Scanning Electron Microscopy

Volume 1985
Number 2 *Part II*

Article 26

3-19-1985

Microstructural Matrix-Crystal Interactions in Calcium Oxalate Monohydrate Kidney Stones

J. Stacholy
University of Florida, Gainesville

E. P. Goldberg
University of Florida, Gainesville

SCANNING ELECTRON MICROSCOPY/1985/II (Pages 781-787)
SEM Inc., AMF O'Hare (Chicago), IL 60666 USA

0586-5581/85$1.00+.05

MICROSTRUCTURAL MATRIX-CRYSTAL INTERACTIONS IN CALCIUM OXALATE MONOHYDRATE KIDNEY STONES

J. Stacholy and E. P. Goldberg*

Department of Materials Science and Engineering
University of Florida, 217 MAE
Gainesville, Florida 32611

(Paper received January 26 1984, Completed manuscript received March 19 1985)

Abstract

The role of the proteinaceous matrix in the formation of calcium oxalate kidney stones is still not well understood. Simple scanning electron microscopy (SEM) has been of somewhat limited value in visualizing the organic and inorganic microstructure due to difficulties in obtaining detailed structural information for cut or fractured surfaces.

To help clarify matrix-crystal microstructure, serial sections from 10-20 mm calcium oxalate calculi were partially demineralized with ethylenediamine tetraacetic acid (EDTA) and examined by SEM. Sections etched by EDTA showed a radial crystal structure composed of "microcrystal" subunits. Sections simultaneously EDTA etched and fixed with glutaraldehyde to insolubilize all matrix mucoprotein showed interesting forms of matrix structure: an amorphous sometimes membrane-like material, and a fibrous material that exhibited an apparent affinity for the inorganic crystalline phase. These observations give evidence for a more important etiological and structural role for the matrix than may be suggested by the relatively low matrix concentration in stones (2-6 wt. %).

Key Words: Kidney stones, Matrix, Calcium Oxalate, Mucoprotein, Scanning Electron Microscopy, Biomineralization.

*For reprints and other information please contact E.P. Goldberg at above address.
Phone no.: (904) 392-4907

Introduction

Kidney stone etiology and the role of the proteinaceous matrix in kidney stone formation remain controversial questions. Physical and chemical relationships between the inorganic crystalline hydrated calcium oxalate phase and the organic matrix phase are still not clearly understood. Conventional scanning electron microscopy has been helpful but of somewhat limited value in visualizing microstructure and matrix-crystal interactions in kidney calculi.

The problems in structural characterization have been due in large measure to difficulties in the preparation of kidney stone samples in which individual crystals of the calculous framework are readily observed. Most previous work has involved freeze-fracturing calculi and examining the fracture and external surfaces [5]. Freeze-fracturing may produce distortion artifacts and does not permit control over fracture location. Use of a cutting instrument results in a surface with little information on crystal habit, even when cut with a diamond blade designed to provide a polished surface with minimal sample damage.

We have therefore investigated chemical etchant methods to provide an improved technique which circumvents some of these problems. Because chemical etching is diffusion controlled, crystals tend to dissolve evenly and preferentially along grain boundaries thereby affording good topographical relief with accentuated visualization of crystal habit. This paper summarizes our SEM work on calcium oxalate monohydrate stone surfaces which have been EDTA etched to afford some interesting observations on matrix-crystal microstructure.

Materials and Methods

Air-dried kidney calculi, characterized as primarily calcium oxalate monohydrate (COM), were obtained courtesy of L.C. Herring Laboratories, Orlando, Florida. Serial sections were cut from 10-20 mm stones on a Buehler Isomet low speed saw equipped with a diamond wafering blade. Methanol was used as a blade lubricant. The sections ranged from 0.64 to 1.53 mm thick. Twenty stones were used in this study and more than 100 have been examined in this laboratory during the past 4-5 years.

