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Adhesion Properties of Uric Acid Crystal Surfaces

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Supporting Information

ABSTRACT: Two key steps in kidney stone formation crystal aggregation and attachment to renal tissues—depend on the surface adhesion properties of the crystalline components. Anhydrous uric acid (UA) is the most common organic crystalline phase found in human kidney stones. Using chemical force microscopy, the adhesion force between various functional groups and the largest (100) surface of UA single crystals was measured in both aqueous solution and model urine. Adhesion trends in the two solutions were identical, but were consistently lower in the latter. Changes in the solution ionic strength and pH were also found to affect the magnitude



of the adhesion. UA surfaces showed the strongest adhesion to cationic functionalities, which is consistent with ionization of some surface uric acid molecules to urate. Although hydrogen-bonding and van der Waals interactions are usually considered to be dominant forces in the association between neutral organic compounds, this work demonstrates that electrostatic interactions can be important, particularly when dealing with weak acids under certain solution conditions.

INTRODUCTION

Recent estimates suggest that 8-12% of men (4-6% of women) in the United States^{1,2} and United Kingdom³ will develop kidney stones at some time in their lives. Kidney stones vary in their composition and size, but in general can be characterized as heterogeneous aggregates of micrometer-sized crystals held together by a small amount of organic matrix. The assembly of these macroscopic entities is not well understood but must include several key steps, including crystal nucleation, growth, aggregation, and attachment to renal tissue. Approaching the problem of kidney stone formation from a physical-chemical perspective requires detailed knowledge of the surface structure(s) of the individual crystalline building blocks and their adhesion properties in relation to other species present in the physiological fluid.

Over 200 different inorganic and organic crystalline phases have been identified in human kidney stones.⁴ Anhydrous uric acid (UA) is the most abundant organic crystalline phase, having been identified as the major component in 10% of stones and a minor component in 2% of mixed composition stones.⁵ High incidence rates of uric acid stones have been correlated with metabolic syndrome^{6–9} and as a result of chemotherapy treatment,¹⁰ but can also occur in otherwise healthy groups. Several other phases of uric acid, including the dihydrate, monohydrate, and various urate salts, have also been identified in human kidney stones, though typically as minor components.^{4,11–14}

The crystal structure of UA was first determined in the 1960s: space group $P2_1/a$ and unit cell dimensions a = 14.464(3) Å, b = 7.403(2) Å, c = 6.208(1) Å, and $\beta = 65.10(5)^{\circ.15}$ UA crystals grown from pure aqueous solution deposit as clear, colorless rectangular plates with large (100)

faces bound by (210), (201), (001), and sometimes (121) faces (Figure 1).¹⁶ Crystal sizes typically do not exceed \sim 200–300



Figure 1. Molecular structure and typical platelike morphology of anhydrous uric acid (UA) crystals grown from acidic aqueous solutions. Scale bar 100 μ m.

 μ m in the largest dimension. Physiologically derived crystals can have more varied morphologies, but typically are also platelike. Micrometer-sized crystals are handled effectively by properly functioning renal systems; however, macroscopic aggregates above a certain size (\geq 5 mm)¹⁷ become problematic. The adhesion properties of UA surfaces are therefore

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clearly important in both the attachment of crystals to renal epithelial cells and their aggregation to other crystals and/or matrix en route to stone formation.

Chemical force microscopy (CFM)¹⁸ has been used to study the adhesion properties of a broad range of materials, though the number of molecular crystal surfaces probed by CFM have to date been somewhat limited.^{19–27} Studies by Sheng et al.^{22,23} demonstrated that tips with ionic terminal groups had a significantly higher adhesion to the most prominent (100) face of calcium oxalate monohydrate (COM) than all calcium oxalate dihydrate (COD) surfaces. This observation was consistent with the greater propensity for COM to form stones. Adhesion studies in the presence of citrate and other urinary species further supported the role of urinary macromolecules in face-specific binding to COM surfaces.²² CFM methods have also been used to assess chemical functionality on cholesterol single-crystal surfaces.²⁰

In this study we used CFM to investigate adhesion on the largest plate face of UA single crystals under well-defined aqueous conditions and in model urine solutions. The adhesion forces between UA (100) and tips modified with various hydrophobic, hydrophilic, and charged groups were assessed in an effort to elucidate the most significant types of interactions on the surfaces that can occur under simulated physiologic conditions and how crystal aggregation, which is mediated by a matrix, occurs in vivo.

