Morphological and Phase Changes in Calcium Oxalate Crystals Induced by Sulfated Polysaccharide Extracted from Algae *Eucheuma striatum*

Jian-Ming Ouyang*[†,††] and Xiu-Mei Wu[††]
[†]*Institute of Biomineralization and Lithiasis Research, Jinan University, Guangzhou 510632, P. R. China*
[††]*Department of Chemistry, Jinan University, Guangzhou 510632, P. R. China*

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The sulfated polysaccharide (SPS) isolated from marine algae *Eucheuma striatum* can inhibit the growth of calcium oxalate (CaOxa) crystal, prevent the aggregation of calcium oxalate monohydrate (COM), and induce the formation of calcium oxalate dihydrate (COD) crystals. All these factors can prevent the formation of CaOxa urinary stones. It indicated SPS may be a potential green drug to CaOxa urinary stones.

Urolithiasis is a very common disease occurred in the worldwide area. The recurrence rate is more than 80%, with a moderate improvement in recurrence rate by conventional therapies. Calcium oxalate (CaOxa) is the major mineral component of most human urinary stones.[1,2] However, the mechanism about the formation of urinary stones is not yet clearly understood and a number of questions about the promoting and inhibiting factors still remain unanswered. Moreover, the therapeutic efforts are neither clinically nor scientifically satisfactory.[3]

Many seaweed polysaccharides, such as agar and carrageenan, are used extensively in industry. In recent years, the medical potential of seaweed polysaccharides has drawn more and more attention to scientists. Antitumor, antivirus, anticoagulant, and antihyperlipidemia biological activities have been found in seaweed polysaccharides, some of which have been developed as new drugs.[4,5] However, there is no report about the application of sulfated polysaccharide (SPS) isolated from marine algae such as *Eucheuma striatum* in the inhibition of CaOxa urinary stones.

In urine, there are glycosaminoglycans (GAGs), including chondroitin sulfate A (C_4S), chondroitin sulfate C (C_6S), heparin, heparan sulphate (HS), dermatan sulfate (DS), keratan sulfate (KS), hyaluronic acid, etc. Most of these GAGs have similar molecular structure as algae polysaccharides. Especially the sulfated glycosaminoglycans such as C_4S, C_6S, HS, DS, and KS can inhibit the growth of urinary stones.[6,7] With this in mind, the inhibitive action of SPS on crystallization of CaOxa was investigated in this work.

All solutions were prepared with reagent-grade chemicals that were purchased from Shanghai Chemicals Co. Double distilled water was used. Sulfated polysaccharide (SPS) was isolated from marine algae *Eucheuma striatum*, which was obtained from Nan-ao Island in southern Chinese Sea and purified following the reference.[8,9] The molecular weight of the SPS used in this experiment is about 20,000. The content of sulfated group in the sample was 18.3%, which was determined by gelatin–$BaCl_2$ method. UV–visible spectrum of SPS shows the characteristic peak of polysaccharide at 200 nm. No peak at 260–280 nm indicated that there is no protein and no nucleic acid in SPS.

The supersaturated CaOxa subphase was prepared according to reference.[10] The original concentration of $CaCl_2$ and Na_2Oxa are 10.0 mmol/L, and the final concentration of both Ca^{2+} and Oxa^{2-} equal 0.30 mmol/L. The concentration of SPS investigated in this work is 0, 0.03, 0.10, 0.50, and 1.0 mg/mL.

The crystallization experiments of CaOxa were carried out at temperature $37 \pm 1\,^{\circ}C$ in a constant temperature chamber according to literatures.[3,11] The morphology of CaOxa was measured by scanning electron microscopy (Phlips XL-30 ESEM) at an operating voltage of 10 kV. X-ray diffraction (XRD) results were recorded on a D/max-γA X-ray diffractometer (Rigaku, Japan) using Ni-filtered Cu Kα radiation ($\lambda = 1.54$ Å) and a scanning rate of 2° min^{-1} at 40 kV, 30 mA. Fourier transform infrared (FT–IR) was carried out with a Bruker IFS25 spectrometer (Bruker Spectrospin, Wissembourg, France) between 4000 and 400 cm^{-1} with a resolution of 4 cm^{-1}.

