of *in vitro* calcium oxalate (CaOx) crystallization as well as urinary CaOx stone formation. GAGs have carboxyl and sulfate (except in hyaluronic acid) groups that enable them to bind to calcium ions in solution and to the surface of calcium-containing crystals. However, GAGs concentrations in urine, as well as those used in *in vitro* experiments, are too low to cause a significant reduction of calcium in solution. Therefore, GAGs are expected to exert inhibition through a mechanism by binding to the crystals. Nevertheless, the extensive *in vitro* studies about the inhibitory effects of GAGs on the CaOx crystallization have employed indirect measurement methods; only a few of those papers have reported on the resulting crystals [3, 9, 14]. Moreover, the inhibitory effects of GAGs and the mechanism underlying these effects still remain unclear because diverse experimental model systems have been used; also, there is uncertainty in the literature about the target crystal form, CaOx-monohydrate (COM), -dihydrate (COD) or a mixture of them, and the crystallization processes (nucleation, growth or aggregation of crystals). Hence, our studies have been directly focused on COM crystals. By using a mild condition, a gentle shaking instead of stirring, we could grow highly reproducible COM crystals with characteristic morphology depending upon additives and their concentrations [15, 16, 17, 18, 19, 20]. The changes in morphology of COM crystals seemed to be a consequence of the inhibition of crystal growth through the interactions between GAG molecules and COM crystals. In this paper, we review these recent studies and discuss the interaction modes between GAG molecules and COM crystals.

Materials and Methods

Crystal formation *in vitro* was performed under conditions close to the *in vivo* situation: 37°C, pH 5.7 and physiological ionic strength under gentle shaking. Reagent grade chemicals and high purity water (15 MΩ.cm

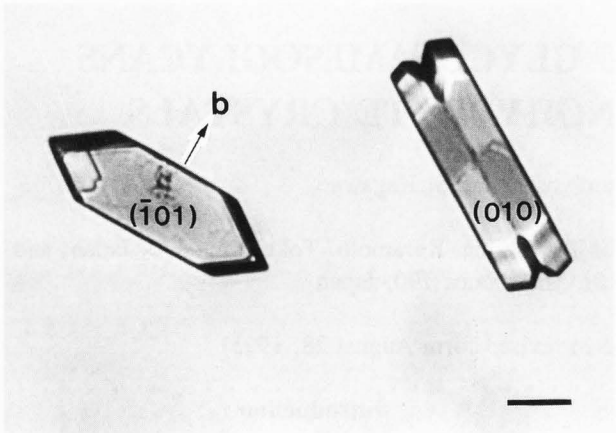

Figure 1. Differential interference micrograph of typical COM crystals formed in supersaturated CaOx solution without any additives, as control crystals of COM. Left, upper (101) face view of crystal with a hexagonal face elongated along [101] direction. Right, side (010) face view of other COM crystal with a double nock face divided four areas under polarized light. Bar = 10 μm.

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resistance) were used. Stock solutions of calcium chloride, sodium oxalate, GAGs and dicarboxylates were prepared using 50 mM sodium acetate buffer (pH 5.7) containing 0.1 M sodium chloride and were filtered through a 0.22 μm Millipore filter. Four ml of 10 mM calcium chloride stock solution, appropriate volumes of additive stock solution and acetate buffer (to bring total volume to 9 ml) were added in a plastic well (35 mm diameter), containing a few small glass plates, and were preincubated for 30 minutes at 37°C. Then, one ml of 2 mM sodium oxalate stock solution was added slowly to initiate crystallization which progressed under gentle shaking with a rotary shaker at 37°C (except as noted otherwise). In our experiments, supersaturated solutions of CaOx initially contained 4 mM calcium and 0.2 mM oxalate in the acetate buffer, so that the initial molar concentration product and the ionic strength of the solutions were 0.8×10^{-6} M^2 and 0.162, respectively. COM crystals grown in the well were observed by Normaski differential interference microscopy (OLYMPUS IMT2 NIC) and micrographs were taken at regular intervals. After 5 to 6 days, the glass plates on which the crystals formed were removed, washed with deionized-distilled water several times, and air-dried. Finally, the specimens were either coated with gold/palladium (10 nm in thickness) for scanning electron microscopy (SEM, using a HITACHI S-800) or coated with carbon (20 to 30 nm thick) for X-ray microanalysis (using a Kevex 7000 energy dispersive X-ray microanalyzer attached to a HITACHI H-500 SEM). The crystals formed in this experiment were examined by X-ray powder diffraction analysis (Rigaku Denki RINT 1300).

