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THE NUCLEATION AND GROWTH OF CALCIUM OXALATE MONOHYDRATE ON SELF-ASSEMBLED MONOLAYERS (SAMS)

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Abstract

A physical chemical approach was used to study calcium oxalate monohydrate (COM) nucleation and growth on various organic interfaces. Self-assembling monolayers (SAMs), containing derivatized organic functional groups, were designed to mimic various amino acid residues present in both urine and stone matrix macromolecules. Derivatized surfaces include SAMs with terminal methyl, bromo, imidazole, and thiazolidine-carboxylic acid functional groups. Pronounced differences in COM deposition were observed for the various interfaces with the imidazole and thiazolidine surfaces having the greatest effect and the methyl and bromo groups having little or no nucleating potential.

Key Words: Calcium oxalate, self-assembling monolayers, nucleation, urolithiasis.

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Introduction

Understanding the mechanisms of biomineralization continues to be an important area of research in biology, chemistry, medicine, and dentistry [11, 19, 21]. Crystal growth processes in nature are especially interesting and challenging since it is often difficult to understand the role of solution speciation, ionic strength, and impurity concentrations in the various physiological environments. Pathological mineralization may result in clinical conditions such as urinary stones, dental and salivary calculi, and gallstones. Additionally, crystal growth processes are involved in the formation of hard tissues such as bone and teeth [7, 17].

The crystallization of sparingly soluble salts in the physiological realm usually involves heterogeneous nucleation onto preexisting surfaces. For example, the formation of hard tissue begins with the deposition of biopolymers and/or protein molecules, which provide the framework for subsequent mineral deposition. Similar mechanisms may also be operative in pathological urolithiasis. However, the underlying causes of idiopathic urinary stone formation are still not completely understood. Stone formation is thought to be caused, in part, by the high supersaturation of urine with respect to the stone forming minerals including calcium oxalate mono- (COM), di-(COD), and tri-(COT) hydrates. Another important component of renal stones is the organic matrix, a mucoid substance which has been found to contain mucoproteins, mucopolysaccharides, calcium, phosphate, and oxalate ion bound to the matrix [5]. The role of the matrix in the formation of stones has been the subject of considerable discussion. Initially, urinary macromolecules were believed to be important in the deposition of the mineral phase, thereby producing the banded organic/inorganic layering found in many stones [16]. Later, coprecipitation with the mineral phase was suggested as an important relationship between matrix and mineral components. However, recently the active matrix theory [2-4, 8, 20] proposed that the organic material, which usually accounts for 2-5% of the stone mass, is the source of mineral nucleation.

Although only partial characterization of the organic matrix of stones has been accomplished, the

isolated macromolecules usually tend to be water soluble and contain a large percentage of polar functional groups. The mucoprotein component contains both aspartic and glutamic acid residues as well as a small amount of γ-carboxyglutamic acid, which has a high calcium binding affinity [14]. The mucopolysaccharide component of the matrix has been found to be composed, in part, of glycosaminoglycans (GAGs) which contain either glucosamine or galactosamine as well as monosaccharide residues possessing R-N-SO_3H and/or R-O-SO_3H moieties [9, 15]. In addition to these macromolecules, there are many polypeptides and amino acids which have been identified in the stone matrix, several of which are listed in Table 1. As important as the type of functional groups found in the urinary macromolecules, the molecular structure and arrangement of sites may also be important. These parameters govern the orientation and/or the availability of active ion binding sites. The particular arrangement of functional groups may facilitate adsorption and/or binding of cations/anions at the organic interface, thus offering sites for subsequent nucleation and growth [6].

Clearly, there is still much to be learned about the mechanism of biological calcification involving calcium oxalate phases as related to problems of urolithiasis. Our approach is to understand the role of proteins and other molecules on the nucleation and growth of calcium-containing salts *in vitro* since this should provide insight on the relationship between the inorganic and organic phases involved in biological mineralization *in vivo*.

To accomplish this, model organic interfaces, in which the chemical functionality and site density is known, are required in order to understand the complex chemical nature of interactions between the mineral and organic phases of renal stones. The use of self-assembled monolayers (SAMs), which form highly ordered, two dimensional arrays due to van der Waals interactions, offers such a system [18]. Surface functional group type as well as site density can be systematically altered using this technique. Once synthesized and characterized, the SAMs can then be used to study the effects of various surface properties (acidic, basic, polar, nonpolar) on COM nucleation and growth.

