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Regulation by macromolecules of calcium oxalate crystal aggregation in stone formers

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Abstract Based on the structure of kidney stones, it is likely that they form as aggregations of preformed crystals, mostly calcium oxalate monohydrate (COM). In this study, we examined the ability of a macromolecular mixture isolated from the urine of normal individuals and stone formers to inhibit aggregation of preformed COM seed crystals in a simple ionic solution using measurements of changes in the particle size distribution (PSD) of preformed COM crystal aggregates. We also examined the effect in this assay of a number of synthetic homopolymers, naturally occurring urine macromolecules, and binary mixtures thereof. The macromolecular mixtures from urine of normals and most stone formers reduced the degree of aggregation of the seed crystals, whereas 22% of stone former urine macromolecules either did not disaggregate or actually promoted further aggregation. Stone formers within one family shared this property, but a non-stone forming sibling did not. Polyanions, either synthetic or naturally occurring, induced disaggregation to an extent similar to that exhibited by normal urine macromolecules, while polycations had no effect on the PSD. However, mixing a polyanion, either poly-aspartate or osteopontin, with the polycation poly-arginine, changed their behavior from disaggregation to aggregation promotion. The disaggregating behavior of normal urinary macromolecules provides a defense against aggregation, but a minority of stone forming individuals lacks this defense, which may contribute to stone formation.

Keywords Nephrolithiasis · Urolithiasis · Polyanions · Polycations · Urine macromolecules · Osteopontin

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Introduction

Kidney stones form as onion-like layered polycrystalline aggregates with proteins, lipids, polysaccharides and other cellular components incorporated between microcrystals within, as well as between layers [4, 6, 8]. It is likely that these structures arise from incorporation of preformed crystals or aggregates into the stones. Clinical studies of crystals found in urine samples revealed greater aggregation in stone formers than in healthy adults[13, 15]. We propose, therefore, that aggregation of preformed crystals plays an integral role in stone formation.

Normal urinary macromolecules inhibit crystal aggregation, although prior research efforts have failed to identify a urine test that reliably identifies stone formers. Also, effective therapies to retard stone formation have been designed almost universally to lower supersaturation with respect to CaOx. Recent studies of urine or macromolecular mixtures isolated from urine suggest that weak aggregation inhibitory activity or frank aggregation promotion is a significant feature of stone formation in some patients [1, 5, 9, 21]. The nature of these macromolecules and their interactions with COM crystals are largely unknown.

Several individual urinary macromolecules, principally proteins, have been identified as important to stone formation, because they associate strongly with COM crystals and inhibit COM crystallization kinetics in vitro. These include osteopontin (OPN, also known as uropontin), nephrocalcin, urinary prothrombic fragment-1 (UPTF-1), bikunin, and, perhaps, others [2, 10, 12, 13, 14, 16, 17, 24]. Many of these macromolecules also inhibit aggregation when tested individually. They all share the characteristics of being polyanionic in physiologic solutions by virtue of the aspartic acid and glutamic acids in their primary structures [19], as well as through the post translational modifications of phosphorylation or glycosylation.

In the experiments described below, we test the relative abilities of the urinary macromolecular mixtures from idiopathic calcium oxalate (CaOx) stone formers and normal, healthy adults to inhibit aggregation of preformed COM crystals in a simple ionic solution at approximately the saturation concentration. We also report the results from testing several individual native macromolecules and some synthetic proteins as model inhibitors, as well as the effects on aggregation of simple mixtures of these macromolecules. We demonstrate that cationic macromolecules can abrogate the inhibitory effect of anionic macromolecules, and certain combinations can actually promote COM aggregation.

Materials and methods

Urine collection

Fresh random urine specimens were collected from normal individuals and stone former patients with an EDTA free protease inhibitor tablet (Roche Applied Science, Indianapolis, IN). Normal urine (NU) was obtained from individuals without a personal or familial history of stones. Stone former urine (SFU) was obtained from patients with recurrent calcium oxalate stones from the Stone Clinic at the Froedtert Memorial Lutheran Hospital in Milwaukee and the Nephrology Clinic the Milwaukee Veterans Affairs Medical Center. Both groups had normal renal function, were negative for protein by urine dipstick and were of comparable ages. Individuals on medications that could affect renal function (NSAIDS, ACEI, ARB), diabetics, and individuals with proteinuria (protein/creatinine >200 mg/g) were excluded from this study. The Institutional Review Board and the Research Committee of the respective institutions approved the studies. The characteristics of the groups are given in Table 1.