Figure 2 (on the facing page). Scanning electron micrographs of COM crystals formed in the presence of 30 μg/ml individual GAGs. (**A**) HA; (**B**) ChS-C; (**C**) ChS-A; (**D**) DS; (**E**) HS; (**F**) HP. The top part of each figure shows an upper (101) face view of a crystal and the bottom part shows a side (010) face view of another crystal. The morphology of these crystals differs from each other. Bars = 10 μm.

Results and Discussion

Morphological effects of GAGs on COM crystals

COM crystals grown in the supersaturated CaOx solutions in the presence or absence of GAGs were observed by optical microscopy and SEM. They show a wide variety of crystal forms such as hexagonal plates, interpenetrating twins, mulberry, and aggregates.

Figure 1 shows the control crystals of COM, grown in the absence of additives; these are the dominant forms. They have an elongated hexagonal (101) face and a double nock (010) face with reentrant corners, indicating its contact twin which is also evidenced by four divisions observed under polarized light. Consequently, these crystals do not appear to be real single crystals in a crystallography sense, but in our papers, we have treated them as "single crystals" and observed changes in their morphology caused by additives.

Addition of GAGs affected the morphology of COM crystals; the results showed characteristic shapes which varied with different types of GAGs. The morphological effects of increasing GAGs concentrations from 1 to 100 μg/ml were very pronounced: the crystals decreased in thickness, suggesting that GAGs inhibit crystal growth [15, 16, 18, 19]. Figure 2 shows scanning electron micrographs of the crystals formed by addition of different GAGs at concentrations of 30 μg/ml.

In the presence of low concentrations (1 μg/ml) of hyaluronic acid (HA), the crystals showed hexagonal morphology very similar to the control crystal; but at higher concentrations (100 μg/ml), the morphology of the crystals was dramatically altered to fine and thin lamellae (results not shown).

The crystals formed in the presence of chondroitin-6-sulfate (ChS-C), at all concentrations, showed the more elongated shape along the [101] direction and the disappearance of the reentrant corners of the twins; Figure 2B presents a typical example for a ChS-C concentration of 30 μg/ml. This morphology appears to result from the enhancement of crystal growth along the [101] direction, especially at the reentrant corners.

The presence of chondroitin-4-sulfate (ChS-A) produced less elongated crystals habits (Fig. 2C) than in the case of ChS-C (Fig. 2B).

