Urol Res (2007) 35:1–14
DOI 10.1007/s00240-007-0078-4

ORIGINAL PAPER

The importance of a clean face: the effect of different washing procedures on the association of Tamm–Horsfall glycoprotein and other urinary proteins with calcium oxalate crystals

Rosemary Lyons Ryall · Phulwinder K. Grover · Lauren A. Thurgood · Magali C. Chauvet · David E. Fleming · Wilhelm van Bronswijk

Received: 3 August 2006 / Accepted: 8 January 2007 / Published online: 3 February 2007

Abstract This study was undertaken to determine whether the use of different washing procedures could explain dissident findings in published studies examining the role of urinary macromolecules in urolithiasis. Calcium oxalate monohydrate (COM) crystals were deposited from or added to the same sieved urine, washed with copious or limited amounts of distilled water, or with methanol, and examined by field emission scanning electron microscopy (FESEM). Demineralized extracts were analysed by SDS-PAGE and Western blotting for Tamm–Horsfall glycoprotein (THG), human serum albumin (HSA), osteopontin (OPN) and prothrombin fragment 1 (PTF1). Synchrotron X-ray diffraction (SXRD) with Rietveld whole-pattern peak fitting and profile analysis was used to determine non-uniform crystal strain and crystallite size in crystals generated from inorganic solutions in the presence of increasing concentrations of THG and prothrombin (PT). HSA and PTF1 were present in all demineralized crystal extracts, confirming their inclusion within COM. OPN was present in all extracts except those derived from pure inorganic COM crystals, because of its occlusion within small numbers of calcium oxalate dihydrate (COD) crystals contaminating the COM population. THG was absent from the demineralized extracts of all crystals washed copiously with water, but present in those washed with methanol or limited amounts of water. FESEM showed extraneous organic material associated only with crystals whose extracts contained THG, confirming that the protein does not bind permanently to the COM crystal surface and is not occluded within the mineral bulk. This was confirmed by SXRD, which showed that non-uniform strain and crystallite size remained unaltered in crystals grown in the presence of increasing THG concentrations. However, non-uniform strain increased and crystallite size decreased with increasing PT concentrations, demonstrating unambiguously that PT is included in COM crystals. It was concluded that scrupulous care must be taken to ensure the complete removal of extraneous THG adventitiously associated with CaOx crystals in order to avoid inaccurate analysis of crystal matrix protein content and possible misinterpretation of experimental data.

Keywords Tamm–Horsfall glycoprotein · Calcium oxalate · Urolithiasis · Intracrystalline protein · Osteopontin

R. L. Ryall (✉) · P. K. Grover · L. A. Thurgood · M. C. Chauvet
Department of Surgery, Flinders Medical Centre, Flinders University School of Medicine, Bedford Park, SA 5042, Australia
e-mail: rose.ryall@flinders.edu.au

D. E. Fleming · W. van Bronswijk
Department of Applied Chemistry, Curtin University of Technology, Perth, WA, Australia

Introduction

The presence of any protein in a renal stone implies that it has played some role in its formation—directly or indirectly, passively or actively. The detection of Tamm–Horsfall glycoprotein (THG) in stones [1], coupled with the fact that it is the most abundant protein in healthy human urine, has ensured that the protein has remained the subject of intense scientific and clinical

scrutiny for many years. Still, despite its distinction as the most extensively studied protein in stone research, the nature of the involvement of THG in stone formation remains as elusive as ever.

Recently, the potential role of intracrystalline proteins in stone genesis has prompted studies to determine whether THG is part of the organic matrix of CaOx crystals. The issue is not trivial: dissolution of calcium oxalate monohydrate (COM) crystals following their internalization by renal cells has been proposed to offer routine protection against stone pathogenesis [2–5]. The presence of proteins within crystals is known to alter their texture, as well as their chemical and physical properties, by stabilizing discontinuities in the lattice and thereby increasing their anisotropy [6]. We have proposed [7–11] that crystals containing proteins would be more vulnerable to attack by proteinases and thus more susceptible to intracellular dissolution than would crystals composed only of mineral. This hypothesis is supported by our demonstration that COM crystals precipitated from centrifuged and filtered urine are more rapidly degraded by MDCKII cells than inorganic crystals [11]. Whether or not THG is occluded within urinary crystals therefore has important implications for its putative role in stone formation.

Because THG was not detected in demineralized extracts of urinary CaOx crystals that had been thoroughly washed to remove any surface-bound proteins [12], we have previously concluded that it is not incorporated into the mineral bulk, and consequently, that it does not bind irreversibly to the CaOx crystal surface. However, those conclusions have since been called into doubt by several studies reporting that THG is present in demineralized extracts of CaOx crystals either formed in [13–17] or added to urine [18–21]. While all those studies present data that directly or indirectly question our contention that THG is not a component of CaOx crystal matrix, some also have ramifications for its role in crystal-cell attachment [18–20]. Others question our observation [9] that urinary macromolecules bind selectively and differently to COM and calcium oxalate dihydrate (COD) [21], or raise serious doubts about the significance of intracrystalline macromolecules in urolithiasis [21].

The most likely explanation for the discrepant data and conflicting interpretations is that different procedures were used in the later studies to process the urine samples and wash the crystals. Unless meticulous precautions are taken to remove any proteins that are not intracrystalline, but which may still be loosely associated with the crystal surfaces, they will remain in the demineralized extract and appear to originate from the crystal interior. The aim of this study was to determine whether differences in the procedures used to wash crystals prior to dissolution in EDTA can account for divergent findings in the published literature. Although the study was concerned principally with THG, analyses were also performed for certain proteins that we have previously reported to be occluded into CaOx crystals, including prothrombin (PT) [22], osteopontin (OPN) [9] and human serum albumin (HSA) [12].

Materials and methods

Chemicals

All reagents used were of analytical grade purity. Unless otherwise stated, biochemicals were supplied by BDH Chemicals Australia (Kilsyth, Victoria, Australia). Other reagents were obtained from the following sources: EDTA and methanol (Univar-grade, Ajax Chemicals, Auburn, NSW, Australia); sodium azide, sodium dodecyl sulphate (SDS), Tris, silver nitrate and dialysis tubing (Sigma Chemical Co., St. Louis, MO, USA); glycine (MERCK Pty Ltd, Kilsyth, Victoria, Australia); bromophenol blue (Merck, Darmstadt, Germany); glacial acetic acid (VWR International Ltd, Poole, England); COM (Fluka Chemie GmbH CH-9471, Buchs, Switzerland); Nunc tubes (Nalgene Nunc International, Rochester, NY, USA); Hybond-C Extra nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA); ammonium persulphate (May and Baker Ltd, Dagenham, England); 2-mercaptoethanol, 30% solution of acrylamide/bis-acrylamide solution and low range molecular weight markers (Bio-Rad Laboratories Inc., Hercules, CA, USA); *N*,*N*,*N*′,*N*′-tetramethyl-ethylene diamine (Acros Organics, NJ, USA); See Blue® pre-stained markers (Novex, San Diego, CA, USA); prefilters and 0.22 μm filters (Millipore Corporation, Bedford, MA, USA). All solutions were prepared with the highest quality water from a "Hi Pure" water purification system fitted with a 0.2 μm pore-size filter (Permutit Australia, Brookevale, NSW, Australia).

Antibodies

Unless otherwise stated, all immunological studies were performed with polyclonal IgG fractions of the corresponding antiserum. The antibodies were obtained from the following sources: rabbit anti-human THG (kindly donated by Professor John Hoyer); goat anti-rabbit IgG HRP conjugate

(#1706515, Bio-Rad Laboratories Inc., Hercules, CA, USA); rabbit anti-human Osteopontin (#AB 8448, Abcam, Cambridge, MA, USA), rabbit anti-human albumin (#126584, EMD Biosciences Inc., San Diego, CA, USA), our own monoclonal antibody (B19-1) to prothrombin fragment 1 (PTF1) [23]; goat anti-mouse IgG HRP conjugate (#1706516, Bio-Rad Laboratories Inc., Hercules, CA, USA).