Urine macromolecule preparation

Urine macromolecules were isolated by ultradiafiltration (10 kD MiniKros, Spectrum Laboratories, Calif) against a buffer containing 100 mM NaCl. The macromolecular fractions were concentrated five to tenfold during this process, and were constantly in the presence of protease inhibitors (Sigma) from the time of collection. Samples were stored at -80°C until assayed. For patient sample comparisons, we added the quantity of macromolecules that would have been contained in the same volume of the original urine as a given quantity of creatinine (i.e., 1 Creat Eq would be the amount of macromolecules contained in the same volume of the original urine as 1 mg of creatinine). Thus, we were adjusting principally for variations in urine dilution between patients, but the samples may contain different quantities of macromolecules from different individuals according to observed variations in this parameter.

Aggregation assay

The macromolecules were tested for their ability to regulate COM aggregation by analysis of the particle size distribution (PSD) changes. For this experiment, we used 0.25 mM $CaCl_2$ and $Na_2C_2O_4$ in 150 mM NaCl, buffered to pH 7.5 with 10 mM HEPES (supersaturation1.11 [11]) at 37°C. The slight supersaturation was chosen to assure that no dissolution could occur to skew the results, for reasons that will be made evident below. COM seed crystals (300 μg) were added to 5 ml of this solution and allowed to mix for 1 h. Then, the PSD of an aliquot of this mixture was determined.

Data analysis

Aggregation data from these bulk crystallization methods are illustrated below as either PSD data for the entire distribution or as R_D, the ratio of the final weight average particle diameter (D_w) for the PSD to the D_w of the COM seed crystals measured daily. Differences between sample conditions were judged using Student's *t*-test with $P < 0.05$ defining significance.

COM seed crystal preparation

Calcium oxalate monohydrate (COM) crystals were prepared by the simultaneous drop wise addition of 2 l of 10 mM $CaCl_2$ and 10 mM $Na_2C_2O_4$ into a continuously stirred beaker. The solution was allowed to mix for 1 week at 4°C. Crystals were harvested by gravity and subsequent low speed centrifugation (3,000 rpm for 10 min). The resulting crystals were washed twice with water, then methanol, and dried overnight in a 60°C oven and stored at room temperature until they were made into a slurry. The crystal slurry was prepared as 1.5 mg COM/ml HEPES buffer (10 mM HEPES, 150 mM NaCl, 0.01% NaN_3; pH 7.5). The slurry was allowed to mix overnight and was used in assays after tracking stable PSD measurements for at least 1 month. We verified that the PSD of the slurry was stable for 6 months to 1 year as long as the mixture remained free of contamination. X-ray powder diffraction confirmed that COM crystals were formed.

Table 1 Urine panel demographics

	Age	N	Men	Ca/creatinine (mg/g)	Protein/creatinine (mg/g)	No. of stones
NU	46 ± 11	25	20	104 ± 32	73 ± 27	None
SFU	51 ± 13	27	18	170 ± 72	93 ± 49	6 ± 8

Particle size measurements

PSD measurements were made using the AccuSizer 780/SIS (Particle Sizing Systems, Santa Barbara, Calif.). To determine the seed crystal PSD, 15 μg COM were added to 15 ml of sizing buffer (0.225 mM $CaCl_2$, 0.225 mM $Na_2C_2O_4$, 10 mM HEPES; 0.2 μm filtered) and measured in duplicate. The sizing buffer was shown to cause neither significant growth nor dissolution of the COM crystals for up to an hour.

Individual macromolecules

Synthetic homopolymers of lysine, arginine, histidine, aspartate and glutamate were added to the crystal slurry to produce concentrations of 100 nM. Details regarding these polymers are given in Table 2. Purified urinary proteins or their equivalent from other sources were also added to the aggregation assay in a systematic manner. Table 3 lists the natural macromolecules that were tested in the crystal assays, their calculated molecular weight and estimated isoelectric point. Combinations of macromolecules were 50 nM in each, for a total concentration of 100 nM.