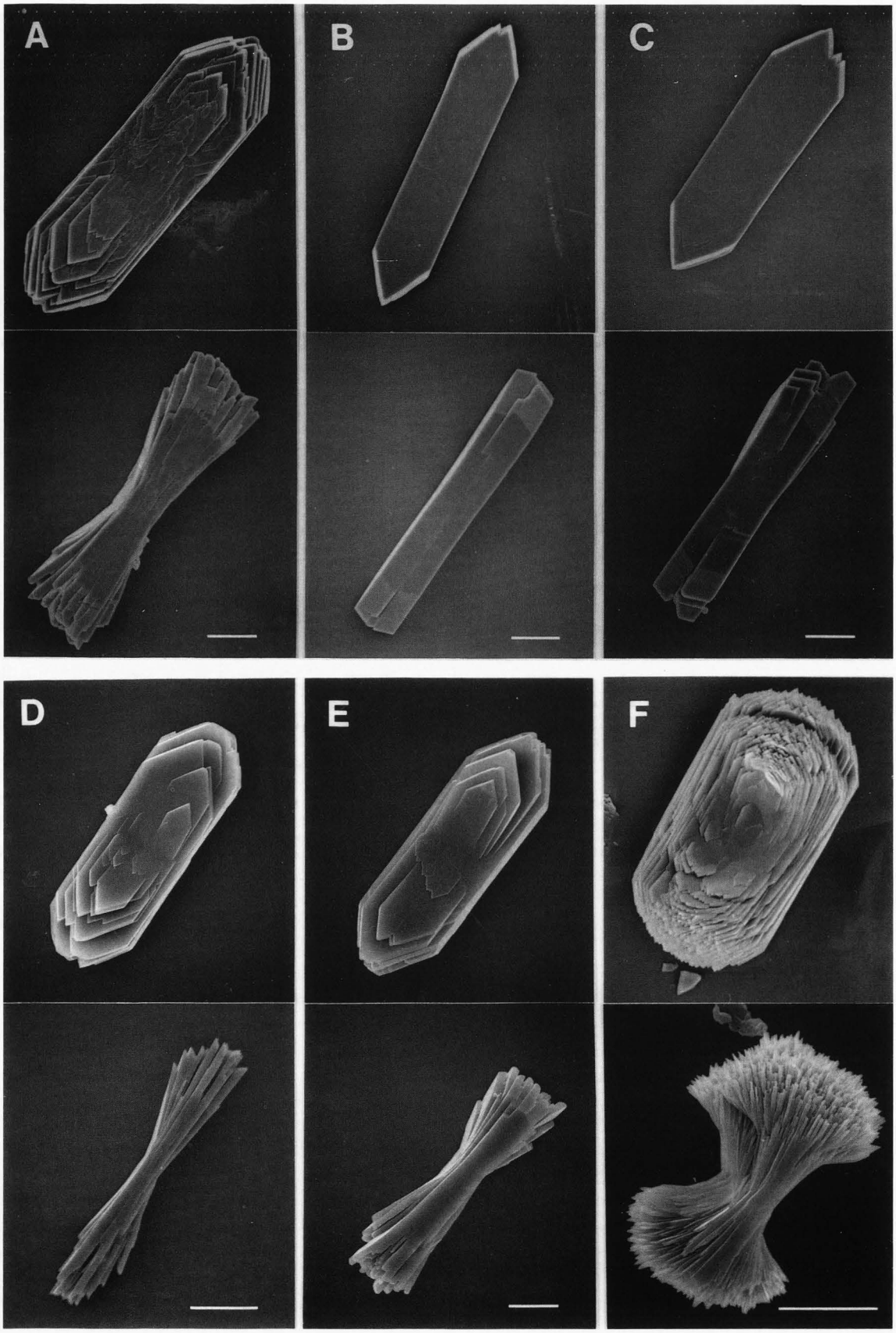
A
B
C
D
E
F

Table 1. Atomic percentages of S and Ca of COM crystals formed in the presence of diverse GAGs at concentrations of 10 μg/ml (statistics: Student's t-test; mean $\pm$ standard deviation, SD).

Elements	Heparin	Dermatan sulfate	Chondroitin 6-sulfate	Hyaluronic acid
S (%)	2.16 $\pm$ 0.77*	1.25 $\pm$ 0.17*	0.47 $\pm$ 0.31	0.58 $\pm$ 0.26
Ca (%)	97.83 $\pm$ 0.75	98.75 $\pm$ 0.17	99.53 $\pm$ 0.27	99.47 $\pm$ 0.27

*: $p < 0.001$ versus hyaluronic acid.

The morphology of crystals in the presence of dermatan sulfate (DS) or heparan sulfate (HS) showed hexagonal plates with slightly rounded vertices and several layers of lamellae. Their side (010) face views show distinguishable features that are thinner layers with DS (Fig. 2D) and a thick (010) face with HS (Fig. 2E).

In the presence of heparin (HP), COM crystals were much smaller in size and appeared as oval {(101) face view} and dumbbell {(010) face view} shapes consisting of stacks of lamellar crystallites (Fig. 2F); these features were evident even at low concentrations of 10 μg/ml (results not shown). These crystal shapes are similar to those observed in urine [23]. They seem to be polycrystalline aggregates resulting from irregularities in crystal growth but not as a result of aggregation of pre-existing crystals.

Mechanism of interactions between GAGs and COM crystals

The feature of the COM crystal morphology, mentioned above, led us to the following speculations: (1) individual GAGs bind to the specific crystal faces with different binding behaviors, the resulting retardation or inhibition of the continuous crystal growth perpendicular to these faces leads to differences in the crystal morphology; and (2) the differences in binding behaviors are related to GAG molecule structures.