One section from each stone was processed for SEM without etching to serve as a control. To examine crystal microstructure, serial sections from the same stone were subjected to chemical etching in 0.25M EDTA for 16 and 36 hours at room temperature. The etching solution was adjusted to pH 7.0 with NaOH. Following the etching period, these samples were gently rinsed with a stream of deionized water to remove loosely adhering material. Adjacent sections from the same large stone were etched in 0.25M EDTA to which 4% glutaraldehyde was added to fix and completely insolubilize the matrix protein. These samples were etched for 24, 50 and 96 hours. Samples fixed in glutaraldehyde were rinsed in 2 washings of 0.01M sodium cacodylate buffer for 20 minutes. Following the rinse they were dehydrated in the same way as unfixed samples in a graded ethanol series (25, 50, 75, and 100%, 2x for 20 min. each). Sections were then critical point dried using CO_2 as the exchange solvent for 30 min. in a Tousimis Samdri PVT-3 critical point dryer. This was followed by mounting on aluminum stubs by gluing with high purity silver paint and sputter-coating with 200-600Å of gold/palladium in a Technics Hummer V. SEMs were obtained using a JEOL JSM-35C run at 25 kV. Polaroid PN55 4x5 black and white film was used for photos. Samples were examined optically on a Nikon Biophot Microphot V series microscope prior to processing for SEM.

Results and Discussion

Urolithiasis has been considered a significant medical problem since the days of Hippocrates [3]. The occurrence of this debilitating and painful disease is high as is hospital bed occupancy for treatment. In the Southeastern U.S.A. the incidence is so high that the term "stone belt" has been coined for this area [1].

Calculi form in response to a wide variety of associated diseases [2] but many cases are considered idiopathic. The majority of kidney stones have a composite structure of a mineral phase in association with 2-6 wt% (5-15 vol. %) of a mucoprotein termed the "matrix". The organic matrix phase has both soluble and insoluble constituents and has not been fully characterized. However, a number of papers have presented amino acid composition data and studies in this laboratory have demonstrated a radial concentration gradient for the matrix protein; the lowest concentrations being found at the stone center [8].

Since calcium oxalate stones are the most common type, they were chosen for this investigation. Calcium oxalate is the predominant crystal phase in 45% of kidney calculi and 15% of all stones are virtually pure calcium oxalate [4, 7]. Of the two hydrate forms, the monohydrate (COM) is more common than the dihydrate (COD). The stones are somewhat porous and usually show concentric laminations and radial striations in their interior structure. The fact that 2-6 wt% protein matrix occurs in most kidney stones (and other biological calcifications) suggests a role for the mucroprotein in stone formation and/or stone growth. Through a better understanding of stone microstructure, it should be possible to clarify the role of the matrix, help find a means to prevent such pathological calcifications, and perhaps also develop improved therapeutic methods for dissolution or disruption of kidney stones.

In previous studies, fractured kidney stones which we have examined by SEM have shown intercrystalline fibrous material bridging adjacent crystals (Figures 1, 2, 3). This has suggested that the matrix may have an important role in maintaining the structural integrity of calculi by acting as an intercrystalline binder. To further clarify the microstructure and matrix-crystal relationships, chemical etching (calcium oxalate dissolution with EDTA) was regarded as a method which would selectively attack the inorganic phase of sectioned stone surfaces to reveal matrix and crystal features not easily seen in the more dense stones.

Serial sections cut from each large stone with a diamond saw were used to provide some control over stone sample variations. Comparison of an unetched surface (Figure 4) with a section etched for 36 hours in EDTA (Figure 5) demonstrated the great enhancement of structure visualized using the chemical etchant. Figure 6 shows improved structural detail of a spherulitic structure in the core of a stone etched 50 hours.