Preparation of CaOx crystals for synchrotron X-ray diffraction

Tamm–Horsfall glycoprotein was purified from human urine as described previously [24]. PT was purified from Prothrombinex-HT (CSL, Victoria, Australia) as described previously [25]. PT was included as a control because it is an intracrystalline component of urinary CaOx crystals [22] and is easier to purify than PTF1, which is the predominant protein found in urinary COM crystals [9, 22]. COM crystals were generated by mixing 5 ml of 0.15 M calcium chloride and 0.15 M sodium oxalate (NaOx) at a rate of 0.4 ml per hour from glass syringes using an infusion pump (Sage Instruments, USA), into 30 ml of distilled water at 37°C. The solutions contained either no added protein (control), PT at final concentrations of 0.6, 4.3, 10.3 and 21.4 mg/l, or THG at final concentrations of 7.6, 21.3 and 34.0 mg/l. The mixture was gently agitated with an overhead stirrer fitted with a glass-stirring rod for 6 h. Precipitated crystals were separated by vacuum filtration, washed with a saturated solution of CaOx and dried under nitrogen.

Synchrotron X-ray diffraction

Synchrotron X-ray diffraction (SXRD) patterns were collected on BIGDIFF, a synchrotron diffractometer installed on Beamline 20B at the Australian National Beamline Facility (ANBF) at the Photon Factory Synchrotron-Radiation Facility within the National Laboratory for High Energy Physics (KEK), Tsukuba, Japan. Gaussian and Lorentzian contributions to X-ray peak profiles were obtained using a Voigt function [26, 27] and used to calculate non-uniform lattice strain, which describes misalignment of crystalline blocks, and average crystallite size (or coherence length) as described previously [10]. Full details of computational methods are reported in Fleming [28]. The X-ray data were subjected to Rietveld refinement using Rietica for Windows 95/98/NT Version 1.72 [29]. Crystals of COM grown slowly in gelatin at room temperature [30] were used as a "strain-free" reference.

Collection and processing of urine

The 24-h urine samples were collected from healthy laboratory colleagues (four women and two men) with no history of kidney stone disease. The samples were collected over sodium azide (final concentration 3 mM) and were refrigerated during the collection period and during storage before use. Absence of haematuria was confirmed by dipstick (Combur-8 Test®, Roche Diagnostics GmbH, Mannheim, Germany). The samples were pooled, sieved (70 μm) and an aliquot (~5 ml) was saved for calcium determination (Modular Analytics SWA, PPE autoanalyser; Roche Diagnostics GmbH, Germany). Another aliquot (~50 ml) of the pooled sample was extensively dialysed (Mr cut-off of 10 kDa) against distilled water at 4°C and lyophilized.

The pooled urine was divided into four portions for separate treatment as follows.

Method 1

Crystallization of CaOx was induced in two 125 ml aliquots by scaling down the method described by Maslamani et al. [16]. A solution of 1.9 ml of 0.1 M sodium oxalate was added dropwise to the urine samples in a shaking water bath and incubated for 3 h at 37°C, thus increasing the final oxalate concentration by 1.5 mMol/l. Crystals were harvested by centrifuging the samples at 10,000 $\times g$ for 25 min at 20°C and the supernatant fluid was removed by aspiration. Crystal washing was performed as follows:

1A One pellet was resuspended by vortex mixing in 2 ml of distilled water and centrifuged as above. These steps were repeated twice more as described by Maslamani et al. [16].

1B The other pellet was suspended in 12.5 ml distilled water, vigorously vortex-mixed and centrifuged at 2,000 $\times g$ for 5 min at 20°C. This washing cycle was repeated nine times. This method is used routinely in our laboratory for washing crystals.

Method 2

This method was based on that of Walton et al. [21], except that pre-formed COM crystals were incubated in real urine, rather than in artificial urine to which isolated urinary macromolecules had been added. The urine was warmed to 37°C and divided into two 250 ml aliquots, to each of which was added 50 mg of commercial COM crystals. The samples were incubated for 2 h at 37°C in a shaking water bath, centrifuged at 2,000

×*g* for 5 min, the supernatants removed by aspiration, and the pellets washed as follows:

2A One pellet was rinsed twice with methanol as described by Walton et al. [21].
2B The other pellet was washed as described in 1B.

Method 3

The metastable limit of the urine was measured and the sample divided into three 250 ml portions. CaOx formation was induced by dropwise addition of a standard NaOx load [31], which increased the final oxalate concentration by 1.5 mMol/l, and the samples incubated in a shaking water bath at 37°C for 3 h [12]. They were then centrifuged at 10,000 ×*g* for 25 min at 20°C, the supernatant fluids removed by aspiration and the pellets washed as follows:

3A The first pellet was washed according to the method of Maslamani et al. [16].
3B The second was washed with methanol as described by Walton et al. [21].
3C The third was washed as described in 1B.

Method 4

The fourth portion of the pooled urine was centrifuged at 10,000 ×*g* for 20 min at 20°C in a Beckman J2-21M/E centrifuge (Beckman Instruments, Fullerton, CA, USA) and filtered through a 0.22 µm filter (GVWP14250, Millipore Corporation, Billerica, MA, USA), and CaOx crystallization as described for Method 3. Crystals were collected and washed as outlined in 1B.

Field-emission scanning electron microscopy

A small sample of crystals from each method was collected by filtration (0.22 µm) at the end of the incubation period and after washing. The filtration membranes were dried overnight at 37°C, mounted on aluminium stubs and coated with platinum to 3 nm thickness using a high vacuum evaporator (DV-502, Denton Vacuum Inc.; Moorestown, NJ, USA). The stubs were examined using a Philips XL30 FEGSEM field-emission scanning electron microscope at 10 kV accelerating voltage and 10 mm working distance. At least ten fields of each sample were examined, and images of crystals that were representative of the majority were recorded. Approximately 90% of the crystals were COM and the remainder, COD.

Demineralization of crystals

After washing, identical weights of each crystal type were demineralized in identical volumes of 0.25 M EDTA (pH 8.0) and the resulting solutions desalted by extensive dialysis against distilled water at 4°C. Each sample was then lyophilized and stored at −20°C.

SDS-PAGE and Western blotting

The lyophilized samples were reconstituted in the same volume of reducing sample buffer and identical volumes analysed by SDS-PAGE and stained with silver as described previously [12]. Immunoblotting [12] used the following antibodies; for THG, 1:500 dilution of rabbit anti-human THG and 1:2,000 goat anti-rabbit IgG HRP conjugate; for OPN, 1:2,000 dilution of rabbit anti-human osteopontin; for HSA, 1:2,000 dilution of rabbit anti-human albumin; for PTF1, 3:1,000 dilution of B19-1 IgG and 1:250 goat anti-mouse IgG HRP conjugate.

Ethical considerations

This study was reviewed and approved by the Flinders Clinical Research Ethics Committee.

Results

SDS-PAGE analysis

Irrespective of the method, crystals precipitated from urine were principally COM with some COD crystals, as would be expected from the calcium concentration (2.7 mM) [9]. Figure 1 shows the SDS-PAGE profiles of the proteins in the pooled sieved urine (lane 2) and in the demineralized extracts of the CaOx crystals prepared and washed as described in Materials and methods. Lane 10 depicts bands in the extract of the crystals precipitated from the centrifuged and filtered urine sample. The sieved urine contains many bands migrating over a broad range of molecular weights and with widely varying staining intensities. In particular, three prominent bands corresponding to molecular weights of ~200, ~100 and ~65 kDa are visible. The identity of that at ~200 kDa is unknown, while the others are THG and HSA, respectively (see below). Fewer bands are seen in any of the demineralized extracts. Although the number and pattern of bands vary depending on the washing methods used, several features are consistent:

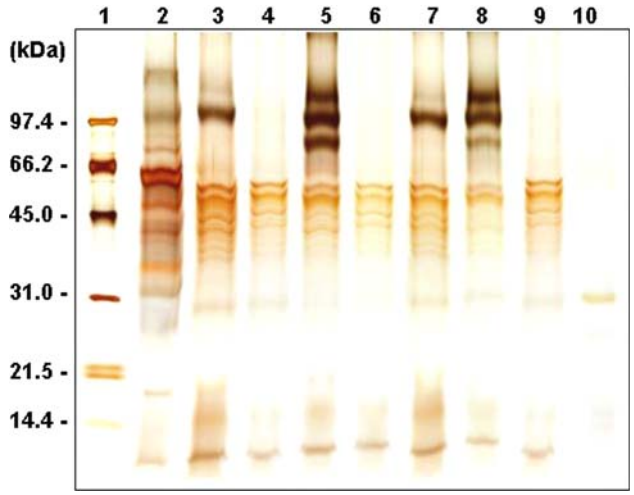

Fig. 1 SDS-PAGE analysis of proteins in sieved urine and in demineralized extracts of CaOx crystals precipitated from or incubated in a sample of the same urine, which were washed using different techniques as described in Materials and methods. *Lane 1* molecular weight markers; *lane 2* sieved urine; *lane 3* crystals prepared and washed according to Method 1A; *lane 4* crystals prepared and washed according to Method 1B; *lane 5* crystals prepared and washed according to Method 2A; *lane 6* crystals prepared and washed according to Method 2B; *lane 7* crystals prepared and washed according to Method 3A; *lane 8* crystals prepared and washed according to Method 3B; *lane 9* crystals prepared and washed according to Method 3C; *lane 10* crystals prepared and washed according to Method 4. See text for details of methods

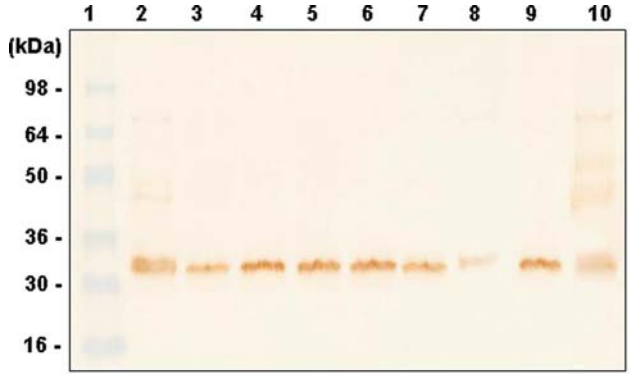

Fig. 2 Western blots, stained for PTF1, in sieved urine and in demineralized extracts of CaOx crystals precipitated from or incubated in a sample of the same urine. *Lane numbers* correspond to the same preparation and washing methods as in the legend to Fig. 1

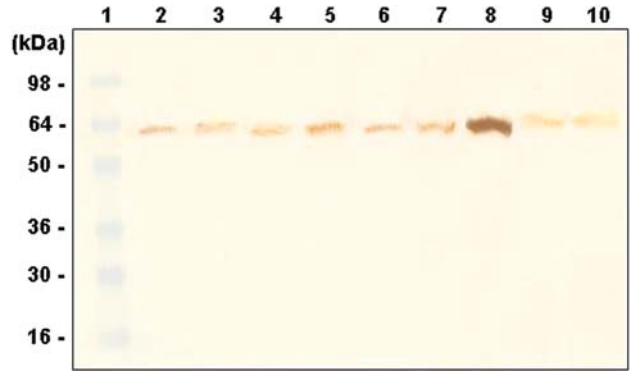

Fig. 3 Western blots, stained for HSA, in sieved urine and in demineralized extracts of CaOx crystals precipitated from or incubated in a sample of the same urine. *Lane numbers* correspond to the same preparation and washing methods as in the legend to Fig. 1

1. A zone of bands corresponding to Mr values of ~35 to 63 kDa, as well as a low Mr band running at the bottom of the gel, is clearly visible in all but lane 10. Because these are absent from the extract of the crystals formed in the centrifuged and filtered urine, they must represent proteins attached to or part of particulate material <70 μm in size, which was not removed by sieving. This was confirmed by electron microscopy (see below).
2. Several prominent bands between ~75 and 150 kDa are in lanes 3, 5, 7 and 8. Since these are completely absent from lanes 4, 6, 9 and 10, which derive from crystals that had been washed copiously with water, they must represent superficial proteins that were not removed by the washing procedures of Maslamani et al. [16] and Walton et al. [21].
3. A band at ~31 kDa is present in all lanes.

Western blotting

Western blotting (Fig. 2) confirmed the ~31 kDa protein to be PTF1. Figure 3 shows the Western blot for HSA. Although the staining intensity varies, the protein is present in all lanes. Western blotting for OPN (Fig. 4) shows a pattern typical of extracts of crystals precipitated from urine collected without preservative [9], extending as a continuous smear from ~50 kDa to greater than 100 kDa. As expected, staining is not present in lanes 5 and 6, since the crystals were COM and OPN is absent from demineralized extracts of COM crystals at low calcium concentrations [9]. Detection of OPN in all remaining extracts reflects the fact that COD crystals were present in all the crystal samples.

Western blotting for THG (Fig. 5) demonstrates a ~85 kDa band in the sieved urine (lane 2). This is less than that in the gel (Fig. 1), which is probably attributable to the different Mr markers used for the two techniques, since a monoclonal antibody that cross-reacts only with THG was used for detection. The same band is also visible in the crystals prepared and/or washed according to Maslamani et al. [16] (lanes 3

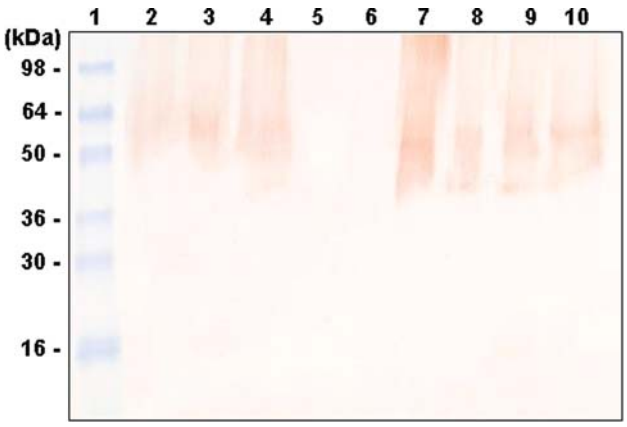

Fig. 4 Western blots, stained for OPN, in sieved urine and in demineralized extracts of CaOx crystals precipitated from or incubated in a sample of the same urine. *Lane numbers* correspond to the same preparation and washing methods as in the legend to Fig. 1

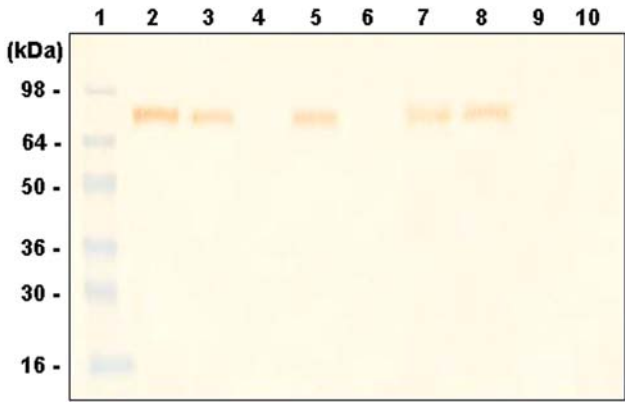

Fig. 5 Western blots, stained for THG, in sieved urine and in demineralized extracts of CaOx crystals precipitated from or incubated in a sample of the same urine. *Lane numbers* correspond to the same preparation and washing methods as in the legend to Fig. 1. Note that THG was absent from extracts of all crystals washed copiously with distilled water (lanes *4*, *6*, *9*) and from the centrifuged urine (lane *10*)

and 7) and Walton et al. [21] (lanes 5 and 8), but is completely absent from all extracts of crystals washed copiously with distilled water (lanes 4, 6 and 9) and from that derived from the centrifuged and filtered urine (lane 10).

FESEM

Crystals generated using the methods of Doyle et al. [12] and Maslamani et al. [16] were principally COM, although some COD crystals were also present. At low magnification large dark patches were seen scattered across the filtration membranes of all unwashed crystals, irrespective of the method used to generate or treat them. They were also visible on the filters of some of the washed crystals (see below). One of these patches is presented in Fig. 6a, which shows unwashed COM and COD crystals precipitated from the pooled sieved urine using Method 3 [12]. At slightly higher magnifications (Fig. 6b) the patches consist of sheets of mucous material. In other views (not shown) clearly evident were fibrous and amorphous cellular debris and membrane fragments, similar to those shown in Fig. 1 of Maslamani et al. [16], whose component insoluble proteins must account for the 35 to 63 kDa protein bands and single low Mr band in lanes 1–9 of Fig. 1, since they were completely removed by centrifugation and filtration (lane 10). Higher magnifications of organic material are presented in Fig. 6c, d, which depict, respectively, unwashed crystals prepared using Methods 1 [16] and 2 [21]. Not all crystals are associated with organic matter: some are covered with mucus, while others located close by are sharply defined because they are denuded of organic material. Large quantities of mucous material were often spread over areas of the filtration membrane where there were few or no crystals and numerous crystals were not associated with organic material.