As a first step towards confirming the validity of above speculations, X-ray microanalysis was performed to determine whether the crystals contained sulfur derived from sulfate residues of GAGs: the results showed that COM crystals formed in the presence of DS or HP contained sulfur in the crystals; and also that, the crystals grown with ChS-C contained the same amount of sulfur as the crystals with non-sulfated HA (Table 1) [18, 19]. All GAGs, including ChS-C, must bind to COM surfaces since they have been reported to shift the zeta potential of CaOx crystals towards more negative values [22] and also been reported to bind in the mode of a monolayer type of adsorption [10]. Since, X-ray microanalysis detects the sum of emergent X-rays (by incident electron beam) in a certain area and depth of the crystals, these results may imply that ChS-C only loosely binds to the crystal surface and is readily removed from it, so ChS-C is not retained in a detectably significant amount in the crystals. That may be the reason why ChS, the most prominent urinary GAG, is notably absent from urinary stone matrix [13]. On the other hand, DS and HP intimately bind to the (101) faces, resulting in their incorporation into the growing crystals through the following mechanism: both GAGs bound to the faces might offer the new nucleation sites and induce oriented crystal nucleation on the faces; thus, they are retained between all stratified crystallites and accumulate in the crystals. These GAGs appear to be able to act not only as growth inhibitors when present in solution, but also as promoters of nucleation when immobilized onto surfaces, as reported in other papers [1, 4, 5], where the dual effect of several molecules, depending on the environment, was mentioned. That may be the possible mechanism of development of the polycrystalline aggregates.

X-ray powder diffraction analysis of the COM crystals grown in the presence of HP or ChS-C was performed. The diffraction intensity of the crystals with HP was much weaker than that with ChS-C, which may also reflect the different interaction modes mentioned above (results not shown).

Individual GAG species have their own chemical names, however, there are only a few differences in their molecular structures, such as the number and location of sulfate groups and the stereoisomerical components of their disaccharide units. Nevertheless, they affected COM morphology differently; therefore, their ability to influence COM crystals seems to relate to the stereochemical structures and the spatial arrangement of carboxyl, sulfate and hydroxyl side groups along the chains. Then, in the next step, the influence of differences in molecular structure of GAGs on COM morphology was studied by using dicarboxylates such as malonate, maleate, fumarate, malate and succinate. Malonate, maleate and malate affected the crystal morphology, that is, the changes in the elongated hexagonal (101) faces to rectangular ones (Figs. 3A, 3B and 3C), whereas the rest of the dicarboxylates did not alter the hexagonal morphology of the faces (Figs. 3D and 3E).

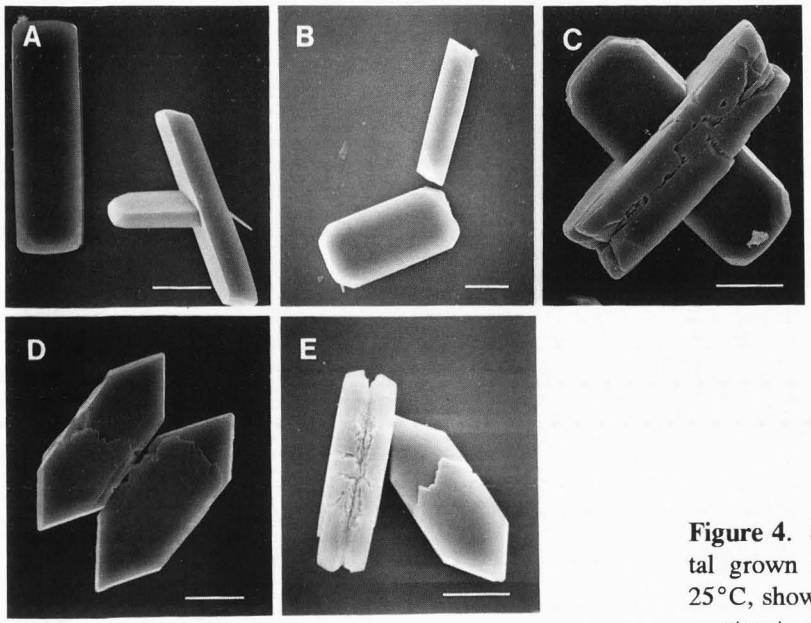

Figure 3. Scanning electron micrographs of COM crystals grown with addition of several dicarboxylates at 10 mM except malate at 4 mM. (**A**) malonate; (**B**) malate; (**C**) maleate; (**D**) fumalate; (**E**) succinate. Crystal (101) faces of upper panels have rectangular shapes, indicating that malonate, malate and maleate affected the crystal morphology. Bar = 10 μm.