Figure 1 shows the SEM images of CaOxa crystals grown in the presence of various concentrations of SPS. SPS can induce the formation of tetragonal bipyramidal calcium oxalate dihydrate (COD) crystals. The percentages of COD crystals increased with the increase of the concentrations of SPS. In the presence of 0.03, 0.10, and 0.50 mg/mL SPS, the percentages of COD crystals are 15, 70, and 90%, respectively.

The average sizes of both the elongated hexagonal calcium oxalate monohydrate (COM) and COD crystals decreased when the concentration of SPS increased from 0 to 0.03, 0.10, 0.50, and 1.0 mg/mL. For example, the average sizes of COM crystals in the presence of 0.03, 0.10, and 0.50 mg/mL of SPS are 9.5×3.3, 3.3×2.7, and $2.0 \times 2.0\,\mu m^2$, respectively. They are apparently less than that in absence of SPS (Figure 1a, about $16.5 \times 5.4\,\mu m^2$). The average sizes of COD crystals decreased

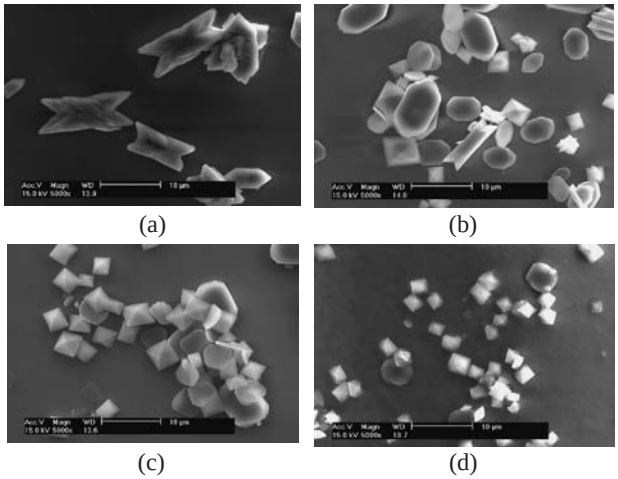

Figure 1. SEM images of CaOxa crystals grown in the presence of SPS of (a) 0, (b) 0.03, (c) 0.10, and (d) 0.50 mg/mL, respectively (the bar: 10 μm).

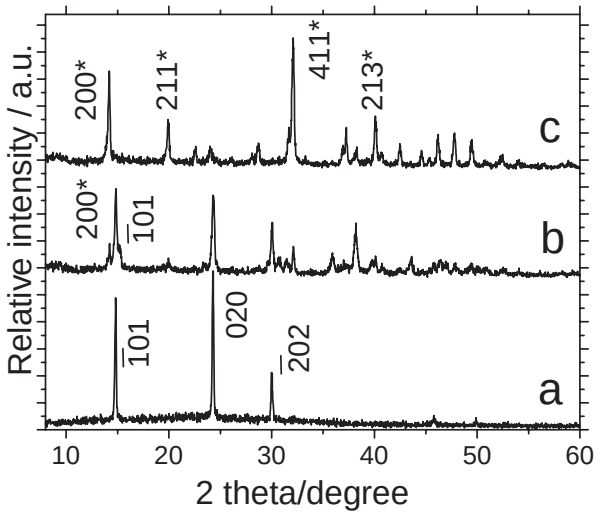

Figure 2. XRD patterns of CaOxa crystals grown in the presence of SPS of (a) 0, (b) 0.03, and (c) 0.50 mg/mL, respectively. The crystal faces with asterisk show COD and those without asterisk show COM.

from 4.8×4.8 to 3.6×3.6 and $2.7 \times 2.7\,\mu m^2$ as the concentration of SPS increased from 0.03 to 0.10 and 0.50 mg/mL, respectively. It indicates that the SPS can inhibit the growth of both COM and COD crystals.