Figure 7 shows images of CaOx crystals generated using the method of Maslamani et al. [16] before washing (Fig. 7a), and after washing using small amounts (Fig. 7b) and large amounts (Fig. 7c) of distilled water. Before washing, abundant quantities of organic material lie as a mantle over the crystals and in thick deposits between them. Although the amount is reduced after washing with small amounts of water (Fig. 7b), a thick layer of organic material is still clearly evident upon the upper surfaces of individual crystals and collected into drifts where they abut or intersect with others. It is also present on the filtration membrane. However, it is not associated with crystals or observed on the membrane surface following extensive washing with water (Fig. 7c).

Crystals prepared and washed using the method of Walton et al. [21] are shown in Fig. 8. Before washing (Fig. 8a), they are associated with large amounts of organic material (indicated by arrows) lying on and between them, as well as on the membrane. After washing with methanol (Fig. 8b), the organic material is still clearly evident (indicated by arrows), although much now appears “stretched” or filamentous, suggestive of drying and/or denaturation. Crystals washed copiously with distilled water (Fig. 8c) are not associated with superficial or interspersed organic material, which is also not on the membrane.

Figure 9 shows crystals precipitated using the method of Doyle et al. [12] before washing (Fig. 9a) and after washing using the method of Maslamani

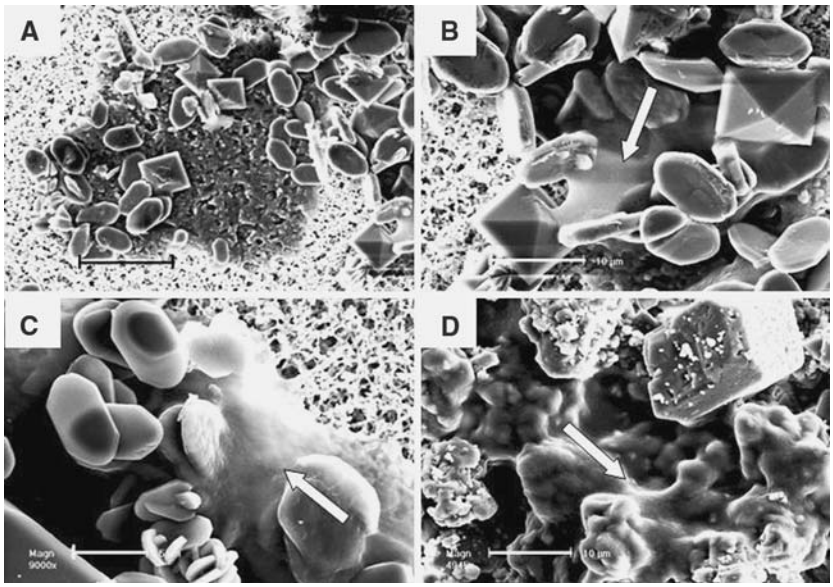

Fig. 6 FESEM images of unwashed CaOx crystals precipitated from sieved urine using Method 3 (**a**, **b**), Method 1 (**c**) and incubated in urine according to Method 3 (**d**). **a** shows that the organic material is present as discontinuous patches on the filtration membrane. Irrespective of the method used to prepare the crystals, organic material (*arrows*) is clearly visible upon and between some (but not all) crystals, which is consistent with its suspension in urine as particles that were not attached to crystals but simply coalesced and co-sedimented with them during centrifugation. *Scale bars* on images are as follows: **a** 20 μm; **b** 10 μm; **c** 5 μm; **d** 10 μm

et al. [16] (Fig. 9b), Walton et al. [21] (Fig. 9c) and copious amounts of distilled water (Fig. 9d). As seen above, crystals are principally COM, although a single COD crystal can be seen. Before washing, profuse quantities of organic material can be seen lying on top of and between adjacent individual crystals, some of which appear to be completely immersed. Although diminished after limited washing with water (Fig. 9b), large amounts are still present. Washing with methanol (Fig. 9c) caused contraction of continuous sheets of organic material into a mottled pattern of smaller, discontinuous or partly connected particles, again indicative of protein denaturation and/or dehydration. In agreement with Figs. 7c and 8c, Fig. 9d shows the complete absence of any organic material. Images in Figs. 7, 8, 9 are consistent with the Western blotting patterns (Fig. 5) and indicate that the organic material is principally polymerized THG [32].

SXRD

Figure 10a, b shows non-uniform strain and crystallite size plotted against the concentration of THG in the solutions from which the crystals were precipitated. Neither alters at THG concentrations from 0 to more than 30 mg/l, which spans the protein's mean concentration range of ~20–40 mg/l in healthy human urine [33]. Figure 10c, d show the same data for crystals precipitated from solutions containing PT at concentrations from 0 to ~20 mg/l. Non-uniform strain rises, and crystallite size decreases relative to the solution concentration of PT, but neither reaches saturation even at the highest concentration tested.

Discussion

It is widely accepted [34–36] that large protein molecules cannot insert into domains of perfect crystallinity. Nonetheless, they can and do occlude into the mineral bulk at the boundaries delineating ordered mosaic blocks [34, 35, 37]. In fact, foreign inorganic inclusions within crystalline structures are common in nature, the most spectacular examples being macroscopic needles of titanium oxide scattered throughout rutilated quartz. For at least 40 years [38], it has been known that organic macromolecules can also become trapped inside individual calcite crystals comprising a large number of natural biominerals, an observation that spearheaded the study of intracrystalline proteins in ancient fossils [39] and extant biominerals, the latter being exemplified by a series of elegant studies by Addadi, Weiner and their colleagues [6, 37, 40].

Though some urinary proteins, including HSA, have been reported to be present within CaOx crystals [12, 41], Doyle et al. [12] concluded that THG is not. Believing that Doyle et al. [12] had used centrifuged and filtered urine, Atmani et al. [13, 14] carried out

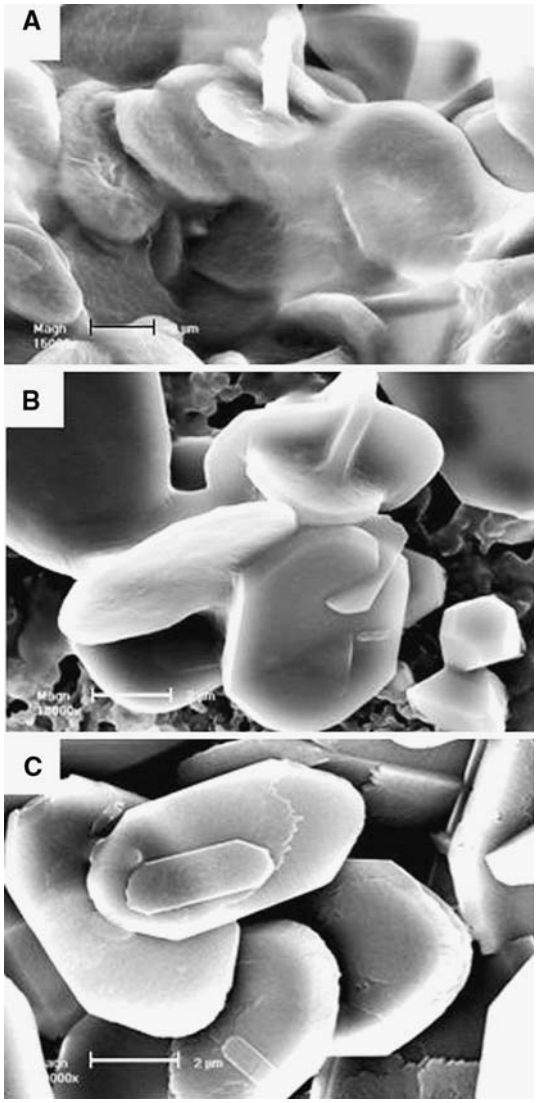

Fig. 7 FESEM images of crystals precipitated from sieved urine according to [16], before washing (**a**) and after rinsing with limited (**b**) or copious (**c**) quantities of distilled water. *Scale bars* on all images indicate 2 µm