Although, this is certainly a simplistic view of the mechanisms involved in urolithiasis, it offers a methodology that divides the complex role of nucleation proteins down into more fundamental chemical components. Thus, the interactions between nucleation proteins and the forming mineral may be more clearly understood.

Materials and Methods

SAM Synthesis

The attachment of SAMs to silicon wafers (polished on one side with a uniform oxide layer 20-25 Å thick) was accomplished as follows. Prior to SAM formation, the wafers were cut into approximate sizes (1.25 x 2.5 cm), placed into teflon racks and ultrasonicated 2-3 minutes in chloroform to remove bulk organic contamination. Any residual trace level of organic contamination was then removed by exposure to an air plasma for 10 minutes. Hydroxylation of the clean wafers was performed by treatment with 0.1 M KOH solution for several minutes. The resulting siloxides were then protonated by soaking in a dilute HNO_3 solution for 10 minutes. Following the acid soak, the wafers were thoroughly washed with deionized water and blown dry with a stream of dry nitrogen. The wafers were used promptly thereafter.

SAM synthesis was accomplished using a trichlorosilane coupling agent. A 15-20 mM solution of a bromine terminated alkyltrichlorosilane, of the desired chain length, in dry cyclohexane (dried over H_2SO_4 and distilled from CaH_2) was prepared and protected from atmospheric moisture in an oven-dried mason jar. The wafers were soaked in this solution for 30-60 minutes at room temperature with stirring. Upon removal from the chlorosilane solution, the silicon substrates were immediately immersed in 2-propanol (Aldrich) in order to quench any superficially associated chlorosilane. The wafers were then sonicated in chloroform for 5 minutes, resulting in mirror bright surfaces. The bromine end group then functions as an intermediate for S_N2 displacement reactions for the desired functional end groups. SAM surfaces were characterized by contact angle measurements, ATR-FTIR (attenuated total reflectance - Fourier-transformed infra-red spectroscopy), X-ray photoelectron spectroscopy (XPS), and ellipsometry.

Table 1. The acid/base characteristics of selected amino acid residues and SAM functional groups.

Molecule	pKa Values[c]	Log K_f[d]
Amino acid		
Aspartic acid	2.09 (α-carboxyl) 3.86 (β-carboxyl) 9.82	1.60[a]
Glutamic acid	2.19 (α-carboxyl) 4.25 (γ-carboxyl) 9.67	1.43[a]
Histidine	1.82 6.00 (imidazole) 9.17	0.95[a]
Proline	1.99 10.60	
SAM		
Imidazole	7.01[b]	0.1
Thiazolidine	1.51[b] 6.11[b]	1.66[a]

[a]from reference 1; [b]from reference 12;
[c]from reference 10; [d]K_f=formation constant.

COM Nucleation

Solutions prepared using reagent grade chemicals (Fisher Scientific) and deionized, reverse osmosis (Millipore), CO_2-free water were filtered {0.22 μm Millipore (Millipore, Bedford, MA) filters} before use. The filters were prewashed to remove any residual wetting agents or surfactants. Calcium ion concentrations were determined by ion exchange using a cation-exchange column (Dowex) followed by the potentiometric titration of the exchanged hydrogen ion with standardized potassium hydroxide solution.

Nucleation and growth experiments were carried out in sealable glass containers placed in a constant temperature bath (37 $\pm$ 0.1°C). A calcium ion selective electrode (Orion) was calibrated by the addition of aliquots of calcium chloride solution to deionized, CO_2-free water in the reaction vessel. Supersaturated solutions ($T_{Ca} = T_{Ox} = 4.00 \cdot 10^{-4}$ mol/l, pH = 6.5-7.0) were prepared by the slow addition of potassium oxalate solution to the calcium chloride solution. An ionic strength of 0.15 mol/l was maintained by the addition of stock sodium chloride solution. When equilibrium had been attained, as apparent by a constant emf value from the calcium ion selective electrode, a rack containing the derivatized SAM surfaces was placed into the solution. The reaction vessel was then sealed and placed into the constant temperature bath and stirred throughout the experiment (24-72 hours). At the conclusion of the crystallization experiment, the surfaces were removed from the mineralizing solution, rinsed and ultrasonicated in deionized water and blown dry with a stream of dry nitrogen gas. Samples were then cut into sections and examined by scanning electron microscopy (Electroscan) and energy dispersive X-ray analysis (Link Analytical).

Periodically, aliquots of the reaction mixture were withdrawn and examined for bulk solution growth by optical and scanning microscopy. If particles were present, the experiment was terminated. For the experiments reported in this paper, no bulk precipitation was observed throughout the reaction time.