The morphology of the COM crystals was also affected by the concentration of SPS. In the control experiment, most of the COM crystals are three-dimensional crystals, and about 20% of the crystals are aggregates. However, a few aggregates were observed in the presence of SPS. It indicated that SPS prevents the aggregation of COM crystals.

Figure 2 showed the XRD patterns of CaOxa crystals. In control test, the XRD pattern only shows the diffraction peaks at 0.593, 0.365, and 0.298 nm, which can be assigned to the $(\bar{1}01)$, (020), and $(20\bar{2})$ planes of COM.[2,11] However, in the presence of SPS, new diffraction peaks at 0.618, 0.442, 0.278, and 0.224 nm appear. The new diffraction peaks suggest a preferential alignment of the (200), (211), (411), and (213) crystal planes of COD. That is, in a low concentration of less than 0.03 mg/mL, SPS mainly inhibits the growth of COM crystals. In a high concentration range of more than 0.10 mg/mL, SPS mainly induce the formation of COD crystals.

FT-IR studies further supported the results obtained by XRD. From standard spectra of COM and COD, the main antisymmetric carbonyl stretching band ($\nu_{as}(COO^-)$) specific to the oxalate family occurs at 1618 cm^{-1} for COM, and at 1648 cm^{-1} for COD. The secondary carbonyl stretching bands, the metal–carboxylate stretch, $\nu_s(COO^-)$ is located at 1317 cm^{-1} for COM, and is shifted to 1329 cm^{-1} for COD. That is, the shifts from 1317 to 1329 cm^{-1} and from 1618 to 1648 cm^{-1} depended on the proportion of the two components in the mixture.[12]

When 0.03 mg/mL SPS was added, $\nu_s(COO^-)$ and $\nu_{as}(COO^-)$ was located at about 1321 and 1622 cm^{-1}, respectively. It indicated that COM was the dominant phase. However, when 0.50 mg/mL SPS was added, $\nu_s(COO^-)$ is located at 1325 cm^{-1}. Considering the fact that COM provides two or three times more intense vibrations than COD,[12] this indicates that COD is the dominant phase. The peaks in the finger print region at about 916 and about 616 cm^{-1} supported this conclusion, since this specific absorption bands for COM crystals located at about 948 cm^{-1} and about 668 cm^{-1}, respectively.[12]

The dramatic changes in morphology and phase composition of CaOxa crystals were due to the template effect of the SPS to CaOxa nucleus formation as well as the strong electrostatic interactions between the Ca^{2+}-rich $(\bar{1}01)$ crystal faces of COM and the polyanionic SPS. SPS is a linear polysaccharide polyanion. Each repeating disaccharide unit has a negatively charged sulfate group ($-OSO_3^-$). Ca^{2+} ions can generate the sulfate salt of SPS and interact with other polar groups in SPS. The complexation between SPS and Ca^{2+} ions not only decreases the supersaturation of CaOxa, but also closes the active sites for crystal growth. The former makes the size of the COM crystals grown in the presence of SPS be obviously smaller than that in control experiment, the latter further enhances the inhibitory activity of SPS to COM and thus induce more COD crystals.

Since COM was found to adsorb ten to fifteen times more than COD, i.e., COM crystals might adhere most strongly to the tubule cell surface in kidney sections.[13] In the animal models of urinary stones, COM was the principal crystalline constituent adhered to the tubule cell surfaces in kidney sections.[11] Theoretical calculations suggest that COM may have a stronger affinity to the cell membranes than COD.[14] So if any additive can induce more percentages of COD in crystallization of CaOxa, this reagent should be developed as a urolith inhibitor.

In conclusion, since SPS can decrease the size of CaOxa crystals, inhibit the aggregation of COM crystal, and induce the formation of COD crystals, so SPS can decrease the danger of stone forming. This experiment indicated that SPS maybe a potential inhibitor to CaOxa urinary stones. Further experiment is in progress.

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