EXPERIMENTAL SECTION

Materials. Water was purified by passage through two Barnstead deionizing cartridges followed by distillation. McIlvaine buffers²⁸ with controlled pH and ionic strength were prepared from $C_6H_8O_7$ ·H₂O (99.0%, EMD), Na₂HPO₄ (99.5%, Fisher), and KCl (99.0%, Sigma). Artificial urine solution²⁹ was prepared from Na₂SO₄ (99.9%, Sigma), KCl (99.0%, Sigma), NH₄Cl (99.8%, EM Science), MgSO₄·7H₂O (98–102%, EM Science), Na₂HPO₄ (99.5%, Fisher), Na₂HPO₄·H₂O (99.1%, Fisher), NaCl (99%, EM Science), Na₃C₆H₅O₇·2H₂O (certified, Fisher Chemical), and urea (certified ACS, Fisher Chemical).

1-Dodecanethiol (DD) (\geq 98%, Aldrich), 11-mercapto-1-undecanol (MU) (97%, Aldrich), 11-mercaptoundecanoic acid (MUA) (95%, Aldrich), 4-mercaptobenzoic acid (MBA) (97%, Aldrich), 4-mercaptophenol (MP) (97%, Aldrich), 4-mercaptoaniline (MA) (97%, Aldrich), and (mercaptoethyl)guanidine (MEG) (\geq 98%, Sigma) were used in the preparation of chemically modified atomic force microscopy (AFM) tips. All chemical reagents were used as received without further purification.

Uric Acid Sample Preparation. UA single crystals were grown by dissolving 18-20 mg of uric acid (>99%, Sigma) in 100 mL of boiling distilled water.³⁰ The solution was buffered to pH 4.0 with sodium acetate (99%, EMD) and acetic acid (99.7%, EMD) and placed in a 37 °C (±0.1°) water bath for 48 h. UA crystals deposited as rectangular plates typically ~200-300 μ m in their longest dimension with large (100) faces (Figure 1).

UA crystals were mounted on 15 mm diameter coverslips with Loctite 5 min epoxy (Henkel Corp.). The quality and orientation of the crystal were established using conoscopy³¹ on an Olympus BX-50 polarizing microscope. The coverslip was then fixed to an AFM sample disk using epoxy and mounted in a small-volume liquid cell in a Digital Instruments Multimode Nanoscope IIIa instrument. All contact mode imaging was conducted at room temperature.

Chemical Force Microscopy. Commercial V-shaped 100–200 μ m Si₃N₄ cantilevers (Digital Instruments, Santa Barbara, CA) were coated by sputtering a 20 Å layer of chromium followed by 200 Å of gold. The gold-coated cantilevers were then functionalized by immersion in 2–3 mM ethanolic solutions of various thiols for 22 h (Figure 2).¹⁸ The cantilevers were subsequently rinsed with absolute ethanol and dried under nitrogen. The spring constant of individual



Figure 2. Chemical structures of thiols used in this study: 1-dodecanethiol (DD), 11-mercapto-1-undecanol (MU), 11-mercaptoundecanoic acid (MUA), 4-mercaptobenzoic acid (MBA), 4-mercaptophenol (MP), 4-mercaptoaniline (MA), (mercaptoethyl)-guanidine (MEG).

tips was determined using the reference cantilever method³² against a CLFC-NOBO tipless rectangular cantilever (Veeco Metrology) of known spring constant. Chemically modified tips had an average spring constant of 0.25 \pm 0.05 N/m.

All experiments on single-crystal UA were performed in unbuffered distilled water, artificial urine,²⁹ or McIlvaine buffer.²⁸ Topographical images of the UA (100) surface under fluid environments were obtained prior to force measurements. Individual force–distance curves were acquired at a rate of 2 Hz in relative trigger mode with a trigger threshold set to 20 nm. Over 500 individual force–distance curve measurements were acquired for each type of modified tip on at least 10 different locations per crystal. Individual deflection versus *Z*-position curves were converted into force–separation using Scanning Probe Image Processor (SPIP) software from Image Metrology (Lyngby, Denmark). Adhesion data were plotted in a histogram with the normal distribution curve defined by the average and standard deviation.