The sample in Figure 7 was etched 36 hours. This typical stone shows an unorganized core structure (arrow) which becomes highly organized about 100μm from the center with regular radial and concentric striations. Close examination (Figures 8, 9, 10) revealed these radial crystals to be composed of individual substructures, possibly held together by matrix material. These individual substructures and the concentric laminar morphology suggest time dependent variations in the urinary environment of the stone with progressive stone growth, with the likelihood of intermittent rather than continuous crystal deposition. The precise nature of these substructures has not been determined. The structure may be a sandwich-like arrangement of alternating layers of matrix and crystal. Another possibility is that the etched platelike appearance is due to preferential etching along grain boundaries between aggregated microscrystalline material that in turn makes up the larger radial crystalline structure.

Glutaraldehyde was added to the etchant in one group of samples to better fix all the matrix in the stone structure. Some portion of the mucoprotein matrix is somewhat soluble and therefore lost during conventional EDTA demineralization, a point which has been noted in earlier studies (e.g. by Lian et al. [6] and in this laboratory [8]) but not adequately considered in the kidney stone literature. Under optical microscopy, the matrix appeared as a thin brown gel on cut surfaces and peeled off in membranes from external surfaces. After dehydration, the matrix exhibited several morphological types. For example, the glutaraldehyde fixed sample shown in Figures 11, 12, and 13 was etched

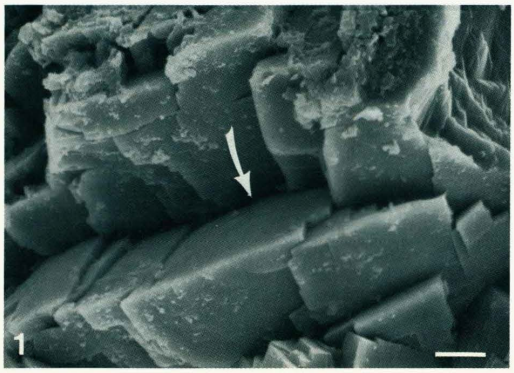

Fig. 1 Fracture surface. Arrow shows site of intercrystalline material enlarged in Fig. 2. Bar = 10 μm.

Fig. 2 Intercrystalline material indicated by arrow. Bar = 1.0 μm.

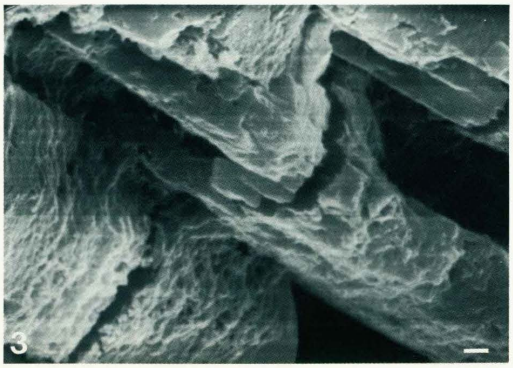

Fig. 3 Fracture sample (dihydrate) showing intercrystalline amorphous material. Bar = 1.0 μm.

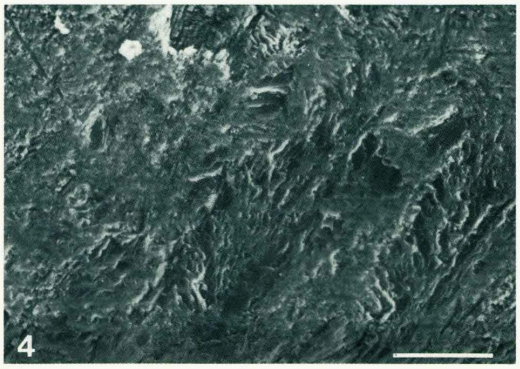

Fig. 4 Surface resulting from wafering with a diamond blade; affords little information. Bar = 100 μm.

Fig. 5 Increased structural definition resulting from 36 hrs. EDTA etch of wafered sample. Bar = 100 μm.

Fig. 6 Enhanced detail of a spherulitic structure in a wafer etched 50 hrs. Bar = 10 μm.