Table 2. Molecular structure of four chondroitin sulfates.*

	component	location of sulfate groups		sulfur contents (%)
ChS-A	Gal N	4-O (80%),	6-O (20%)	6.2 - 6.6
ChS-C	Gal N	4-O (10%),	6-O (90%)	6.4 - 6.8
ChS-D	Glc A	2-O (30%),		7.1 - 7.7
	Gal N		6-O (100%)	
ChS-E	Gal N	4-O (100%),	6-O (60%)	8.2 - 9.0

*Data from Seikagaku Kogyo Co., Ltd. (Japan)

These results demonstrate that the appropriate distance between two carboxyl groups is required for them to cause morphological effects, and that a hydroxyl group also plays a part as a binding site [20], while Grases *et al.* [8] have related the hydroxyl groups to the molecular rigidity. Their interactions with COM faces were probably through cooperative binding of both carboxyl groups [11] and/or carboxyl and hydroxyl groups. Therefore, the side groups in GAG molecules might have the specific arrangements which depend on the GAG species, that cause differences in binding behaviors.

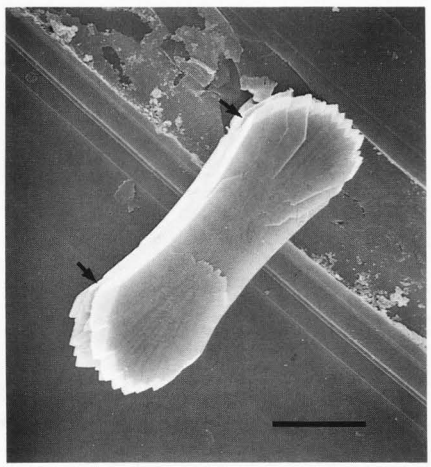

Figure 4. Scanning electron micrograph of COM crystal grown in the presence of ChS-C (30 μg/ml), at 25°C, showing a dumbbell-shape with serrated ends and reentrant corners (arrows). Bar = 10 μm.

The effects of ChS on the COM crystal morphology and growth

ChS-C had a peculiar effect on COM crystal shapes, the elongated morphology and the disappearance of reentrant corners of neat crystals. ChS-C was not detected on the crystals by X-ray microanalysis even though it contains the same amount of sulfur as DS. We became specially interested in ChS in terms of its molecular structure because L-iduronic acid in DS molecule and D-glucuronic acid in ChS-C are stereoisomers. The effects of the number and location of sulfate groups in ChS molecules on the crystal morphology were examined by using four types of ChS (-A, -C, -D and -E) (Table 2). Morphological features of the crystals divided them into two groups, having mainly the ester sulfate groups in the C-4 position or in the C-6 position on the galactosamine (Figs. 2C and 2B). The ratio of length {[101] direction} to width {[010] direction} on the (101) face of the crystals in the case of the former (ChS-A and -E) or the latter (ChS-C and -D) were 3.6 $\pm$ 0.2 or 4.6 $\pm$ 0.3, respectively. The results indicate that the location of sulfate groups plays more important roles on the crystal shapes rather than the sulfate contents in ChS molecules.

The morphological effect of ChS-C on the COM crystal was changed by decreasing the experimental temperature to 25°C. The elongated morphology of the crystals (Fig. 2B) changed to a dumbbell-shape with serrated ends and reentrant corners (Fig. 4). The changes

