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SCANNING ELECTRON MICROSCOPIC STUDY OF EFFECT OF VARIOUS AGENTS ON URINARY CRYSTAL MORPHOLOGY

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Abstract

Crystals seen in human urinary stones namely whewellite, weddellite, brushite, octocalcium phosphate, apatite, struvite and newberyite were grown in vitro in silica gel medium. The crystal growth medium was reproduced with addition of known inhibitors of crystallisation namely tartaric acid and citric acid and urine samples of stone patients and normal controls. The size and shape of the crystals were studied in the original setup and on addition of various agents as observed under the scanning electron microscope (SEM). The SEM appearance of crystals was uniformly reproducible. Addition of known inhibitors produced alteration of crystal habit and stunting of growth. Urine of stone patients produced changes in crystal appearance. Normal urine samples produced reduction in size of crystals and altered shapes. It is surmised from the observations that normal urine contains inhibitors of crystallisation and these are absent in the urine of certain stone formers.

Key Words: Urolithiasis, crystals, silica gel, inhibitors, tartaric acid, citric acid, crystal growth, scanning electron microscopy of crystals.

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Introduction

Urinary stones have been noted to contain various minerals in their crystalline forms namely whewellite (calcium oxalate monohydrate), weddellite (calcium oxalate dihydrate), brushite (calcium hydrogen phosphate dihydrate), octocalcium phosphate (calcium hydrogen phosphate pentahydrate), apatite (calcium magnesium phosphate), struvite (magnesium ammonium phosphate hexahydrate) and newberyite (magnesium hydrogen phosphate pentahydrate).(5). Silica gel media have been used modifying the conventional gel method for growth of inorganic crystals in vitro.(7). Such a modified method has been utilised in the present study for growing the crystals which constitute urinary stones. The scanning electron microscope (SEM) has been utilised successfully for qualitative estimation of crystal pattern of urinary stones.(4,6,12). Such identification of crystals has been confirmed using elmental X-ray microanalysis. (9). Though several studies have implicated inhibitors of crystallisation in normal human urine to protect against urinary stone formation, a clear picture of their role has not yet emerged. The present study has been undertaken to identify the effect of inhibitors on crystal morphology and assess the difference between the actions of urine of stone patients and normal controls on the crystals. The scanning electron microscopic appearances of the crystals grown in vitro and the effects produced by the addition of inhibitors or urine samples are presented.

Materials and Methods

U tubes of 1.7 cm. diameter and 16.5 cm. height were used for growing whewellite and weddellite crystals. Hane's tubes were used for growing brushite, octocalcium phosphate, apatite, struvite and newberyite crystals. Once gellation of the first nucleating substance occurred, the second nucleating solution was poured on top of the gel. The first and second nucleating substances varied with the type of crystal grown. Where needed, the test agent or urine sample was added to the top solution.

Procedure for growing different crystals.

Whewellite and weddellite. In U tubes, 1.03

density sodium metasilicate was taken and pH adjusted to 6 with 3 M acetic acid and allowed to set. After gellation, 1 M oxalic acid was poured into the left limb and 1 M calcium chloride into the right. This was set aside for three to four weeks for growing whewellite. For growing whewellite and weddellite simultaneously, after gellation of sodium metasilicate, 1 M oxalic acid was poured into the left limb and equal quantities of 1 M calcium chloride and 1 M magnesium acetate poured into the right.

Brushite, octocalcium phosphate and apatite. Sodium metasilicate gel was prepared as above in Hane's tubes, adding 0.3 M phosphoric acid, pH adjusted to 5 and allowed to set. After gellation, 1 M calcium chloride was layered on top and set for two weeks. For growing brushite alone, 3 M phosphoric acid was incorporated in the gel and 0.5 M calcium acetate added on top at pH 6.

Struvite. After preparing sodium metasilicate as above in Hane's tubes, pH was adjusted to 7, 0.5 M ammonium dihydrogen phosphate incorporated and allowed to set. After gellation, 1 M magnesium acetate was layered over and left for one week.

Newberyite. Sodium metasilicate was prepared, pH adjusted to 7, 0.5 M magnesium acetate incorporated and allowed to set. After gellation, 1 M ammonium dihydrogen phosphate was poured over and set for three weeks.

All experiments were carried out at room temperature. At the end of the experiments, the contents of the tubes were washed in distilled water to isolate the crystals.