RESULTS AND DISCUSSION

UA crystallizes in a layered structure (Figure 3). Each layer in the *bc* plane consists of parallel ribbons of uric acid molecules hydrogen-bonded head-to-head $(O_2 \cdots H - N_1, 1.826 \text{ Å}, 175.0^\circ)$ and tail-to-tail $(O_8 \cdots H - N_7, 1.734 \text{ Å}, 155.8^\circ)$ with the ribbon plane nearly perpendicular to the (100) surface. No hydrogen bonding exists between ribbons within a layer, though ribbons in adjacent layers are also hydrogen bonded to one another to create a 3D network. The (100) surface therefore presents a 2D array of edge-on uric acid molecules with both H-bond donor (N-H) and H-bond acceptor (C=O) groups projecting from the surface. Previous in situ AFM work³³ showed that the (100) surface topography is fairly smooth with a high proportion of unit cell height steps (14 Å) and multiples thereof aligned parallel to the crystallographic *b* direction.

Adhesion force measurements between single-crystal UA (100) surfaces and seven different types of functionalized probe tips (Figure 2) were obtained under aqueous and model urine conditions. Accurate average forces can be derived from the statistical analysis of numerous force-distance curve data obtained under identical conditions. Statistical treatment of the data minimizes variations in the individual forces measured for



Figure 3. Crystal packing diagram for UA constructed from fractional coordinates in ref 15. (Top) Layers in the bc plane viewed down the c axis or parallel to the (100) plane. (Bottom) UA packing viewed normal to the (100) plane in which adjacent layers are colored blue and red to better show their relative orientation and near perpendicular orientation relative to the (100) surface.

any given tip-surface combination due to minor variations in tip shape and radius^{18,34} as well as difficulties in quantifying the exact geometry between the tip and the sample.³⁵

In our experiments, a minimum of 500 individual force curves were assessed for each tip—sample combination under a given set of solution conditions. At least three different UA crystals were used for each type of tip, and approximately 10 force curves were obtained at each point with a minimum of 10 points per UA sample. Most individual adhesion values fell within the normal distribution curve with only a few outliers with unusually high forces, which are presumably due to multiple contacts between the tip and UA sample. The average adhesion forces and standard error of the mean reported herein were calculated from all measured values.

Tips terminated with hydrophobic (methyl), hydrophilic (hydroxyl, amino), and ionic (amidinium, carboxylate) groups

cover a range of potential binding interactions that can occur in vivo between biomolecules and the UA surface. DD-, MU-, MEG-, and MUA-coated tips mimic alanine, serine, arginine, and glutamic acid side chains, respectively. Interactions with arenethiol functionalities (i.e., MBA, MP, MA) were also examined to assess whether steric factors contribute to the adhesion forces measured.

Adhesion Force Measurements in Distilled Water. Adhesion force measurements between UA (100) and the variously functionalized tips were first carried out in distilled water (pH 6.5 ± 0.4). Representative histograms appear in Figure 4 (the rest appear in the Supporting Information). A comparison of the adhesion forces in water also appears in Figure 5 (blue bars). Overall, the average adhesion force measured for the different tips varied by a factor of 3. The cationic MEG tip (2.22 nN) had an average adhesion force ~33% higher than that of any other type of tip. The three tips with ionizable groups, MA, MBA, and MUA, had the next highest forces in a similar range (1.62–1.70 nN). The two hydroxyl-terminated MP and MU tips were similar (1.29–1.34 nN), and the lowest adhesion was obtained from methyl-terminated DD tips (0.78 nN).