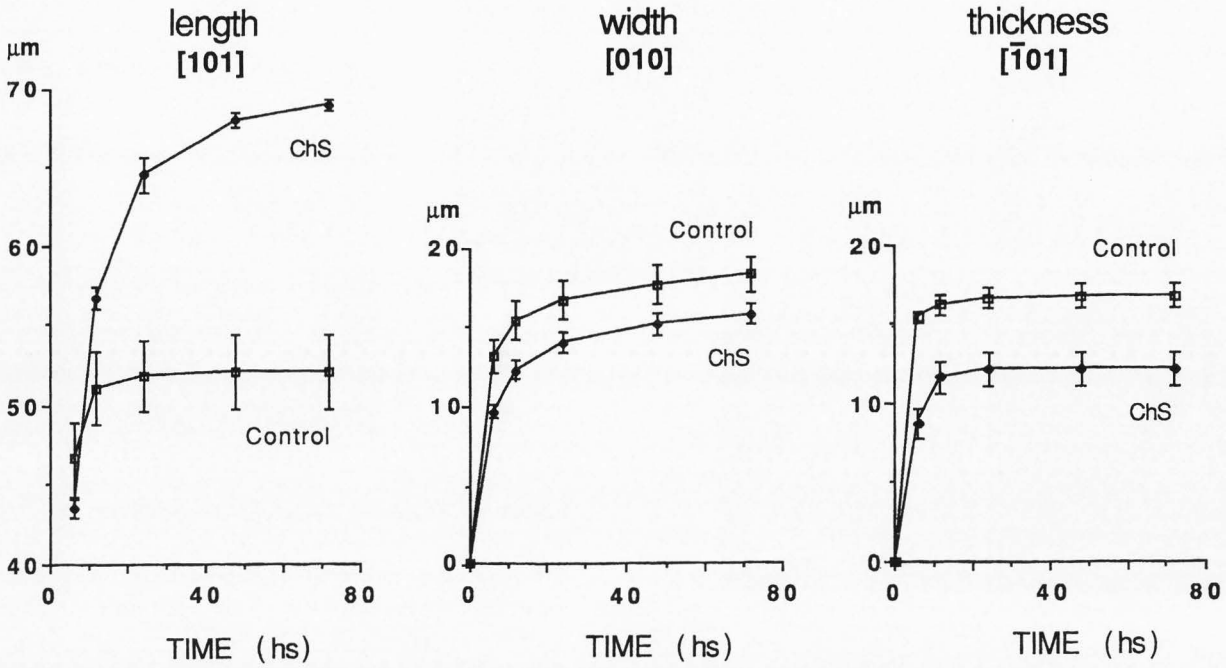

Figure 5. Effect of ChS on COM crystal growth. The sizes in length, width and thickness of the crystals formed in the presence (solid circles) or absence (hollow squares) of ChS-C (100 μg/ml) were measured from the optical photographs taken at indicated times after starting crystallization and plotted.

in the conformation of ChS-C molecule and consequent binding behavior to the crystals might occur because of a change in temperature causing a conformational alteration of macromolecules [12]. This observation might imply that the molecular conformation is one of the important factors affecting crystal morphology [21].

The effect of ChS-C on the COM crystal growth was also determined. The growth rates in length, width and thickness {[101] direction} of the crystals were obtained from the optical photographs of the crystals taken at 6, 12, 24, 48 and 72 hours after start of crystallization with or without ChS-C (Fig. 5). The results showed a retardation of crystallization (nucleation and/or growth) in the early stages, and a continuous enhancement (with a high rate) of crystal growth along the [101] direction in the presence of ChS-C. The growth rates along the [101] and [010] directions were lower in comparison with those of control crystals, resulting in relatively elongated morphology [21]. Moreover, ChS bound to COM may exhibit a strong calcium-concentrating effect [2], increasing calcium concentrations around the crystals, that could explain the continuous enhancement of crystal growth along the [101] direction. These findings suggest that ChS-C binds selectively to the (101) and (010) faces, and consequently has a dual, but quite the opposite, effect on the crystal growth depending upon directions.

In addition, the elongated shape of neat crystals showed little change at any concentration of ChS-C, which might be related to some possible biological functions of ChS which controls the crystal shapes [1].

Conclusions

Methods: Although the observation of the crystal morphology is a very simple experiment, we can follow the growth of COM crystals *in vitro* and observe resulting crystals directly. Our results indicate the importance of direct observation. For example, the effect of ChS-C may not be evaluated correctly by the Coulter counter method because that method assumes the particles to be spherical, whereas the crystals formed in the presence of ChS-C are elongated. Any other method, besides direct observation, cannot estimate such a dual effect of ChS-C on the crystal growth depending on the directions. Conclusively, the careful observation of the crystal morphology is an essential method and can introduce new concepts into the studies.