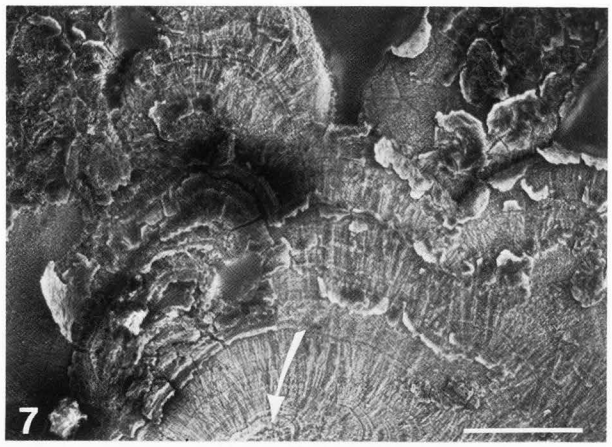

Fig. 7 Overall view of wafer etched 36 hrs. Arrow at center of stone. Bar = 1000 μm.

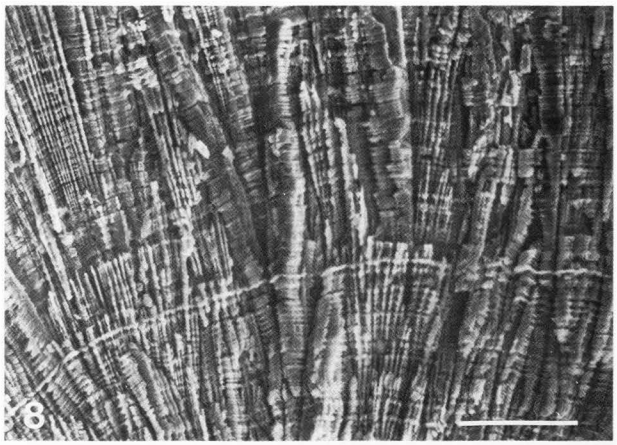

Fig. 8 Radial crystal structure seen in 36 hr. etched wafer. Bar = 100 μm.

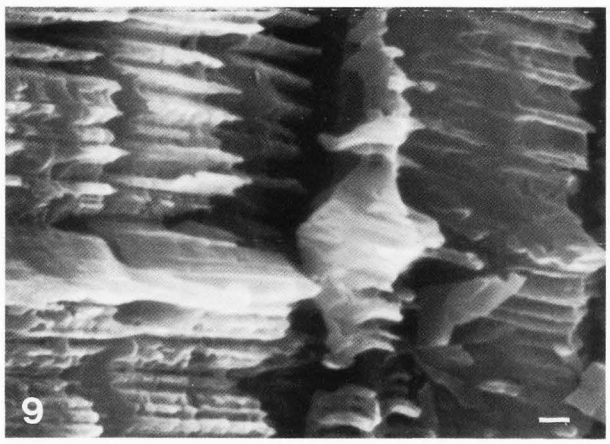

Fig. 9 Detail of radial crystal substructure. Bar = 1.0 μm.

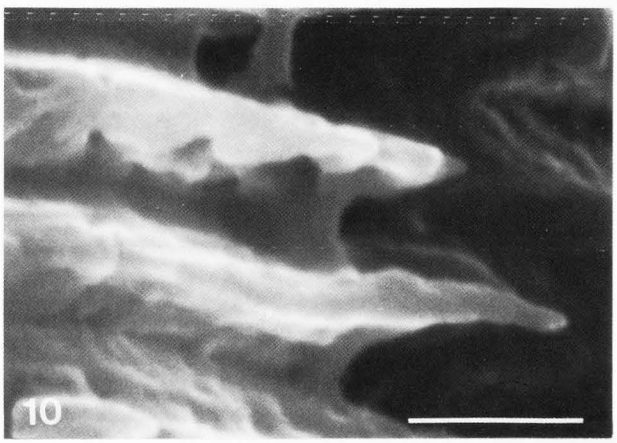

Fig. 10 Increased magnification of radial crystal substructure shows material bridging subunits. Bar = 1.0 μm.