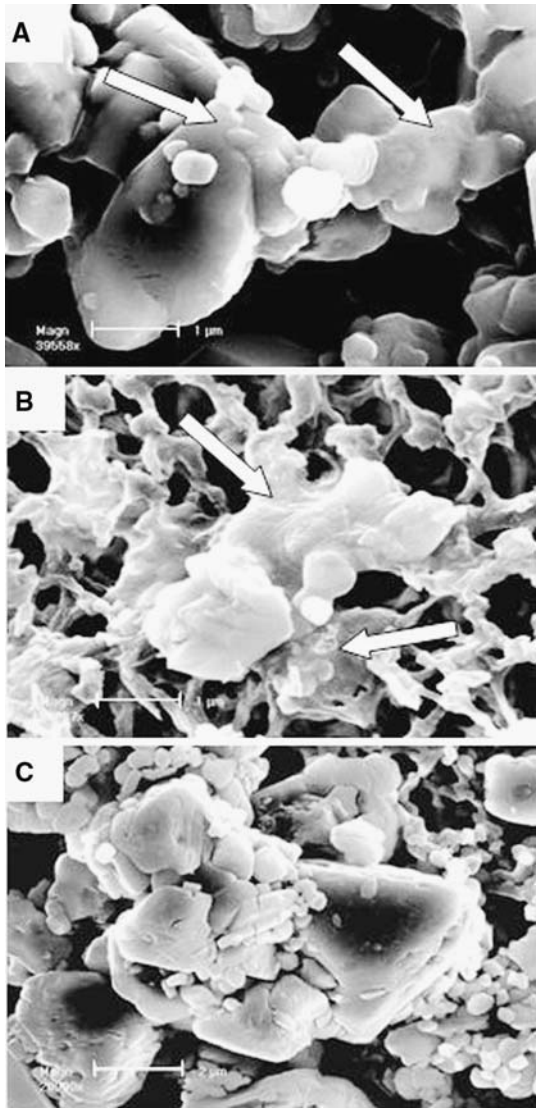

Fig. 8 FESEM images of commercial COM crystals incubated in sieved urine according to [21], before washing (**a**) and after rinsing with methanol (**b**), or with copious quantities of distilled water (**c**). The *scale bars* on images are as follows: **a** 2 µm; **b** 1 µm; **c** 2 µm

similar studies using whole human urine. Detecting THG, albumin and other proteins in extracts of all crystals that had not been washed with NaOH, they questioned the suggestion that protein adsorption is selective [12, 41]. More importantly, they regarded both proteins as major components of the crystal matrix, a notion that was reinforced in two further papers [15, 16]. Indeed, Maslamani et al. [16] stated that filtration and centrifugation of urine caused a "substantial reduction in THP inclusion" and that their results "clearly demonstrate(d) the incorporation of both THP and albumin in the crystal matrix", despite their observation that THG was associated only loosely with the crystals. The use of "inclusion" and "incorporation" is puzzling, given other reports from the same group suggesting that THG is not present within the crystals [42, 43]. In all their studies [13–16], the crystals were generated in urine and collected by centrifugation. Under those circumstances, *any* undissolved extraneous material present in the urine would inevitably have co-sedimented with the crystals, even if it had not actually been adsorbed to their surfaces, including cellular debris, THG and polymeric HSA [44]. Furthermore, the crystals were purposely not washed exhaustively with water or NaOH, which would have removed superficial proteins, in the belief that they should not be "discarded but need to be taken into consideration with other proteins which are included in the crystal interstices" [15]. Given the apparent contradictions in

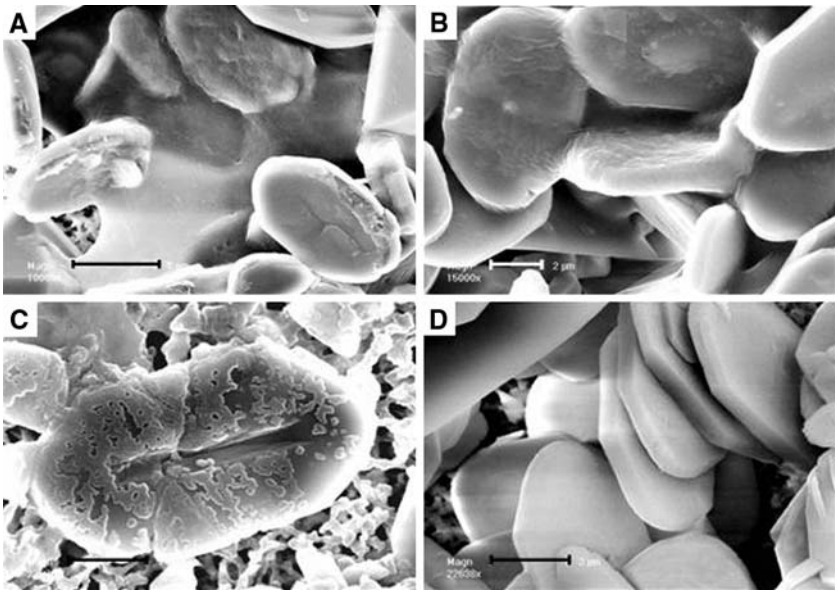

Fig. 9 FESEM images of crystals precipitated from sieved urine according to [12], before washing (**a**) and after rinsing with limited amounts of water (**b**), with methanol (**c**) or with copious quantities of distilled water (**d**). *Scale bars* on all images indicate 2 μm

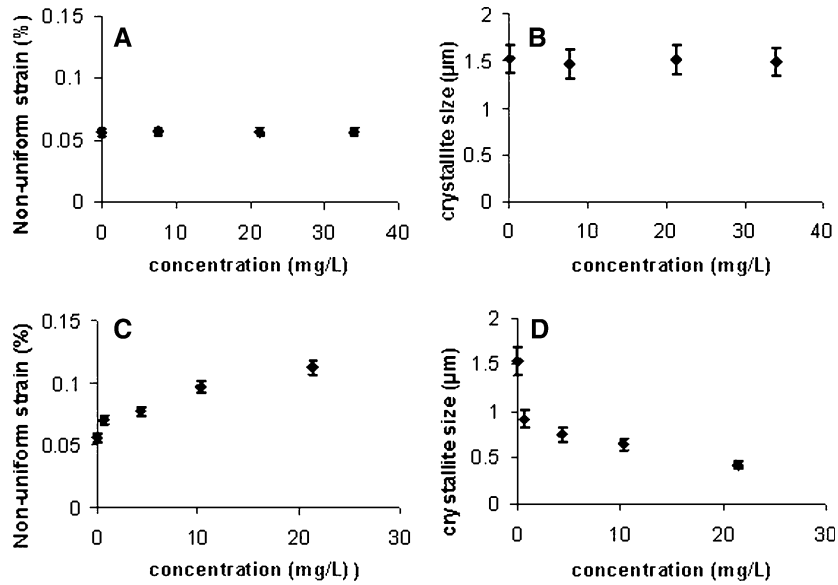

Fig. 10 Non-uniform strain (**a**) and crystallite size (**b**) plotted as a function of protein concentration for THG, and corresponding data for PT (**c**, **d**)

the foregoing discussion, there is a clear need for a precise definition of the term "crystal matrix".