Results and Discussion

In order for crystallization to occur, the solution must be supersaturated with respect to the precipitating mineral phase. For an inorganic, ionic salt, the crystallization driving force may be expressed as:

$$\Delta G = -RT \ln S \qquad (1)$$

where ΔG is the Gibbs free energy of formation, R is the gas constant, and S is the supersaturation. In the case of calcium oxalate monohydrate, the supersaturation may be written in terms of the lattice ion activities as:

$$S = (IP/K_{sp})^{1/2} \qquad (2)$$

where $IP = (Ca^{2+})(C_2O_4^{2-})$ and K_{sp} is the solubility product ($2.28 \cdot 10^{-9}$ l^2 mol^{-2} at 37°C). The relative supersaturation, σ, can then be expressed as:

$$\sigma = S\text{-}1 = (IP/K_{sp})^{1/2} - 1. \qquad (3)$$

To determine the activities of ionic species, calculations were performed as described by Nancollas [13] using expressions for mass balance, electroneutrality, and the equilibrium and ion pair association constants for the formation of various species including calcium oxalate (CaOx) and sodium oxalate ($NaOx^-$) ion pairs.

Mineralization experiments were done on a variety of SAM surfaces possessing different chemical functionality and site density. Figure 1 shows the molecular structure of the terminal end of the SAM surfaces used in this study. The thiazolidine molecule which has carboxyl, thiol, and amine functionality as well as a chiral carbon is a derivative of the amino acid proline. Imidazole contains a secondary amine and is similar in structure to the amino acid histidine which has been identified in urinary proteins. The methyl terminated SAM is similar to the methyl group found in the alanine amino acid residue. Additionally, another non-polar surface group, bromine, was investigated. Although this molecule is not biologically relevant, its chemical characteristics may add additional insight into the important parameters for calcium oxalate nucleation. Also, both the methyl and bromine terminated end groups provide non-polar and surfaces in contrast to the polar, hydrophilic thiazolidine and imidazole surfaces.

The efficacy of a surface to induce mineralization was related to the number of crystallites formed per unit area of the surface. For this experiment, the surfaces were placed into the same supersaturated calcium oxalate solution in order to assure that the driving force for nucleation, fluid dynamics, and temperature were the same for each substrate. Figure 2 shows a graph of crystallite density for the bromine, methyl, imidazole and thiazolidine SAM surfaces. The apparent differences in particle density may be due to the type of active site at the organic interface. Both thiazolidine and imidazole are polar, hydrophilic molecules as demonstrated by their contact angle with water (pH = 7) of 30° and 65° $\pm$ 1°, respectively. Both the bromo and methyl terminated SAMs are hydrophobic and have water contact angles (pH = 7) of 81° and 110° $\pm$ 1°, respectively. Thus it appears the nucleation of COM is affected by the polar nature of the organic interface. It is interesting however, that the thiazolidine surface, with its acidic carboxyl group and lower contact angle, did not induce COM mineralization to the extent of the imidazole surface. This may be due, in part, to the orientation of the carboxylic acid group on the thiazolidine ring. Examination of molecular models shows that the carboxylate group, attached to the carbon alpha to the nitrogen atom, is most probably positioned down away from the surface and thus would be partially shielded from solution calcium ions. Contact angle data supports this theory since the thiazolidine surface has a much higher contact angle with water (30°) as compared to zero degrees for monolayers in which the carboxyl group is orientated on the outermost layer [16].