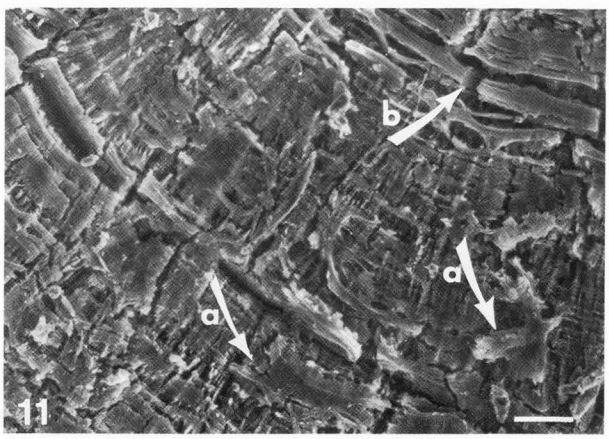

Fig. 11 Results from simultaneous etch/fixation 24 hrs. Arrows to amorphous (a) and membranous (b) forms of matrix. Bar = 100 μm.

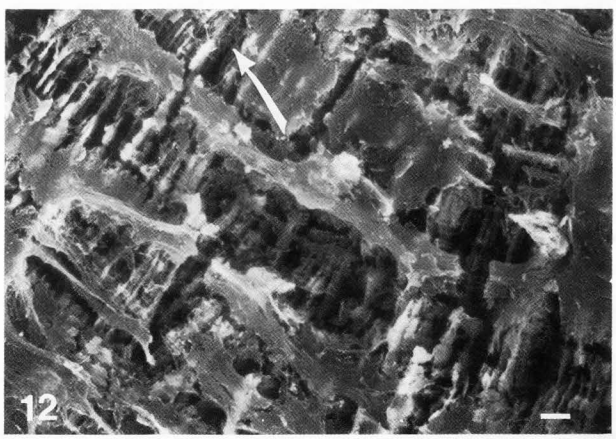

Fig. 12 Simultaneous etch/fixation 24 hrs. shows fibrous matrix (arrow) adhering to crystals. Bar = 10 μm.

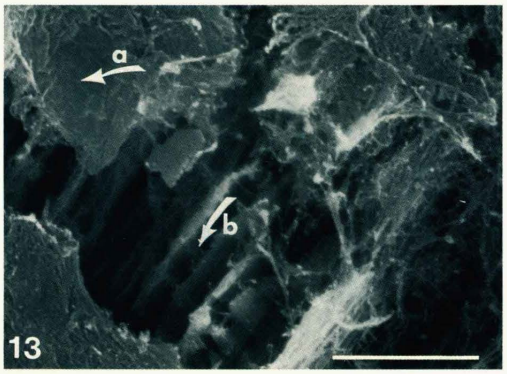

Fig. 13 Simultaneous etch/fixation 24 hrs. Showing amorphous (a) and fibrous (b) matrix. Bar = 10 μm.

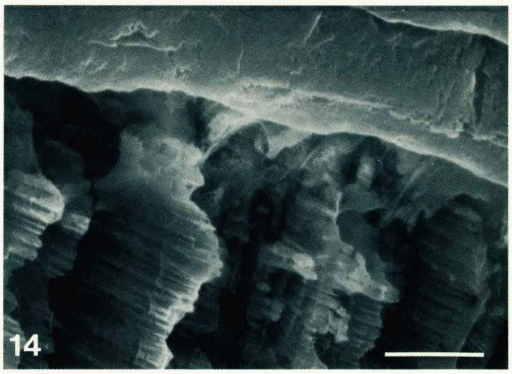

Fig. 14 Wafer etched and fixed 50 hr. has rigid crystalline appearance. Bar = 10 μm.

Fig. 15 Wafer etched and fixed 50 hrs. Bar = 1 μm.

Fig. 16 Detail of 50 hrs. etch/fixation sample. Bar = 1.0 μm.