Figure 11 illustrates various ways in which organic material can associate with crystals precipitated from unprocessed or sieved urine, or with crystals in a kidney stone. In Fig. 11a, crystals with macromolecules adsorbed to their surfaces, but which are not interred within the bulk, are shown in yellow. OPN would be such a macromolecule, which is perhaps best termed "superficial", since it actively binds to the surface of growing inorganic COM crystals, [45], but is absent from demineralized extracts of thoroughly washed COM crystals precipitated from urine [9]. Texturing (coloured blue in Fig. 11b), which represents macromolecules incarcerated within the COM mineral bulk, is depicted in all remaining crystals. Appropriately entitled "intracrystalline" [34, 36], these are not removed by exhaustive washing, even with NaOH [12] or bleach [36] (Fig. 11b). Examples of such macromolecules are PT and its fragments possessing the so-called Gla domain [9, 13, 15, 22]. Figure 11c represents crystals with macromolecules such as OPN adsorbed upon their surfaces, but also containing others interred within, while Fig. 11d shows the same crystal aggregate but now associated with loosely bound organic material (green) that completely or partially covers some of its component crystals, as well as others located nearby. This material, consisting of undissolved flocculent proteins such as THG [32] or subcellular particles [46], which may settle upon some crystals when they are centrifuged or filtered following their precipitation from, or exogenous addition to unprocessed urine, can

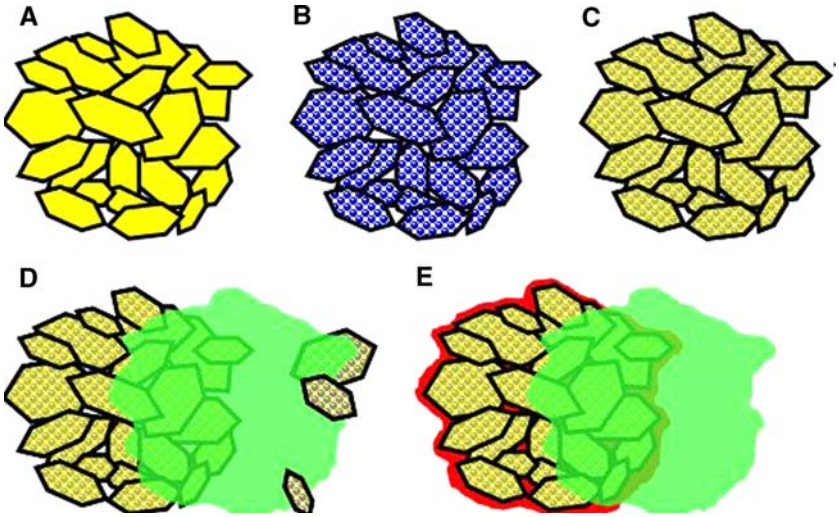

Fig. 11 Diagram illustrating the difference between macromolecules associated with clusters of COM crystals. Intracrystalline macromolecules are shown as texture in all crystals. **a** Aggregate associated with superficial macromolecules only (*yellow*). **b** The same aggregate containing intracrystalline macromolecules only (*blue texture*). **c** The same aggregate containing both superficial and intracrystalline macromolecules. **d** The same aggregate as in **c**, plus extrinsic organic material, such as THG or normal urinary cellular material (*green*), which completely or partially covers some of the crystals in the cluster, as well as others located nearby. Note that not all crystals are covered with organic material, which can settle when the crystals are centrifuged or filtered. **e** A small stone, whose component crystals contain intracrystalline macromolecules, but which are also covered with superficial macromolecules. In addition, the structure is associated with extrinsic macromolecules that surround the stone or are trapped inside by successive crystal layers. Extrinsic macromolecules can include those resulting from damage caused by the stone itself (*red*), as well as "normal" urinary macromolecules or urinary sediment (*green*), which here are displaced to the right to expose the red ones, which would otherwise be concealed

be seen in Fig. 6. Such organic material and macromolecules, which have no chemical or physical affinity for the surfaces of the crystals, and whose association with them is therefore purely coincidental, can be defined as "extrinsic". Although they would not bind irreversibly to the surfaces of crystals, they might nonetheless become trapped in the spaces between them, under which circumstances they could be described as "intercrystalline" [36]. However, they are clearly not intracrystalline; nor can they be described as "crystal matrix". Nonetheless, they could still be part of "stone matrix". If the crystal aggregate shown in Fig. 11e was a stone, the organic material remaining after dissolution of the component crystals would include all the associated macromolecules, whether intracrystalline (texture), superficial (yellow) or extrinsic (green), as well as macromolecules released by tissue injury caused by the stone (shown in red). Having been trapped within the structure by successive crystal layers, they would not be removable by even the most aggressive washing procedures. Therefore, the terms "crystal matrix" and "intracrystalline" should be reserved specifically for molecules that are embedded *inside* the crystal and which cannot be removed by exhaustive washing. While the use of such specific definitions might seem pedantic, failure to distinguish between inaccessible organic molecules sealed within crystals and accessible ones excluded from the mineral bulk has hampered study of the role of macromolecules in biomineralization [36]. As seen below, similar failure has confused our understanding of the roles of macromolecules in stone pathogenesis.

Osteopontin was detected in extracts of all crystals except those treated according to the method of Walton et al. [21], which consisted of pure commercial COM, in keeping with our previous report [9] that OPN does not bind irreversibly to COM, in urine, at low calcium concentrations (2 mM). It is also supported by atomic force microscopy studies confirming that although OPN binds to growing COM crystals [45], the advancing growth front moves beneath the adsorbed molecules. OPN is a low affinity Ca-binding protein [47], whose conformation is profoundly affected by the ambient concentration of free Ca^{2+} ions, which it binds with relatively low Kd [48]. Only ~25 of the 50 potential Ca-binding sites of OPN are occupied at a [Ca] of 1–2 mMol/l [48] and saturation requires a calcium concentration of between 5 and 10 mMol/l [47]. OPN would therefore be expected to bind only relatively weakly at the concentration of calcium used here (2.7 mM). In the present study, our detection of OPN in crystals precipitated from urine using the methods of Doyle et al. [12] and Maslamani et al. [16] is almost certainly attributable to small numbers of

COD crystals, which were commonly observed amongst the more numerous COM crystals.

As we have reported previously, both HSA [12, 22, 23, 49] and PTF1 [9, 12, 22, 23, 49] were consistently detected in the extracts of all crystals, irrespective of the washing technique used, indicating that both must bind irreversibly to the crystal surface and be interred within the mineral bulk. In the case of PTF1, this conclusion is supported by the SXRD data, which showed that increasing concentrations of its parent molecule, PT, caused a proportional increase in non-uniform strain and a concomitant reduction in crystallite size. This would have resulted from the strong adsorption of PT on to the crystallites, which inhibited their growth and consequently increased their misorientation. These changes can be attributed to increasing quantities of the protein interred within the mineral, since SDS-PAGE and Western blotting have shown that the amount of PTF1 in COM crystals precipitated from urine containing increasing amounts of crystal matrix extract increases directly with the extract concentration [9]. In addition to confirming our previous report that the protein is occluded into the COM mineral [10], these results demonstrate the existence of a dose-response relationship between the amount of material incorporated into the bulk and indices of crystal texture, which, to our knowledge, has not been reported previously for any mineral. Conversely, the synchrotron data furnish unequivocal proof that THG, all of which would have been in solution under the conditions used, is not occluded into the mineral, since the same parameters remained unchanged with increasing concentrations of the protein. This conclusion was supported by the SDS-PAGE and Western blotting studies.

Tamm–Horsfall glycoprotein was absent from demineralized extracts of COM crystals exhaustively washed with water, regardless of whether they were generated from urine [12, 16] or added exogenously to it [21]. In contrast, it was present in extracts of crystals washed using the method of Maslamani et al. [16] and Walton et al. [21]. The findings were corroborated by electron microscopy, which showed organic material closely associated only with crystals whose demineralized extracts contained THG. In urine, THG exists as polymerized flocs that are easily removed by low speed centrifugation and filtration [32, 50]. However, the protein is soluble at high pH, low ionic strength and low concentration [50, 51] and would therefore be easily removed from precipitated urinary crystals by exhaustive washing with distilled water, in which it is soluble [50]. Its presence in extracts of crystals washed according to Maslamani et al. [16] can therefore be attributed to its incomplete removal by the limited quantities of water used, which also explains its association with COM crystals in other reports from that group [13–15], especially in calcium phosphate crystals [14, 15] which are exceedingly small and consequently far more likely to retain THG and other extrinsic proteins trapped within crystal aggregates. Collectively, our SDS-PAGE, Western blotting, field emission scanning electron microscopy and SXRD findings show conclusively that THG is not an intracrystalline component of CaOx crystals. Reports of its presence in demineralized extracts of COM crystals must therefore result solely from a combination of its co-sedimentation and co-filtration with the crystals during their isolation and subsequently, its incomplete removal—factors that have also frustrated the interpretation of other studies.