To the silica gel crystal growth set up, 1cc each of 0.125 M tartaric acid, 0.5 M citric acid, urine of stone patients, urine of age and sex matched controls and distilled water was added to the growth media.

The crystals grown in the different situations were isolated, dried and taken up for SEM study. Crystals larger than 1 cm were trimmed to fit the size of the brass studs. After fixing with silver paste, the surfaces were made conductive by sputtering with gold to 100 Å thickness and studied under a JEOL JSM 35 C scanning electron microscope. Relevant fields were photographed.

Results

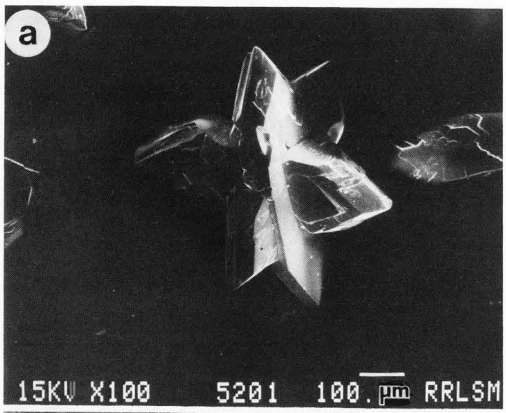
The crystals grown in vitro had characteristic appearances which were reproducible. Fig. 1 shows the scanning electron microscopic appearances of the various crystals grown. Fig. 1a is the picture of whewellite (COM) crystal grown in silica gel medium showing multifaceted crystal growth presenting the prismatic habit of the COM crystals. They did not conglomerate to form large crystals. Fig. 1b is the picture of a weddellite crystal with dipyramidal shape. The surface appeared homogeneous on small magnifications. Fig. 1c shows the brushite crystal with layers of plate like crystal growth. Fig. 1d shows the spherical octocalcium phosphate having a smooth surface and fine markings. Fig 1e shows apatite crystals grown in clusters with dense aggregation. Fig 1f shows the coffin lid shaped struvite crystals tending to get adherent to each other. Fig. 1g is the appearance of the newberyite crystal showing the parchment like appearance on a level surface.

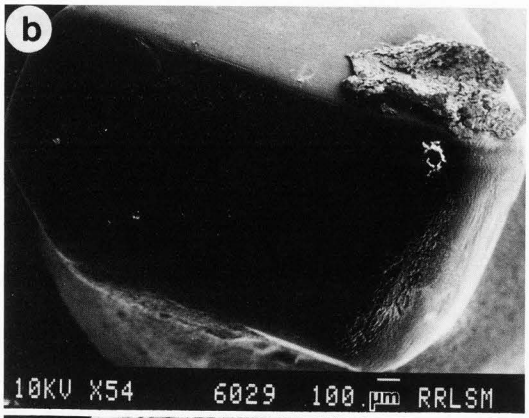
Figs. 2a and 2b are pictures of whewellite formed after addition of tartaric acid and citric acid respectively in the medium compared to Fig. 1a showing whewellite formed without adding the agents. It is seen that tartaric acid produced a reduction in size of the COM crystals and the crystal edges were blunted. Citric acid produced significant distortion of crystal architecture. Figs. 3a and 3b show weddellite crystals grown after addition of tartaric acid and citric acid respectively in the growth medium. Compared to the control (Fig. 1b), addition of tartaric acid produced a similar shaped fragile crystal, the surface of which cracked and the interior of the crystals exhibited negative spaces with partition walls on which typical dipyramidal weddellite crystals were seen. Citric acid produced distorted crystals with irregular shape. Figs. 4a and 4b are pictures of brushite crystals grown in the gel media after addition of urine samples of stone patients and control subjects respectively. Both situations produced crystals entirely different from those grown originally. While the urine of stone patients produced aggregates of tall platelike columns of crystals, normal urine produced square shaped crystals without any aggregation. Figs. 5a and 5b show high magnifications of the surface of octocalcium phosphate crystals grown in media incorporating urine of stone patients and normal controls respectively. While the urine of patients produced crystals not very different from the original, the normal urine produced distinctly fibrous strands without any evidence of adhesions. The other crystals studied namely apatite, struvite and newberyite did not show much difference in habit, when patient's urine was added. Normal urine however produced distinct differences in certain situations. Fig. 6 shows the distinctly distorted characteristics of apatite crystal compared to the original (Fig. 1e), when normal urine was added. Fig. 7 depicts the totally irregular configuration of the struvite crystal on addition of normal urine (compare with Fig. 1f). Fig. 8 however shows that addition of normal urine did not produce any significant alteration in the crystal habit of newberyite compared to the original crystal seen in Fig. 1g.