Results

Seed crystal PSD

The initial seed particles were highly aggregated structures, as illustrated in Fig. 1. These particles, which were generated from an initially highly supersaturated solution, were expected to be aggregates based on previously published crystal growth experiments in hydroxyapatite, [3] as well as more recent experiments on COM formation in our own laboratory (unpublished results). The individual particle boundaries are not well defined in this SEM micrograph; it is difficult to tell whether the clusters are lying next to one another or if they are physically attached after depositing them unto the SEM grid and drying the sample. The apparent dimensions of the single crystals within the aggregates appear to be of the order of 1–2 μm. The aggregates shown here are roughly 3–5 single particle diameters in linear dimension, which is an important reference point for judging the output from the Accusizer.

Table 2 Synthetic macromolecules tested in aggregation assay. Molecular weight was determined by low angle light scattering

Polymer	Source	MW	pK_R
Poly-aspartate (pD)	Sigma P5387	12 kDa	3.9 β-COOH
Poly-glutamate (pE)	Sigma P4636	14 kDa	4.07 γ-COOH
Poly-histidine (pH)	Sigma P9386	20 kDa	6.04 imidazole
Poly-lysine (pK)	Sigma P2658	28 kDa	10.54 ϵ-NH_3^+
Poly-arginine (pR)	Sigma P4663	12 kDa	12.48 guanidino

An example of the seed crystal PSD from the Accusizer is illustrated in Fig. 2 in a plot of volume fraction (or, equivalently, weight fraction) as a function of particle diameter. Since the operating principle of the Accusizer is based on sizing individual particles as they traverse the optical path, each distribution is actually a histogram calculated by assigning each individual particle as a single count to a channel number based on the measured size for that particle. The operating range of the instrument (from 0.5 to 400 μm) has been divided into 512 channels to obtain the distributions illustrated in Figure 2. The diameter assigned to each particle is that of the equivalent spherical particle (one having the same light scattering and obscuration signals as the measured particle), and consequently is not required to match the dimensions as seen in Fig. 1. However, if one calculates the number-averaged particle diameter, which would be a better reflection of the distribution of sizes observed in a random photograph, there is fair correspondence between the average dimensions recorded by the Accusizer (5.5 μm) and those seen in SEM images. Unfortunately, the aggregated character of the initial particle limits our ability to visually assess the changes in aggregation state by microscopic methods, and we will discuss only the quantitative assessment of PSD using the Accusizer in this report.

Comparison of individual macromolecules

Initially, we sought to characterize the COM crystal aggregation behavior under a variety of experimental conditions to gauge the sensitivity of the experiment to various parameters, using synthetic polymers as models. The control condition of seed crystal, but no macromolecules, added to the test solution showed a non-significant trend toward increasing particle size ($R_D = 1.03 \pm 0.10$, $n = 68$ trials from three different COM slurries), consistent with some growth taking place under the slight supersaturation of the test solution. As seen in Fig. 2, the addition of pD induced a decease in average particle size or disaggregation of the original seed PSD. Having previously established that the PSD of the seed was invariant in the time scale of months, when stored in a saturated solution environment, the time dependence of the response to adding pD was explored. We found that at 10 min and at 1, 2 and 3 h of exposure to the polymer yielded identical changes in PSD. Finally, the affect of macromolecules on the aggregation of preformed particles must certainly depend on the polymer concentration relative to the crystal surface area. We tested this concept in two ways, by varying pD and pE concentrations at constant seed crystal addition and by varying the seed crystal addition at constant pD concentration. We found essentially no difference in D_w between the 100 nM and 1,000 nM pD and pE concentrations added to 300 μg of COM seed crystal in suspension, while the 10 nM pD and pE samples showed approximately 70% of the disaggregating effect

Table 3 Urinary macromolecules tested in aggregation assay. Molecular weight and pI were estimated by calculation from amino acid backbone (SWISS-PROT Database); bOPN was a gift from E. Sorenson, University of Aarhus, Denmark

Macromolecule	Source	MW	PI
Transferrin (Tf)	Amersham no. 1058772	77 kDa	6.8
Tamm-Horsfall glycoprotein (THP)	BTI no. 381	70 kDa	5.1
Ceruloplasmin (CP)	Sigma C2026	122 kDa	5.4
Mouse osteopontin (mOPN)	Kidney cell media [24]	32 kDa	4.3
Bovine osteopontin (bOPN)	Milk [20]	31 kDa	4.6
Human serum albumin (HSA)	Sigma A8763	69 kDa	5.9
Chondroitin sulfate (CS)	Sigma C7571	22 kDa	NA

of the higher polymer concentrations. Similarly, the PSD was unaffected by the quantity of seed added to a 100 nM pD solution in the range of 25–600 μg of COM seed added. We concluded that a relatively small quantity of polymer was required to achieve essentially complete disaggregation of COM. We have used the 100 nM macromolecule concentration as a test condition for comparing different macromolecules to one another, because it is well away from a condition where observed changes might be due to the polymer concentration, and because the macromolecule concentrations expressed on a mass/volume basis fall in the range of typical macromolecule concentrations in the urine (1–10 μg/ml).