Crystals: It is a well-known law that crystals consist of atoms with an orderly arrangement which is different in different faces. Especially, in the case of the interactions involving crystal-surface-related processes, the atomic arrangement of the interacting faces of COM should be considered of great importance. There are coplanar calcium and oxalate in the (101) and (010) faces of COM crystals [7]. According to Addadi and Weiner

[1], however, the former face seems to be more interactive than the latter one. _Therefore, GAGs might have mainly bound to COM (101) face.

Moreover, in spite of having the same chemical components, COM and COD are quite distinct substances in terms of crystallography, monoclinic and tetragonal, respectively. The various morphologies of COM crystals indicate that the crystal surfaces of COM is susceptible to the influence of additives, whereas COD crystal morphology appears identical regardless of additives, suggesting that the COD crystal surface is barely influenced by any of these substances [17] Thus, the specific phase of CaOx, on which the effects of GAGs were examined, should be stated clearly in order to avoid confusion.

GAGs: GAGs in solution have been reported to adopt random-coil conformations [6] However, the highly reproducible morphology of COM crystals suggests that GAGs molecules at 37°C seem to adopt structural conformations, in which a certain set of the side groups in the molecules may cooperatively take part in binding to specific COM faces and the other set of them may act as a nucleation site or exert a calcium-concentrating effect. Although the above stated mechanisms are highly speculative, they should be taken into consideration for understanding the actual effects of GAGs. In conclusion, we propose that GAGs might perform not only inhibitory but also promotive roles in the crystallization processes.

Acknowledgments

The authors would like to thank Professor Kichiro Koto for his helpful comments on crystallography, and Mr. Akira Kanaya for performing X-ray microanalysis.

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Discussion with Reviewers

A.A. Campbell: Did the authors perform any modelling of the additives? There is much discussion about stereochemistry and random-coil orientations without any basis for making these assumptions. Assumptions are made about specific binding of the additives to the crystals. Any modelling to explore the additive stereochemistry and internal spacing of functional groups with the location of calcium ions on the respective crystal faces?

Authors: We discuss the interaction modes based on the stereochemical knowledge of GAG molecules and also based on the results obtained from our experiments using four types of ChS and dicarboxylates. We performed the modelling of the dicarboxylates and referred to Deganello and Piro [6] for the crystal structures of the COM faces.

A.A. Campbell: Is a calcium-rich crystal face important?

Authors: Yes, we think so and an interactive one is important as reported by Addadi and Weiner [1], as stated in the **Conclusions**.

F. Grases: It is stated that GAGs bound to the faces might offer new nucleation sites and induce nucleation on the faces forming polycrystalline aggregates. Could this mechanism be responsible of COM primary aggregation during renal stone development?

Authors: According to your paper [25], which gives the definition of the primary aggregation of COM crystals, our stated mechanism may be responsible for COM primary aggregation, though we do not have any direct evidence concerning renal stone formation.

S. Deganello: The symmetry of the $(\bar{1}01)$ is not hexagonal. Please note that COM crystallizes in the monoclinic system (P21/n or P21/c).

Authors: Although the word "hexagonal" may not be an accurate one in crystallography, it is found in many papers as the morphological expressions of COM $(\bar{1}01)$ face (e.g., Campbell *et al.* [5], Antinozzi *et al.* [24]).

W.C. de Bruijn: What is the influence of the organic to inorganic ratio on your **Conclusions**? Is it better, for clarity of the process to estimate these ratios in terms of mol/l rather than as mg/ml since these GAGs are rather large macromolecular complexes?

Authors: We wish to emphasize that the influence of the organic components (GAGs) on the COM crystal morphology depends more on their structural conformations rather than their concentrations. It is shown that the increase in GAGs concentrations strengthens their effect on the crystal morphology though.

Urinary concentrations of GAGs have been reported in weight (μg/ml or mg/day) rather than on molar basis. Moreover, these chemicals are commonly shown to have a wide range of molecular weights in their explanations (for example ChS-C: 4-8 x 10^4). We, therefore, used them in μg/ml basis, although it might be better to use molar concentrations in the experiment.

W.C. de Bruijn: How were your experimental conditions related to urinary conditions?

Authors: The crystallization solutions were prepared with physiological ionic strengths), pH 5.7 and within the normal urinary concentrations of calcium and oxalate, and the crystallization took place at 37°C and under a gentle shaking instead of a vigorous stirring which is impossible *in vivo* but is ordinarily used in the *in vitro* experiments.

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