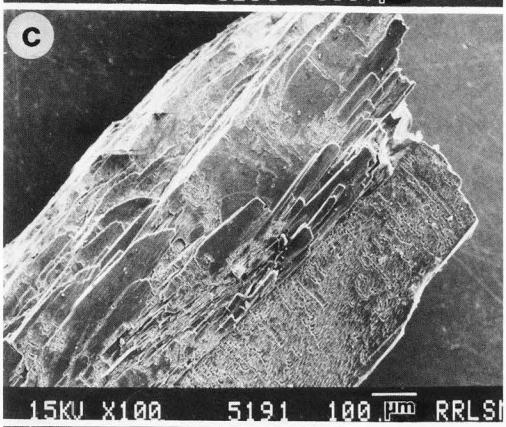
Discussion

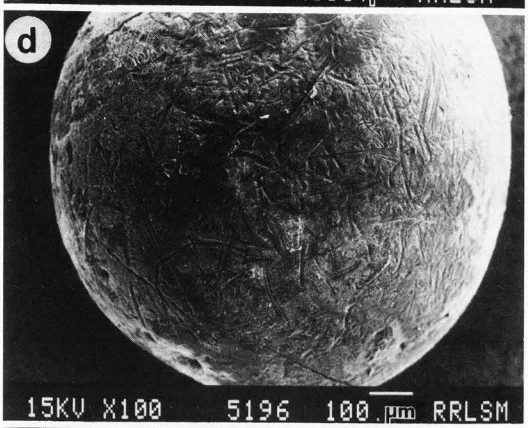
Urinary stones are seen to be composed of minerals and organic matrix. Whether the mineral structure or the organic matrix initiates the process of calculogenesis still remains a controversy. Studies to identify the physicochemical aspects of calculogenesis are confronted with the problems of development of realistic models to mimic human urinary crystal growth patterns. Khan et al. (8) have made calcium oxalate monohydrate crystals by mixing equal volumes of calcium chloride and potassium oxalate monohydrate solutions. Growing crystals in silica gel medium as shown in the present study has the possible advantage that the crystals grow and take their original shapes as they float in the gel.

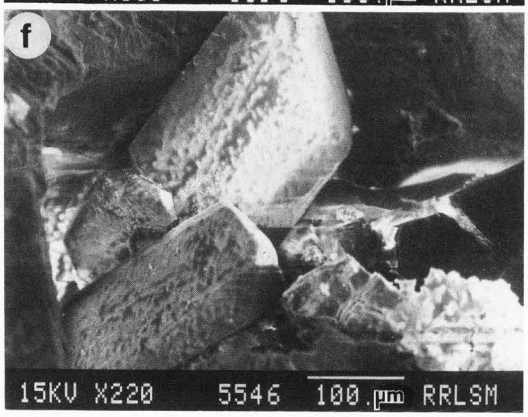
Finlayson et al. (2) suggest that adhesive











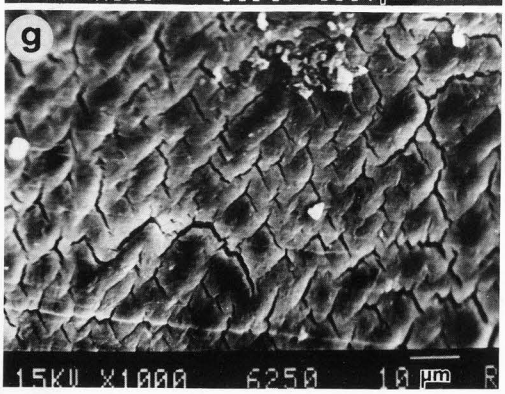


Fig. 1a. Whewellite crystal grown in silica gel.
Fig. 1b. Weddellite crystal grown showing dipyramidal habit.
Fig. 1c. Brushite crystal showing platelike habit.
Fig. 1d. Octocalcium phosphate showing spherical habit.
Fig. 1e. Apatite crystals showing clusters.
Fig. 1f. Struvite crystals showing coffin lid shape.
Fig. 1g. Newberyite crystal showing parchment appearance.