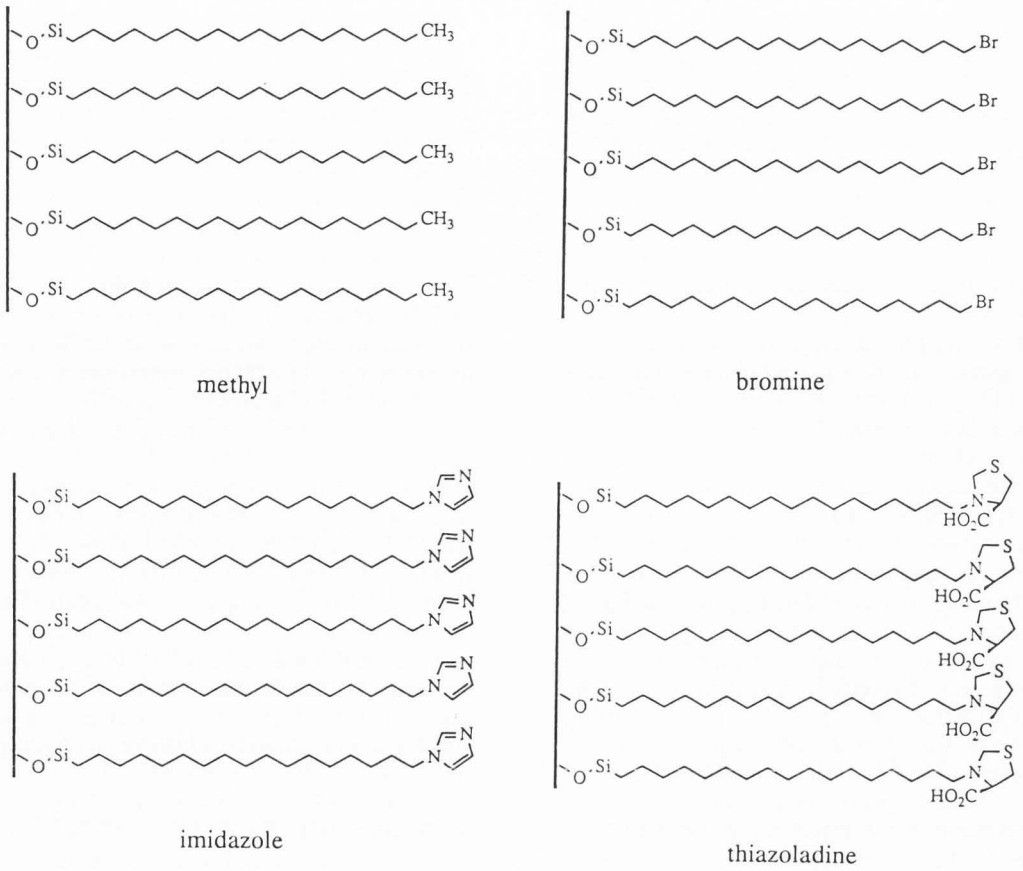


Figure 1. Schematic drawings of SAM interfaces used in the calcium oxalate monohydrate nucleation experiments.