The significantly higher adhesion observed between cationic tips (MEG) and UA (100) we attribute to both charge-assisted hydrogen-bonding and electrostatic interactions. The pK_{a} of uric acid is 5.5.³⁶ In solutions where the pH > pK_a , one expects the majority of uric acid molecules in solution to be ionized to urate by loss of a proton at the N3 position.³⁷ Molecules in the bulk of a UA crystal must still be protonated regardless of the solution conditions; however, given the near perpendicular orientation of uric acid molecules relative to the UA (100) plane, it should be feasible to deprotonate at least some of the surface molecules, thereby imparting a partial negative charge to the surface. Previous electrophoretic mobility studies on UA particles indicate that UA crystal surfaces under some conditions bear a small negative charge.³⁸ It follows that cationic tips would therefore have the highest adhesion to these types of surfaces. The potential for an MEG tip to interact with surface uric acid molecules with varying protonation states on a given UA (100) surface may also help to explain the comparatively larger number of individual adhesion measurements that were 2+ standard deviations above the mean.

The ionization state of amino-terminated thiols is dependent on both the solution pH and whether they are free in solution or bound to a substrate. The pK_1 of protonated MA in solution is 4.3; however, when bound to a surface, the pK_a is estimated to shift to 6.9 ± 0.5 .³⁹ Under the CFM conditions used (pH 6.5), the MA tip is presumably neutral. This would enable it to act as a hydrogen bond donor at the tip-crystal interface but not form complementary charged pairs with surface urates. Aliphatic carboxylic acids such as MUA typically have a pK_{a} of ~4.8 in solution, but reportedly shift to higher values of ~5.2 when bound to a surface.⁴⁰ The pK_a of aromatic carboxylic acids such as MBA is 5.5 in solution but shifts to 7.0 when bound to a surface. 41 In a pH 6.5 solution, one expects MBAcoated tips to be protonated but MUA-coated tips to be partially or fully ionized to carboxylate. The different ionization states should affect the type of interactions at the tip-crystal interface, with only the former able to act as both a hydrogen bond acceptor and a hydrogen bond donor. However, the overall adhesion properties for these two tips were similar.

Tips with hydroxyl end groups (i.e., MP and MU) can hydrogen bond to surface uric acid molecules, though the



Figure 4. Frequency distribution of adhesion forces between the UA (100) face and different thiol-coated tips performed in distilled water with pH \approx 6.5: (a) MEG, 2.22 \pm 0.04 nN, (b) MUA, 1.62 \pm 0.02 nN, (c) MU, 1.29 \pm 0.04 nN, (d) DD, 0.78 nN \pm 0.02.



Figure 5. Adhesion forces measured between UA (100) and different functionalized tips measured in distilled water (blue bars) and in artificial urine solution (red bars).

strength of the alcohol hydrogen bonds tends to be weaker than that of the carboxyl hydrogen bonds. That there are only minor differences in adhesion between aliphatic and aromatic tips suggests that steric factors do not significantly affect the adhesion measurements. The lowest mean adhesion force of 0.78 nN observed with the DD methyl-terminated tip was expected, given the polar nature of the UA surface and the nonpolar nature tip.

Adhesion Force Measurements in Model Urine Solution. In an effort to better assess the adhesion properties under physiologic conditions, adhesion was reexamined in model urine solution. Urine is a complex fluid whose composition and concentration vary greatly depending on a variety of factors, including diet, exercise, and degree of hydration. The model urine used in this study was derived from an established standard²⁹ consisting of Na₂SO₄ (14.9 mM), KCl (92.6 mM), NH₄Cl (65.1 mM), MgSO₄·7H₂O (6.7 mM), Na₂HPO₄ (1.8 mM), Na₂HPO₄·H₂O (39.6 mM), NaCl (213.9 mM), Na₃C₆H₅O₇·2H₂O (2.7 mM), and urea (291.4 mM). The model urine solution had a pH of 5.0–5.10, and its ionic strength was ~0.5 M.

Over 1200 individual force curves between each type of thiol and UA (100) surfaces were next collected in artificial urine solution. At least six different crystal samples were used for each type of tip. The values obtained are plotted in Figure 5 (red bars), and histograms for each tip-surface combination are found in the Supporting Information. Adhesion forces measured in artificial urine were all lower than the corresponding forces in aqueous solution. In most cases, adhesion was reduced by 38-47% in model urine, the exception being that for the DD tips, which decreased by only ~13%. Aqueous solution and model urine differ in two key respects: the latter has a lower pH (5 vs 6.5) but a much higher ionic strength. Notably, despite the differences in the solutions, the trends in the relative forces were identical in water and model urine (e.g., MEG > MA, MBA, MUA > MP, MU > DD).