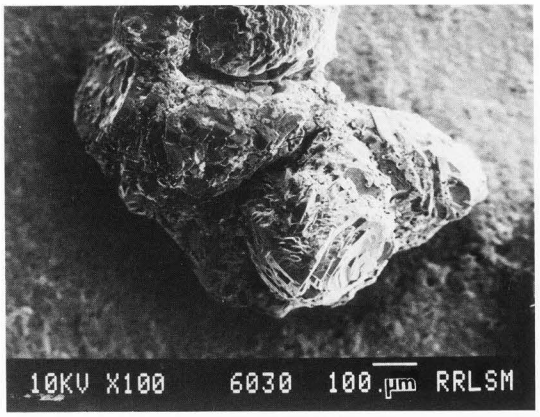


Fig. 2a. Whewellite crystal on adding tartaric acid showing stunting of crystal edges.

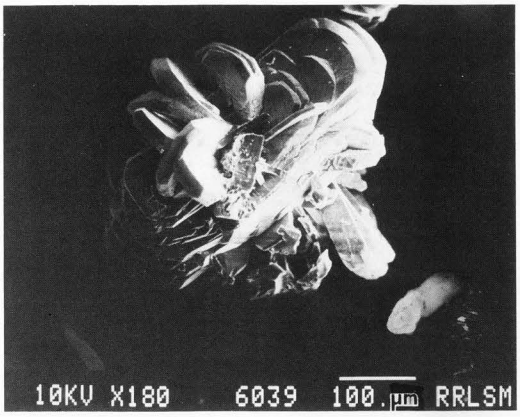


Fig. 2b. Whewellite crystal on addition of citric acid showing distortion of architecture.

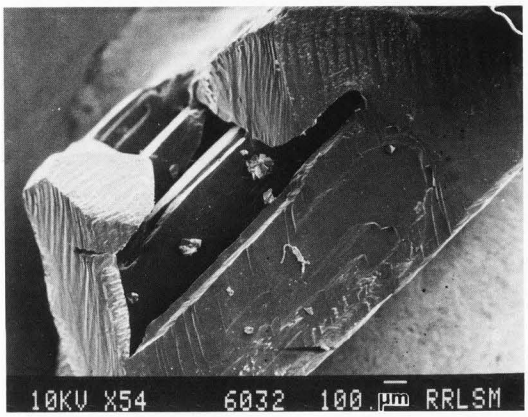


Fig. 3a. Weddellite on adding tartaric acid showing cracked crystal surface with negative spaces.

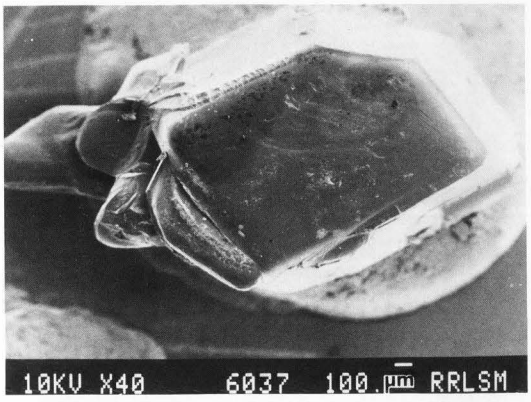


Fig. 3b. Weddellite on adding citric acid showing irregular shape of crystal.

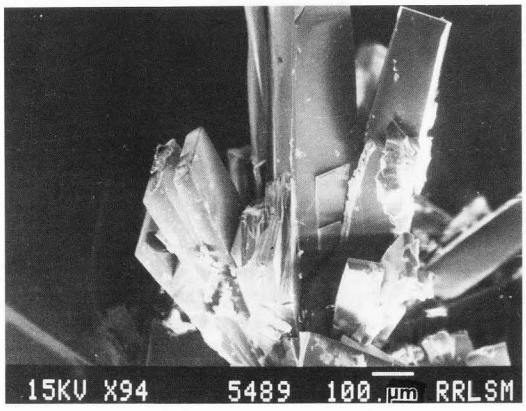


Fig. 4a. Brushite crystal on adding urine of stone patient showing platelike columns.