All samples were tested at 100 nM total polymer concentration, and the binary mixtures of polyanions with polycations contained equimolar quantities of the two macromolecules with total polymer concentration of 100 nM. The results are illustrated graphically in Fig. 3, showing R_D for both the individual macromolecules and the binary mixtures. The individual macromolecules either induced disruption of preexisting aggregates (polyanions, which includes most native proteins) or had little effect on these aggregates (polycations and Tf). Mixtures have been prepared of polyanions and polycations, because of the potential for adhesive electrostatic interactions between these oppositely charged macromolecules. As seen in Figure 3, the mixture of pD with pR promoted aggregation, while the other five mixtures had little effect on aggregation. In addition, mOPN, a native polyanionic protein, when mixed with pR also promoted aggregation.

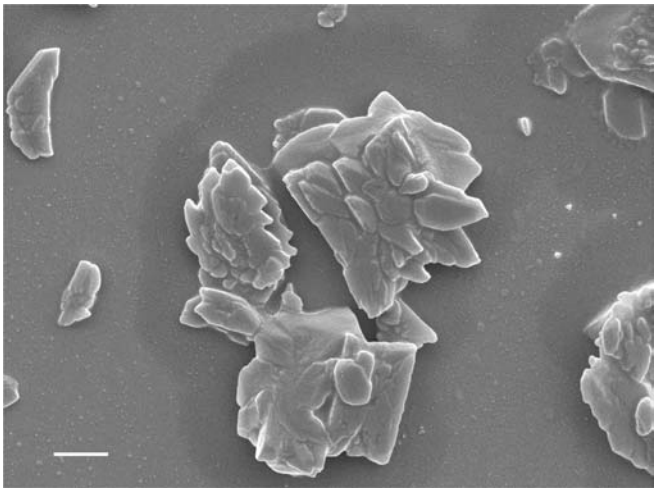

Fig. 1 SEM micrograph of typical COM particle from seed crystal slurry, showing evidence of substantial aggregation. The *scale bar* represents 1 μm

Urinary macromolecular mixtures

Data from aggregation assays using patient urinary macromolecules are summarized in Fig. 4 as scatter plots of R_D showing the measured values for each individual sample, separated into categories for the 27 stone formers and the 25 normals included in this study. The normal data cluster relatively tightly about the

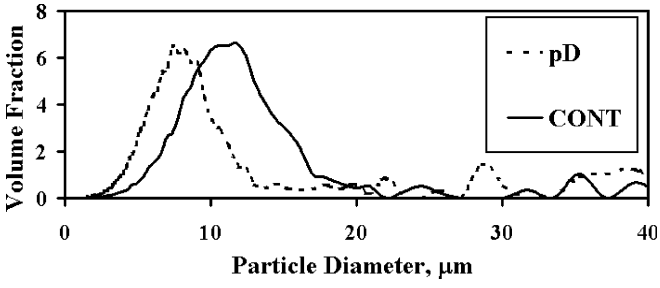

Fig. 2 Typical particle size distributions are shown as a plot of volume fraction vs particle diameter for the CONT (no polymer added) and for a 100 nM pD sample. The pD containing sample illustrates disaggregation behavior as seen with the addition of most anionic macromolecules. Initial seed crystal is not shown since it is not significantly different from the CONT

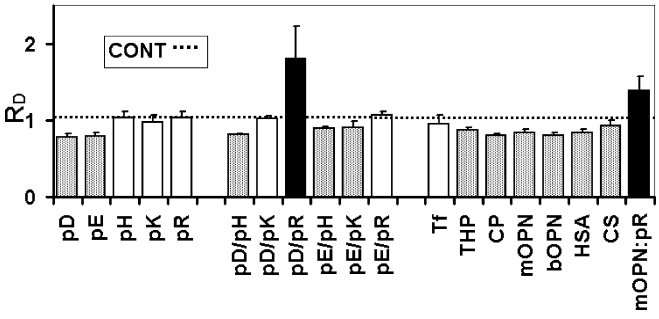

Fig. 3 Histogram of R_D-AGG values for individual macromolecules and binary mixtures. Abbreviations as listed in Tables 2 and 3. *Black bars* have RD values significantly greater than CONT, *grey bars* are significantly less than CONT and *white bars* are not significantly different than CONT