In an attempt to quantify the amount of protein inside CaOx crystals, Walton et al. [17] measured the density of COM and COD crystals precipitated from unprocessed human urine and concluded that it is a not a major intracrystalline component of the mineral structure. This raised doubts that proteins can occupy a significant proportion of the volume of some individual crystals [7, 8] and undermined the hypothesis that intracrystalline proteins assist stone prevention by rendering trapped crystals more susceptible to intracellular degradation [7–9, 10, 11]. However, Walton et al. [17] based their calculations on the total amount of protein associated with the crystals, which had been isolated by centrifugation and rinsed with methanol. Water-miscible alcohols are well known to denature proteins and have been commonly used for many years as precipitants during protein purification [52]. Indeed, addition of methanol to urine induces immediate cloudiness, caused by precipitation of proteins. Not unexpectedly, therefore, Walton et al. [17] detected THG in the extracts of all crystals. Furthermore, two-thirds of the associated protein was removable by washing with NaOH, and organic material was clearly visible on the crystal surfaces (as was also seen in Figs. 8b and 9c of the present study). Their conclusions were therefore based on measurements of protein *associated* with the crystals—intracrystalline, superficial and extrinsic—rather than on the amount of protein actually interred within them.

In a later study [21], Walton et al. incubated inorganic COM and COD crystals separately in artificial urine containing urinary macromolecules [21]. They detected PTF1, OPN and THG in extracts of both COM and COD and concluded that particular proteins do not interact with specific CaOx hydrates as we had previously reported [9]. Again, however, Walton et al.

[21] centrifuged the crystals and washed them with methanol. The extracts would therefore have contained precipitated proteins, especially flocculent THG, which had co-sedimented with them, as was shown by scanning micrographs presented in their paper. Therefore, their observation that urinary proteins bind promiscuously to inorganic COM and COD in artificial urine is not conclusive, and does not discount the possibility that binding of proteins to individual polymorphs *in urine* is selective, especially since they performed all binding studies only at a single calcium concentration (1 mM). Similar doubts can be expressed about another study [20], which reported indiscriminate binding of urinary proteins to COM and COD crystals, but whose results would also have been confounded by the methods used to isolate and wash the crystals.

A failure to remove THG completely from CaOx crystals incubated in urine has also caused confusion in crystal-cell attachment experiments. In early studies, Lieske and his colleagues reported that THG did not affect the binding of COM crystals to MDCK and BSC-1 cells [53], but significantly decreased the cells' ability to internalize them [54] by interacting with the cells, rather than by coating the crystals. In both instances THG purified from human urine was used, being applied either as an aqueous solution directly to the crystals [53] or added to the cell culture binding medium [54]. Hsieh et al. [55] also used purified THG, but added it to the cell culture medium, rather than to the crystals, and showed that it inhibited attachment of COM to MDCK cells. Later work by Lieske's group [18, 19], however, reported that urinary macromolecules, one of which was THG, inhibited the adhesion of COM crystals to MDCK cells by coating their surfaces [18, 19]. Because THG was the major component of the demineralized crystal extracts, they concluded that it is a key inhibitor of crystal attachment, particularly in male stone formers, whose THG exhibited reduced inhibitory activity [19]. Wiessner et al. [20] also deduced that coating of crystals by urinary macromolecules was responsible for reduced binding of both COM and COD crystals to cells and suggested that THG may play a role as a platform for the attachment of other proteins. There can be little doubt that THG inhibits the attachment of COM crystals to renal cells in culture, but this is unlikely to result from its binding directly to the crystals. This ability, exaggerated by its sheer preponderance in urine, has tended to emphasize its role in crystal-cell attachment and perhaps to subordinate the contributions of other macromolecules that inhibit by interacting with the crystal faces. An unidentified 200 kDa protein [56], fibronectin [57], a highly acidic region of nucleolin-related protein [58], osteopontin [53] and an unidentified 39 kDa protein [59] have been shown to inhibit adhesion by coating the crystal surface, but those binding experiments were carried out in the absence of THG, which otherwise might have masked the magnitude of their effects.

Summary and conclusion

Failure to eliminate all unbound THG by adequate washing has created the impression that it coats the surface of COM crystals and produced inaccurate analyses of urinary COM crystal matrix, thus potentially raising uncertainties about its interactions with other urinary proteins, as well as the quantitative protein composition of COM crystal matrix. Consequently, scrupulous care must be taken to ensure the complete removal of extraneous THG adventitiously associated with CaOx crystals in order to avoid inaccurate analysis of crystal matrix protein content and possible misinterpretation of experimental data, as has occurred in the biomineralization field [36].

Acknowledgments We gratefully acknowledge grants from the National Institutes of Health, USA (Grant No. NDDK 1 R01 DK064050–01A1), the Australasian Urological Foundation, The Australian Institute of Nuclear Science and Engineering and the Australian Synchrotron Research Program. David Fleming was the recipient of a Curtin University of Technology Postgraduate Scholarship.

References

1. Grant AMS, Baker LRI, Neuberger A (1973) Urinary Tamm–Horsfall glycoprotein in certain kidney diseases and its content in renal and bladder calculi. Clin Sci 44:377–384
2. Lieske JC, Toback FG, Deganello S (1998) Direct nucleation of calcium oxalate dihydrate crystals onto the surface of living renal epithelial cells in culture. Kidney Int 54:796–803
3. Lieske JC, Norris R, Swift H, Toback FG (1997) Adhesion, internalization and metabolism of calcium oxalate monohydrate crystals by renal epithelial cells. Kidney Int 52:1291–1301
4. Lieske JC, Deganello S, Toback FG (1999) Cell-crystal interactions and kidney stone formation. Nephron 81:8–17
5. Lieske JC, Deganello S (1999) Nucleation, adhesion and internalization of calcium-containing urinary crystals by renal cells. J Am Nephrol Soc 10:S422–S429
6. Aizenberg J, Hanson J, Ilan M, Leiserowitz L, Koetzle TF, Addadi L, Weiner S (1995) Morphogenesis of calcitic sponge spicules: a role for specialized proteins interacting with growing crystals. FASEB J 9:262–268
7. Ryall RL, Fleming DE, Grover PK, Chauvet MC, Dean CJ, Marshall VR (2000) The hole truth: intracrystalline proteins and calcium oxalate kidney stones. Mol Urol 4:391–402
8. Ryall RL, Fleming DE, Doyle IR, Evans NA, Dean CJ, Marshall VR (2001) Intracrystalline proteins and the hidden