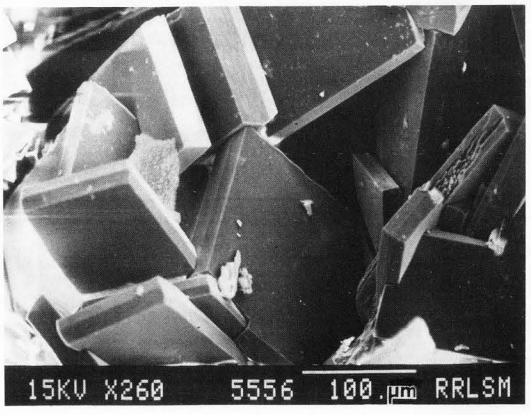


Fig. 4b. Brushite crystal on adding normal urine showing square shaped crystals without aggregation.

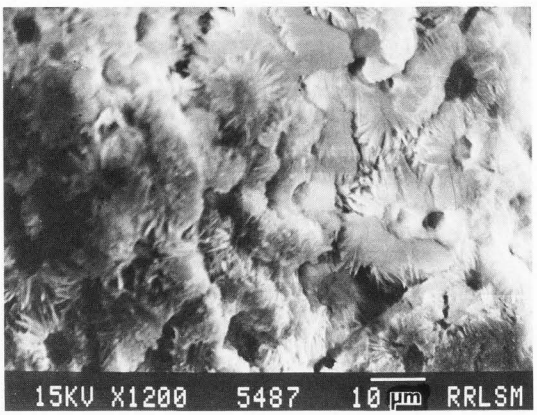


Fig. 5a. Octocalcium phosphate after adding urine of stone patient showing irregular pattern on surface.

Fig. 5b. Octocalcium phosphate after adding normal urine showing fibrous strands.

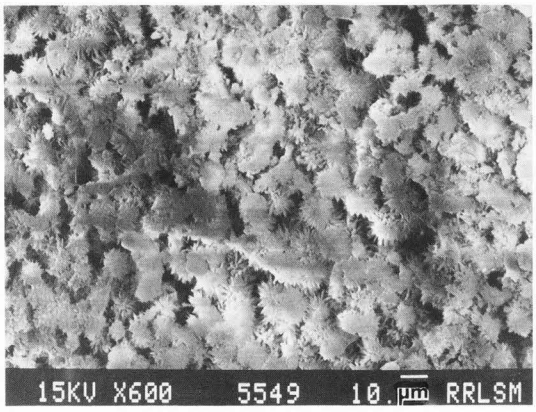


Fig. 6. Apatite crystal on addition of normal urine showing distorted pattern of crystals.

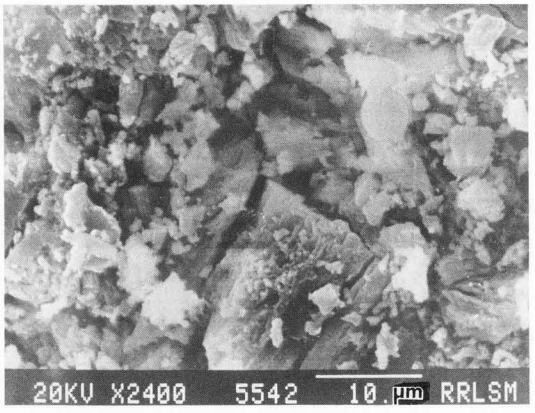


Fig. 7. Struvite crystals on adding normal urine showing totally irregular configuration.

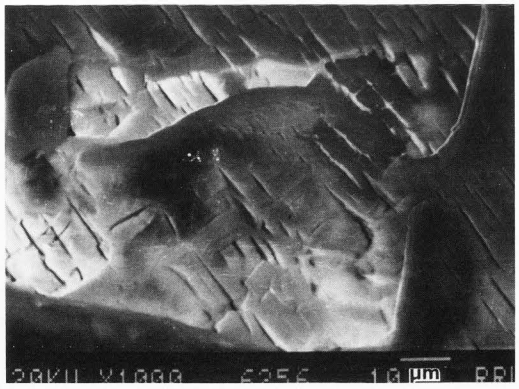


Fig. 8. Newberyite crystal on adding normal urine showing no significant change from original.