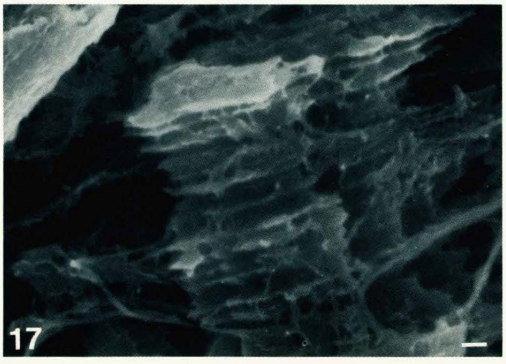

Fig. 17 Etch/fixation at 96 hrs. showing fibrous appearing substructure. Bar = 1.0 μm.

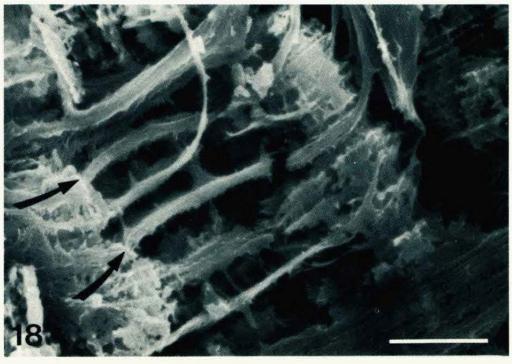

Fig. 18 Etch/fixation at 96 hrs. showing stacked membrane substructure. Bar = 10 μm.

24 hours. An easily peeled, solid, apparently amorphous material covered most of the surface (Figures 11-a, and 13-a). A fibrous matrix material was also found closely associated with the crystals (Figures 12, 13-b). The matrix phase was also seen to take the form of a membrane-like structure corresponding to concentric laminations running through the bulk of the stone (Figure 11-b).

As noted above, the composition of the oriented crystalline substructures of the etched radial crystals has not been determined. In the 50 hour etched and fixed sample (Figures 14, 15 and 16) these substructures are distinct and the protruding ridges appear crystalline. However, in the samples etched and fixed 96 hours, the ridges begin to show a more fibrous structure (Figures 17, 18) suggestive of a predominantly matrix composition. The sample etched 96 hours (Figure 18) also shows what appears to be stacked membranes but the spacing appears to be much greater (5-10 microns) than spacing of the crystal-like substructures (1 micron).

Conclusions

Scanning electron microscopy of fractured calcium oxalate calculi showed fibrous material bridging adjacent crystals. To enhance visualization of microstructure, we utilized a chemical etchant method to provide better topographical relief and accentuate crystal habit. Sections cut and etched in EDTA exhibited radial crystals composed of individual subunits.

Stone sections were also etched and simultaneously fixed with glutaraldehyde to insolubilize all matrix proteins. Several matrix morphologies were apparent. An amorphous-appearing material covered much of the cut surface. A membrane-like form was seen running through the bulk of the stone. There was also a fibrous form of matrix found in close association with the crystals. The fibrous matrix exhibits an apparent affinity for the crystallites. The overall impression is that the organic mucoprotein matrix may play a more important etiological and structural role than suggested by the relatively low concentration in kidney stones (2-6 wt. %; 5-15 vol. %).

References

1. Boyce WH, Garvey FK, Strawcutter HE. (1956). Incidence of Urinary Calculi Among Patients in General Hospitals, 1948 to 1952, J.A.M.A. 161, 1437-1442.

2. Garvey FK, Boyce WH. (1956). Diagnostic and Therapeutic Problems Incident to the Surgical Treatment of "Malignant" Renal Calculous Disease. J. Int. Coll. Surg. 25, 310-326.

3. Hagler L, Herman RH. (1973). Oxalate Metabolism, Am. J. Clin. Nutr. 26, 758-765.

4. Herring LC. (1962). Observations on the Analysis of Ten Thousand Urinary Calculi, J. Urol. 88, 545-562.

5. Kookmin KM. (1983). Diagnostic Electron Microscopy of Urinary Stones, in: Diagnostic Electron Microscopy, B.F. Trump and R.T. Jones (eds.), John Wiley and Sons, N.Y., 227-247.