ultrastructure of calcium oxalate urinary crystals: implications for kidney stone formation. J Struct Biol 134:5–14
9. Ryall RL, Chauvet MC, Grover PK (2005) Intracrystalline proteins and urolithiasis: a comparison of the protein content and ultrastructure of urinary calcium oxalate monohydrate and dihydrate crystals. Br J Urol Int 96:654–663
10. Fleming DE, van Riessen A, Chauvet MC, Grover PK, Hunter B, van Bronswijk W, Ryall RL (2003) Intracrystalline proteins and urolithiasis: a synchrotron X-ray diffraction study of calcium oxalate monohydrate. J Bone Min Res 18:1282–1291
11. Chauvet MC, Ryall RL (2005) Intracrystalline proteins and calcium oxalate crystal degradation in MDCK II cells. J Struct Biol 151:12–17
12. Doyle IR et al (1991) Inclusion of proteins into calcium oxalate crystals precipitated from human urine: a highly selective phenomenon. Clin Chem 37:1589–1594
13. Atmani F, Opalko FJ, Khan SR (1996) Association of urinary macromolecules with calcium oxalate crystals induced in vitro in normal human and rat urine. Urol Res 24:45–50
14. Atmani F, Glenton PA, Khan SR (1998) Identification of proteins extracted from calcium oxalate and calcium phosphate crystals induced in the urine of healthy and stone-forming subjects. Urol Res 26:201–207
15. Atmani F, Khan SR (2002) Quantification of proteins extracted from calcium oxalate and calcium phosphate crystals induced in vitro in the urine of healthy controls and stone-forming patients. Urol Int 68:54–59
16. Maslamani S, Glenton PA, Khan SR (2000) Changes in urine macromolecular composition during processing. J Urol 164:230–236
17. Walton RC, Kavanagh JP, Heywood BR (2003) The density and protein content of calcium oxalate crystals precipitated from human urine: a tool to investigate ultrastructure and the fractional volume occupied by organic matrix. J Struct Biol 143:14–23
18. Kumar V, Farell G, Lieske JC (2003) Whole urinary proteins coat calcium oxalate monohydrate crystals to greatly decrease their adhesion to renal cells. J Urol 170:221–225
19. Kumar V, Peña de la Vega L, Farell G, Lieske JC (2005) Urinary macromolecular inhibition of crystal adhesion to renal epithelial cells is impaired in male stone formers. Kidney Int 68:1784–1792
20. Wiessner JH, Hung LY, Mandel NS (2003) Crystal attachment to injured renal collecting duct cells: Influence of urine proteins and pH. Kidney Int 63(4):1313–1320
21. Walton RC, Kavanagh JP, Heywood BR, Rao PN (2005) The association of different urinary proteins with calcium oxalate hydromorphs: evidence for non-specific interactions. Biochim Biophys Acta 1723:175–183
22. Stapleton AMF, Ryall RL (1995) Blood coagulation proteins and urolithiasis are linked: Crystal matrix protein is the F1 activation peptide of human prothrombin. Br J Urol 75:712–719
23. Stapleton AMF, Dawson CJ, Grover PK, Hohmann A, Comaccio R, Boswarva V, Tang Y, Ryall RL (1996) Further evidence linking urolithiasis and blood coagulation: urinary prothrombin fragment 1 is present in stone matrix. Kidney Int 49:880–888
24. Grover PK, Ryall RL, Marshall VR (1990) Does Tamm–Horsfall mucoprotein inhibit or promote calcium oxalate crystallization in human urine? Clin Chim Acta 190:223–238
25. Grover PK, Ryall RL (1999) Inhibition of calcium oxalate crystal growth and aggregation by prothrombin and its fragments in vitro (1999): relationship between protein structure and inhibitory activity. Eur J Biochem 263:50–56
26. Langford JI (1978) A rapid method for analyzing the breadths of diffraction and spectral lines using the Voigt function. J Appl Crystallogr 11:10–14
27. Langford JI, Cernik RJ, Louer D (1991) The breadth and shape of instrumental line profiles in high resolution powder diffraction. J Appl Crystallogr 24:913–919
28. Fleming DE (2004) Urolithiasis: occurrence and function of intracrystalline proteins in calcium oxalate monohydrate crystals. PhD thesis, Curtin University of Technology. http://www.adt.curtin.edu.au/theses/available/adt-WCU20050124.093851/
29. Hunter BA (1998) Rietica: a visual Rietveld program. International Union of Crystallography, Commission on Powder Diffraction Newsletter, vol 20, pp 18–24
30. Henisch H (1970) Crystal growth in gels. The Pennsylvania State University Press, University Park, pp 21–26
31. Ryall RL, Hibberd CM, Marshall VR (1985) A method for studying inhibitory activity in whole urine. Urol Res 13:285–289
32. Wiggins RC (1987) Uromucoid (Tamm–Horsfall glycoprotein) forms different polymeric arrangements on a filter surface under different physicochemical conditions. Clin Chim Acta 162:329–340
33. Ganter K, Bongartz D, Hesse A (1999) Tamm–Horsfall protein excretion and its relation to citrate in urine of stone-forming patients. Urology 53:492–495
34. Addadi L, Weiner S (1989) Stereochemical and structural relations between macromolecules and crystals in biomineralization. In: Mann S, Webb J, Williams RJP (eds) Biomineralization: chemical and biochemical perspectives. VCH, New York, pp 133–156
35. Berman A, Addadi L, Kvivk A, Leiserowitz L, Nelson M, Weiner S (1990) Intercalation of sea urchin proteins in calcite: study of a crystalline composite material. Science 250:664–667
36. Sykes GA, Collins MJ, Walton DI (1995) The significance of a geochemically isolated intracrystalline organic fraction within biominerals. Org Geochem 11/12:1059–1065
37. Berman A, Addadi L, Weiner S (1988) Interactions of sea-urchin skeleton macromolecules with growing calcite crystals – a study of intracrystalline proteins. Nature 331:546–548
38. Watabe N (1963) Decalcification of thin sections for electron microscope studies of crystal-matrix relationships in mollusk shells. J Cell Biology 1:701–703
39. Towe KM (1980) Preserved organic ultrastructure: an unreliable indicator for Paleozoic amino acid biogeochemistry. In: Hare PE, Hoering TC, King K (eds) The biogeochemistry of amino acids. Wiley, New York, p 73. Cited in [36]
40. Aizenberg J, Ilan M, Weiner S, Addadi L (1996) Intracrystalline macromolecules are involved in the morphogenesis of calcitic sponge spicules. Conn Tissue Res 34:255–261
41. Morse R, Resnick MI (1988) A new approach to the study of urinary macromolecules as a participant in calcium oxalate crystallization. J Urol 139:869–873
42. Gokhale JA, Glenton PA, Khan SR (1996) Localization of Tamm–Horsfall protein and osteopontin in a rat nephrolithiasis model. Nephron 73:456–461
43. Gokhale JA, Glenton PA, Khan SR (1996) Immunocytochemical localization of Tamm–Horsfall protein in the kidneys of normal and nephrolithic rats. Urol Res 24:201–209
44. Wiggins RG, Kshrisagar B, Kelsch RC, Wilson BS (1985) Fragmentation and polymeric complexes of albumin in human urine. Clin Chim Acta 149:155–163
45. Qiu SR, Wierzbicki A, Orme CA, Cody AM, Hoyer JR, Nancollas GH, Zepeda A, De Yoreo JJ (2004) Molecular modulation of calcium oxalate crystallization by osteopontin and citrate. PNAS 101:1811–1815

46. Wiggins RG, Glatfelter A, Kshrisagar B, Brukman J (1986) Procoagulant activity in normal human urine associated with subcellular particles. Kidney Int 29:591–597
47. Chen Y, Bal BS, Gorski JP (1992) Calcium and collagen binding properties of osteopontin, bone sialoprotein, and bone acidic glycoprotein-75 from bone. J Biol Chem 267:24871–24878
48. Denhardt DT, Guo X (1993) Osteopontin: a protein with diverse functions. FASEB J 7:1475–1482
49. Ryall RL, Grover PK, Stapleton AMF, Barrell DK, Tang Y, Moritz RL, Simpson RJ (1995). The urinary F1 activation peptide of human prothrombin is a potent inhibitor of calcium oxalate crystallization in undiluted human urine in vitro. Clin Sci 89:533–541
50. Dawnay ABStJ, Thornley C, Cattell WR (1982) An improved radioimmunoassay for urinary Tamm–Horsfall glycoprotein. Biochem J 206:461–465
51. McQueen EG, Engel GB (1966) Factors determining the aggregation of urinary mucoprotein. J Clin Path 19:392–396
52. Cohn EJ, Strong LE, Hughes WL, Mulford DJ, Ashworth JN, Melin M, Taylor HL (1946) Preparation and properties of serum and plasma proteins. IV. A system for the separation into fractions of the protein and lipoprotein components of biological tissues and fluids. J Am Chem Soc 68:459–475
53. Lieske JC, Leonard R, Toback FG (1995) Adhesion of calcium oxalate monohydrate crystals to renal epithelial cells is inhibited by specific anions. Am J Physiol 268:F604–F612
54. Lieske JC, Toback FG (1993) Regulation of renal epithelial cell endocytosis of calcium oxalate monohydrate crystals. Am J Physiol 264:F800–F807
55. Hsieh N, Shih CH, Chen HY, Wu MC, Chen WC, Li CW (2003) Effects of Tamm–Horsfall protein on the protection of MDCK cells from oxalate induced free radical injury. Urol Res 31:10–16
56. Yamaguchi S, Wiessner J, Hasegawa A, Hung L, Mandel G, Mandel N (2002) Calcium oxalate monohydrate crystal binding substance produced from Madin-Darby canine kidney cells. Int J Urol 9:501–508
57. Tsujihata M, Miyake O, Yoshimura K, Kakimoto KI, Takahara S, Okuyama A (2000) Fibronectin as a potent inhibitor of calcium oxalate urolithiasis. J Urol 164:1718–1723
58. Sorokina EA, Wesson JA, Kleinman JG (2004) An acidic peptide sequence of nucleolin-related protein can mediate the attachment of calcium oxalate to renal tubule cells. J Am Soc Nephrol 15:2057–2065
59. Kumar V, Yu S, Farell G, Toback FG, Lieske JC (2004) Renal epithelial cells constitutively produce a protein that blocks adhesion of crystals to their surface. Am J Physiol Renal Physiol 287:F373–F383