aggregation occurs in stone formation because of extreme insolubility of the stone matrix. Gregory et al. (3) have demonstrated that in rats fed diets supplemented with various crystal forming substances and adjustments of pH, different types of crystals were formed at the papillary tips. Pak et al. (13) have cited evidence of epitactic heterogeneous nucleation by one stone crystal on another. In their experiments, an increasing amount of uric acid added to the system facilitated the precipitation of supersaturated calcium oxalate solution. Several studies (1,8,9,11) have identified that matrix - crystal lattice relationships play an important role in shaping up the final crystal structure. Finlayson et al. (2) feel that there is a reduction in the zeta potential of the COM in urine compared to water probably due to the adsorption of negatively charged urinary constituents like citrate, pyrophosphates and acid polymers. Khan and Hackett (10) have done extensive work on the development of calculi in rats and have described the in vivo inhibitory effect

of pyridoxine, phosphate and magnesium and the promoter effect of uric acid. All these studies show that the process of nucleation and growth of various urinary crystals are influenced by agents promoting or inhibiting crystallisation.

In the present study, the purity of crystals grown was confirmed by Infra-red analysis. It was observed that tartaric acid and citric acid produced distinct distortion of crystal habit of whewellite and weddellite. Presence of human urine in the environment produced significant changes in the morphology of brushite crystals; the significance of the finding is however yet to be deciphered. Octocalcium phosphate, apatite and struvite crystals were significantly altered in their habits and appeared to lose their crystalline character in the presence of normal human urine in the growth environment. This change was evidently lacking in the presence of urine of stone patients. Newberyite crystals did not show any significant change in the presence of normal urine in the growth environment. This crystal is probably not inhibited or otherwise influenced by inhibitors in urine.

These observations make it possible to agree with the opinions of the authors quoted earlier that there is a significant effect of environment on the architecture of the growing crystals even in the absence of the matrix material. Inhibitors like tartaric acid and citric acid significantly altered the in vitro growth of crystals. Many previous studies have centred on calcium oxalate crystals. The present study proves that these inhibitors have a definite influence on the morphology of most crystals usually seen in the human urinary stone. The extent and type of influence, however, varied with the type of crystal.

Even though inhibitors are well described in human urine, their role has not been extensively studied. We have recognised definite inhibitory activity of crystal growth in the urine of normal individuals. This was definitely lacking in the urine of many stone formers. Some of the urinary inhibitors have been already identified, namely magnesium, citric acid, pyrophosphates and glycosaminoglycans, but probably, there are other hitherto unrecognised factors in normal human urine, which prevent crystal growth and aggregation and protect the normal individual from stone deposition in the urinary tract.

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Discussion with Reviewers

A. Hesse: Are the results reproducible?
Authors: The scanning electron microscopic appearances are highly reproducible under standard laboratory conditions.

A. Hesse: Is the spherical form of octocalcium phosphate also typical in urinary stones?
Authors: The habit of the crystals grown in vitro has been different from that seen in stones. The difference in shape of crystals in Figs. 1d and 5a is due to the difference in magnification of the two photographs. High power view of Fig. 1d is not reproduced to avoid repetition.

A.Hesse: Do characteristic crystal forms exist for newberyite on gel crystallisation?
Authors: Yes. The appearances are reproducible as can be shown in Figs. 1g and 8. It is noted however that the newberyite crystals were the most difficult to study by SEM, as the surface cracked very easily under the electron beams.

A. Hesse: Do these investigations permit the conclusion that tartaric acid and citric acid cause an aggregation to larger grouping of crystals of whewellite?
Authors: These investigations do not permit such a conclusion. The study of the different crystals grown in citric acid and tartaric acid show the inhibitory effect. Figs. 2a and 2b have been shown only to demonstrate the alteration of crystal habit.

A. Hesse: Is the change in crystal appearance dependent upon the urine of specific stone patients (type of stone)?
Authors: The material presented in this paper does not include details of urine of different types of stone patients.

Reviewer III: Some of the crystals are fairly large. Are they individual crystals or an agglomeration? Did the authors try to break into them?
Authors: In the crystal growth medium, the crystals grow to larger sizes than they appear in the urine or in the stones, but generally retaining their original habits. Though macroscopically, they appear to have the same habit, the SEM appearances indicate them to be agglomeration of crystals. We have tried to break them and look into them. The internal appearances confirm our view. The growth of large crystals is probably akin to the formation of the large stones in the urinary tract.