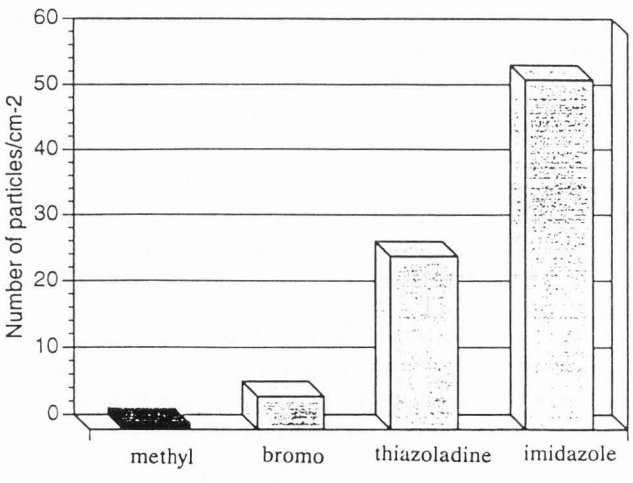
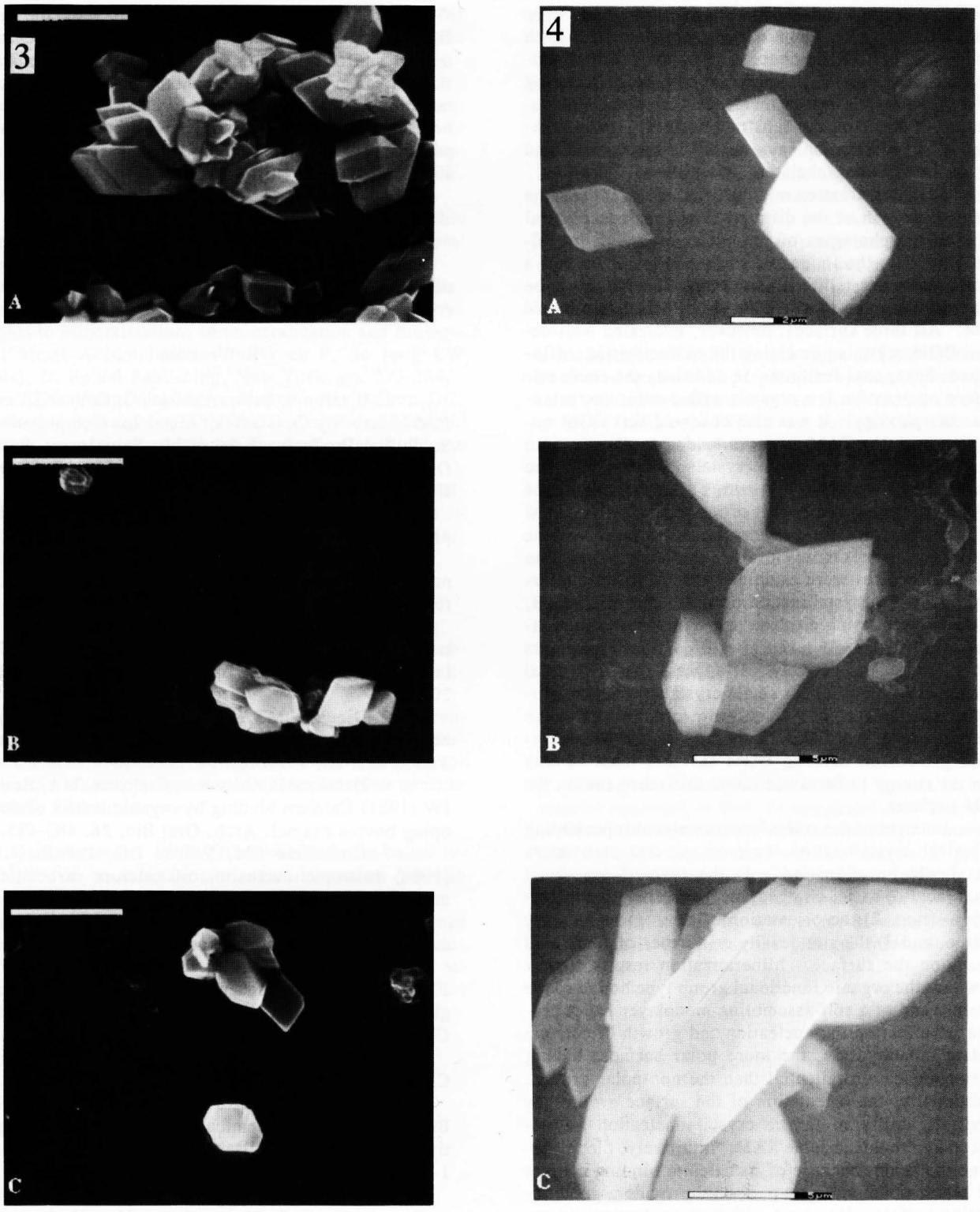


Figure 2. Nucleation and growth of calcium oxalate monohydrate as a function of SAM terminal group type.

A second possible explanation for the increased crystallite growth on imidazole is its calcium binding affinity [1]. Both the pKa's and log K_f of the SAM end group molecules as well as selected amino acids are listed in Table 1. The value of the pKa's for histidine and imidazole are relatively close. When an alkyl chain is substituted for the amine hydrogen on the imidazole molecule, the pKa increases from 7.01 to 7.22 [12]. Thus, at the pH of the mineralization solution the imidazole is not protonated (is uncharged). This is somewhat contrary to the idea that, in order to promote crystal nucleation, it is advantageous to have a charged (either acidic or basic) template. Therefore, the ability of the imidazole surface to nucleate calcium oxalate must be due to some other chemical interaction. As shown in Table 1, the log K_f for the neutral imidazole molecule and Ca^{2+} is 0.1. Although this is a weak interaction, the binding affinity of a surface with a high imidazole loading density may provide large enough interaction with the calcium ion to induce COM deposition. Future experiments are planned to study the effect of loading density of surface functional groups on COM nucleation.



Figures 3-4. Scanning electron micrographs of COM crystals nucleated onto a **(3A)** imidazole, **(3B)** bromine, **(3C)** thiazolidine terminated SAM, and **(4)** imidazole SAM after **(4A)** 13, **(4B)** 38 and **(4C)** 63 hours.
Bars = 12 μm (in 4a), and 5 μm (in rest of the micrographs).

The methyl and bromine terminated SAMs were poor nucleators of calcium oxalate monohydrate. Both surfaces are quite hydrophobic and do not possess functional groups that have any calcium binding or chelating ability. This adds further evidence that, for COM nucleation to occur on the substrate surface, it is advantageous to utilize a molecule which is hydrophilic and has calcium binding/chelating potential.