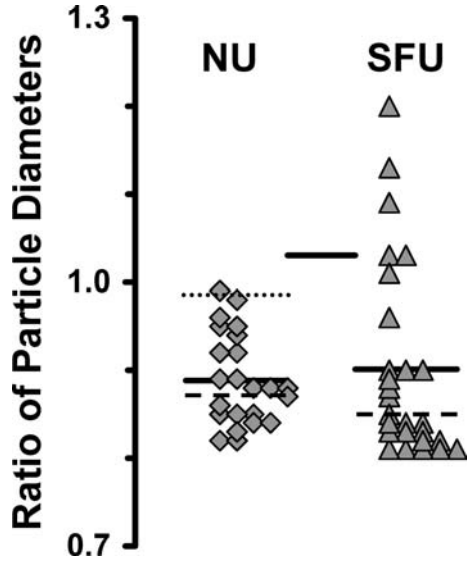

Fig. 4 A plot of all individual data for urinary macromolecular mixtures in the aggregation assay. Note the much wider range of values represented within the set of stone former samples as compared to the normal values. The *solid horizontal bar* in each data set shows the mean values, and the *dashed lines* are the median values. The *solid bar* between the data sets represents the mean of the experimental control values (no macromolecules added) and is significantly different from both NU and SFU groups. The *dotted bar* in the NU column shows the upper value of two SDs from the mean of the values

mean value of 0.89 ± 0.05, demonstrating significant disaggregation of the COM seed compared to the condition without added macromolecules (1.03 ± 0.1). Conversely, the data from stone former samples exhibit a much wider range of R_D values, although the mean (0.90 ± 0.11) is not different from that of the NU samples. However, six of 27 (22%) of SFU samples demonstrate weak disaggregation (3/27) or aggregation promotion (3/27) in this assay. The mean of this group, 1.08 ± 0.07, is statistically different than the mean R_D value from normals ($P < 0.001$), but not from the control group without macromolecules. There are also a few stone former samples that exhibit slightly greater disaggregation effects than the typical normal (six of 27, 22%), but none are less than 2SD from the mean of the normal urine group.

Among patients, there was no relation between the R_D values and the normalized protein values. The average of the normalized protein values for the NU samples was 26 ± 14 μg/mg (protein/creatinine); and for the SFU samples it was 30 ± 20 μg/mg, which were not significantly different from each other. Also, no correlations were observed between R_D values and either age or gender.

If the identifiable defects from this assay reflect genetic predispositions toward stone formation through the elaboration of abnormal urinary macromolecules, then we might expect to find that the stone forming family members of a patient exhibiting a defect in aggregation inhibition in our assay should also exhibit a similar defect, even if the same cannot be said for a randomly selected patient from the stone former pool. We have examined this question in one family cluster consisting of four siblings over the age of 60. The three sisters in this family grouping all form calcium oxalate stones, while their brother has never formed a stone. We found that the R_D values from the samples derived from the stone forming sisters were 1.01, 1.46, and 1.03; all exhibiting weak disaggregation or aggregation promotion, while the sample from the non-stone forming brother gave us a value of 0.86, consistent with the normal adult population. A second collection and retesting of the incident patient, whose first R_D value was 1.01, resulted in an R_D value of 1.51.

Discussion

The studies with model polymers and individual urinary macromolecules provide a number of important observations that facilitate the interpretation of the results from the patient samples. First, the absence of an R_D dependence on concentration, even at one tenth the standard test condition shown in pD, suggests that the differences we have observed reflect the fundamental interactions of each macromolecule with the initial COM seed crystals. As mentioned above, our standard test concentration corresponds approximately to the typical concentrations of previously identified urinary macromolecules in normal urine. Consequently, the effects described both for native individual macromolecules and the macromolecular mixtures from patient samples are also likely to be in a concentration independent region, and the observed differences can be interpreted as related to chemical structural differences between the macromolecules rather than being dependent on sample concentration. Finally, the demonstration of aggregation promotion by some stone formers represents a clearly identifiable pathology with obvious mechanistic correlation to the disease process.