Other Factors. To assess the relative contribution of pH and ionic strength, we examined adhesion of MEG, MUA, and DD tips in a series of McIlvaine buffers ($C_6H_8O_7$ ·H₂O,

 Na_2HPO_4) prepared at pH 5, 6, and 7. The advantage of using this buffer system is that KCl could be added to each solution to maintain a constant ionic strength (IS) of 0.5 M. The IS of human urine typically ranges from 0.3 to 0.6 M.⁴²

Both DD and MEG tips showed little variation in the average adhesion over this pH range under fixed IS conditions. The ionization state of the tips does not change over this range: DD is always neutral, and MEG is always cationic. Adhesion forces between MUA tips and UA (100) decreased by 13% from pH 5 to pH 6. If one assumes the UA surface maintains the same negative surface charge over the pH range examined, the reduction in MUA adhesion at elevated pH can be explained by changes in the ionization of the tip. With increased pH, one expects a greater percentage of the COOH groups in MUA to be deprotonated to COO⁻ and a consequent reduction in this tip's adhesion to a negatively charged UA surface. Other factors may also contribute to the reduction in adhesion at higher pH. Previous studies on UA particles showed that the surface electric potential increases over a pH range of 2.0-6.5, although the increase is fairly minor in the upper pH 5-6.5 range.³⁸ Also worth noting is that there was some difficulty in obtaining measurements at higher pH values since UA solubility increases exponentially when the pH > pK_a .⁴³ The changing uric acid solution concentration at higher pH may also influence the adhesion observed at a given pH. Adhesion measurements obtained in McIlvaine buffer were lower than the analogous measurements obtained in distilled water with equivalent pH and more comparable in magnitude to those obtained in model urine.

We attempted to assess the influence of ionic strength on adhesion by measuring the interactions between a nonionizable MU probe at pH 5 in McIlvaine buffers with ionic strengths ranging from 0.3 to 0.7 M. Only a modest increase in adhesion forces was observed from 0.70 nN (0.3 M) to 0.80 nN (0.5 M) and 1.03 nN (0.7 M). Again, the magnitude of the force in these various ionic strength solutions was more comparable to forces obtained in model urine. Both pH and ionic strength clearly influence the magnitude of adhesion with some types of functionalities. It is well-known that the pH and ionic strength of actual urine can vary significantly. Presumably adhesion to renal epithelial cells and/or aggregation of UA to other particulate matter occurs more/less readily under some local conditions than others.

CONCLUSION

Chemical force microscopy was used to directly quantify the adhesion between UA (100) surfaces and various types of chemical functionalities. Measurements obtained in distilled water and model urine showed similar trends, with the highest adhesion found between UA (100) and cationic surfaces. The magnitude of any force was found to be very dependent on the mediating solution. Ionic strength and pH are clearly influential solution parameters, though other factors may also affect the magnitude of the adhesion.

That UA crystal adhesion to cationic surfaces was higher than that to anionic surfaces highlights a major difference between molecular crystal surfaces (in this case of a weak acid) and most other inorganic biominerals. The latter typically are thought to interact through strong electrostatic interactions with other charged species, both anionic and cationic. For small-molecule organic crystals, the types of intermolecular interactions are usually considered to be weaker, e.g., typically some combination of hydrogen-bonding and/or van der Waals forces. A previous study by Koka et al. on the adhesion of UA crystals to renal epithelial cells⁴⁴ concluded that hydrogen bonding (rather than ionic bonding) plays a major role in UA crystal–cell interactions under conditions where UA is electrically neutral. What the present study reveals is that electrostatic interactions can also be significant, particularly when the solution conditions alter the ionization state of the crystal surface.

ASSOCIATED CONTENT

Supporting Information

Histograms obtained for tip-surface combinations measured in water, model urine, and McIlvaine buffer. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Notes

The authors declare no competing financial interest.

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