Scanning electron micrographs, taken at the same extent of growth of the different SAM surfaces, reveal different morphologies of the nucleated COM crystallites. Clearly, the imidazole surface (Figure 3A) has a much greater crystallite density than either the bromine (Figure 3B) or the thiazolidine (Figure 3C) terminated SAM. All three surfaces, however, nucleated well-defined COM crystals possessing the characteristic coffin-shaped, hexagonal features. In addition, the imidazole surface nucleated a few crystals with a cubic or rectangular morphology. It was also observed that COM nucleation onto the SAM surfaces tended to develop into aggregates rather than single crystals. This may be due to the energy barrier for growth on an existing COM surface being lower than that of nucleation on the SAM surface. In order to examine the development of the forming aggregates more closely, experiments were done in which surfaces were examined at various time intervals during the crystallization process. Figures 4A, B, and C show scanning electron micrographs of an imidazole terminated SAM taken after 15, 38, and 63 hours reaction time, respectively. It appears that the initial COM nucleation produces single crystals evenly distributed across the surface, which grow into clusters as the reaction proceeds. Thus the energy required for epitaxial growth on existing COM surfaces must be less than the energy to form additional critical nuclei on the SAM surfaces.

In terms of the role of macromolecules promoting biological crystallization there are several parameters that should be addressed: **1**) the type of functional group (acidic, basic, charged, etc.) necessary for mineral deposition, **2**) the orientation of these groups on the surface, and **3**) the site density or number of functional groups on the surface. Mineralization results clearly show that the organic functional group type bound to the terminal end of a self-assembling monolayer had a pronounced effect on the nucleation and growth of calcium oxalate monohydrate, the more polar surfaces having greater nucleation potential than the non-polar groups. Additionally, the orientation of the surface group may affect its ability to induce crystal nucleation as evidenced by the thiazolidine SAM. Intuitively, the thiazolidine molecule, because of its calcium binding affinity and acidic functional group, should provide a good nucleating surface. However, although nucleation did occur on the thiazolidine SAM, it was not as effective a nucleating surface as the imidazole SAM. This is probably due to the carboxyl group being oriented down and away from the SAM surface.

These preliminary results clearly show the benefits of using self-assembling monolayers as well characterized, uniform surfaces to study the influence of functionality on crystallization phenomena. Thus, a complex macromolecule may be divided into its simpler components in order to understand the contribution of each aspect of the molecule in the promotion of nucleation from solution.

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Discussion with Reviewers

W.G. Robertson: How long does it take to produce observable nucleation of calcium oxalate under the conditions specified by the authors?
Authors: The amount of time for the onset of observable calcium oxalate nucleation varied between the different surfaces. The fastest being the imidazole and thiazolidine SAMs which showed calcium oxalate after approximately 24 hours.

W.G. Robertson: What effect would the so-called inhibitors of crystallization of calcium oxalate have in such a system?
Authors: We have looked at some amino acid groups both immobilized on a surface as well as free in solution. It was found that when the amino acid was attached to a surface it was an effective nucleator but when free in solution, the same molecule retarded calcium oxalate growth.

J.P. Binette: The early promising results of this study again testify to the correlation of structure and function. As the authors expand this research, would they consider (1) adding complexing amino acids, acidic and basic; (2) adding "inhibitors" (e.g., magnesium) to their *in vitro* system?
Authors: Yes, we have already begun to look at single amino acid groups such as cysteine attached to the SAM surface. We are expanding to other residues as well as dipeptides. It would be very interesting to look at the effect of other ions added to the system to investigate competitive electrostatic interactions at the surface as well as their affects on the overall nucleation and growth characteristics.

S.A. Smesko: Could the technique of SAM fabrication be altered to allow the preparation of monolayers of proteins and macromolecules taken from fractionated urine or stone samples? Would the surfaces be able to be characterized as to functional groups and/or group density?
Authors: We are currently working on the attachment of small proteins and amino acid groups. Functional group characterization is done by X-ray photoelectron spectroscopy and solid state NMR.

S.A. Smesko: Could SAMs having mixed functionalities be prepared so that the competition or synergism among/between functionalities as nucleation and/or growth sites could be addressed.
Authors: We are presently working with the gold thiol SAMs in order to prepare mixed monolayers of various functionalities.

Y. Nakagawa: Many mineralization reports including articles appeared in Ref. 11 suggested that amino acid residues are important for calcium binding. I wonder if the nucleation experiments were carried out at pH 5.7-6.0, which is the actual urine pH, how do these imidazole containing surfaces act for nucleation?
Authors: Mineralization experiments were done at pH 6.2 which is very near the pKa of imidazole (pKa = 7.01).