The fact that no single macromolecules tested caused aggregation promotion reinforces previously published assertions that the native inhibitory macromolecules in urine were polyanionic in character and generally prevent undesirable aggregate formation in COM. Perhaps the most significant observation from the model polymer studies was that combinations of pR with pD, as well as the naturally occurring polyanionic protein OPN with pR, promoted COM aggregation. Certainly, normal urine contains a large number of macromolecules, with a wide range of isoelectric points. The model macromolecule mixture results suggest that emphasis needs to be placed on characterizing the polycationic macromolecules or macromolecules with polycationic regions, which could combine and interfere with the typical polyanions to create the unfavorable environment required for stone formation. Previous protocols isolating and characterizing polyanions may have been missing the critical components.

A subset (22%) of our randomly selected idiopathic calcium oxalate stone formers manifest urine containing a mixture of macromolecules that fails to disrupt this physical aggregation in COM, and in many cases, actually promotes further aggregation of these crystals. We are led to the conclusion that a defect in aggregation inhibition is a common finding in stone former urinary macromolecules with obvious ramifications for the disease process. We also note that the failure of most previous studies, which have focused on population average values, to identify differences between stone formers and normals may simply reflect the diversity of defects within the population, rather than their absence. More recent work has demonstrated findings very similar to ours [1], with a subset of stone formers showing defects in aggregation inhibition, while the overall stone former population averages did not differ from those of the normal adult samples in their studies.

There are also a number of SFU that demonstrate a greater disaggregating effect on the seen crystal than the group of NU. While a deviation in this direction by stone former samples cannot be conveniently interpreted in the clinical disease process, it does suggest that more than one type of urinary macromolecule behavior may exist within the population of stone former samples. It may be, for example, that enhanced disaggregation is an adaptation to a stone-forming tendency due to a different abnormality, hyperoxaluria, for example.

In addition, stone forming siblings of one of the aggregation promoting stone formers also demonstrate aggregation promotion in this assay. The non-stone forming sibling, on the other hand, showed disaggregation of the COM seed crystals typical of normal urine. The correlation of our in vitro result with disease process in this family cluster supports a genetic link, though the nature of the macromolecule(s) involved remains to be determined.

Presumably, identification of macromolecules responsible for inducing aggregation will be critical to understanding stone formation; however, given the complexity of the mixture of macromolecules in urine, with several hundred identifiable components [22], attempting to screen possible combinations randomly is an unmanageably large task. This task can be simplified, if the important chemical structural features and combinations of macromolecular structures can be defined through studies of individual macromolecules, including both synthetic and native macromolecules.

We have previously reported that urinary macromolecules can selectively alter crystal morphology [23]. The direction of crystal growth can also be selectively modified by different macromolecules [7]. These observations are consistent with the idea that macromolecules interact selectively with crystal faces. Certain synthetic polyanions, frequently used as models of urinary macromolecules in COM crystallization assays, demonstrated surface-activity by reducing the adhesion force between a chemically modified AFM probe tip and specific COM surfaces, and this selectivity appeared to derive from the local microstructure (i.e., aspartate vs glutamate residues in a peptide) [18]. Although the aggregates that are formed during the precipitation of COM from a simple ionic solution may follow a different process from the aggregation process in urine, mechanisms such as these may be involved in the generation the ''dumbbell'' geometry of COM crystals that often pass asymptomatically in the urine or of the crystal arrangements observed in stones.

In conclusion, the studies reported here demonstrate that a mixture of macromolecules isolated from the urine of normal individuals uniformly disaggregates COM crystals, whereas a significant proportion of similarly prepared macromolecules from stone former urine does not have this activity and often actually promotes aggregation. Among this group, the trait may be expressed in a familial fashion. A group of anionic macromolecules, both synthetic and natural and including some that are normally present in urine, also produces the disaggregation of COM crystals. Mixing a polyanion with the cationic macromolecules changes their behavior from disaggregation to aggregation promotion. We propose that these behaviors can be explained by interactions between individual macromolecules and specific crystal surfaces, by interactions between pairs of macromolecules, or both. This phenomenon may result in the prevention of crystal aggregation required for stone formation, and its lack may contribute to stone formation in a subset of stone formers.

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