6. Lian JB, Prien L, Jr, Glimcher JJ, Gallop PM. (1977). The Presence of Protein-Bound γ-Carboxyglutamic Acid in Calcium-Containing Renal Calculi, J. Clin. Invest. 59, 1151-1157.

7. Nordin BEC, Hodkinson A. (1967). Urolithiasis, Adv. Int. Med. 13, 155-182.

8. Warpehoski MA, Buscemi PJ, Osborn DC, Finlayson B, Goldberg EP. (1981). Distribution of Organic Matrix in Calcium Oxalate Renal Calculi, Calc. Tiss. Intn. 33, 211-222.

Discussion with Reviewers

P. Malet: Can the authors expound on the etching process? Are only calcium salts being removed or is some protein (perhaps bound to calcium salts) also being removed?

Authors: EDTA leaches calcium by chelation resulting in dissolution of calcium oxalate. However, there is evidence [6] that some matrix protein is also soluble in the EDTA solution. Thus, virtually all EDTA stone treatments reported in the literature have neglected consideration of solubilized protein and somewhat naively assumed that the "matrix" is the unextracted insoluble mucoprotein remaining after EDTA treatment. Our objective in crosslinking the total matrix with glutaraldehyde prior to EDTA treatment was in part to insure retention of all of the matrix in the final etched structure.

P. Malet: What direct proof do the authors have that what they are terming matrix material in the glutaraldehyde-fixed samples is really the protein matrix?

Authors: The term "matrix" is admittedly a rather loose term. However, since prolonged EDTA extraction is well known to substantially demineralize kidney stones by removing most of the calcium oxalate, and only cross-linked protein with perhaps some strongly associated or bound calcium ash can remain insoluble, the residual material, the matrix, must therefore be primarily composed of cross-linked protein. This view is supported by our own and other workers protein and ash analyses showing low inorganic content for the residual EDTA extracted material.

P. Malet: Have the authors performed x-ray microanalysis of the stones' surface before and after etching?
Authors: EDX has been done on representative samples, however not on the specific samples pictured in the text. After partial EDTA demineralization, results showed significant calcium content. However, extensively demineralized residual matrix was not analyzed by EDX.

P. Malet: Could the etching process be shearing away material in between crystal planes thus giving the appearance of crystalline subunits which are, in fact, an artifact of the etching process itself?
Authors: This seems unlikely. Etching (demineralization) should occur predominantly by diffusion controlled attack at crystal edges and boundaries.

K. M. Kim: One of the possibilities concerning the presence of the organic matrix between crystals in a urinary stone is its accidental trapping during stone formation. Please comment.
Authors: The possible adventitious presence of matrix (as an innocent bystander) has of course been a major question. From our studies and other literature, especially work by Leal and Finlayson (1977), it appears much more reasonable to regard the amount, composition and distribution of matrix in calcium oxalate stones as too great and too consistent to be due to simple "trapping".

S. Deganello: Why is the lowest concentration of matrix found close to the center of the stone?
Authors: Reasons for the protein concentration gradient are not well understood but it is certainly an important observation which must be explained to understand the role of the organic matrix. This phenomenon is discussed in our earlier paper [8]. One interesting possibility is that stone nucleation and growth stimulates increased protein production by kidney cells in response to the irritating condition. Such protein may adsorb to the growing stone in the lamellar morphology observed provoking further growth and irritation leading in a cyclic sequence of protein and mineral deposition.

S. Deganello: Have you been able to resolve any contact(s) which could indicate whether the matrix has any control over the oriented growth of the whewellite striations?
Authors: No, unfortunately it is not yet possible to cleary establish that crystal growth is organized by some specific interaction with the matrix substrate.

Additional Reference

9. Leal JJ, Finlayson B. (1977). Adsorption of Naturally Occurring Polymers onto Calcium Oxalate Crystal Surfaces, Investigative Urol., 14, 278-283.