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Attachment of Calcium Oxalate Monohydrate Crystals on Patterned Surfaces of Proteins and Lipid Bilayers

Zhihua An,[†] Soolim Lee,[†] Harry Oppenheimer,[†] Jeffrey A. Wesson,[‡] and Michael D. Ward*,[†]

Department of Chemistry and Molecular Design Institute, New York University, 100 Washington Square East, New York, New York 10003-6688, and Consultant Care Division, Department of Veterans Affairs Medical Center, 5000 West National Avenue (111K), Milwaukee, Wisconsin 53295

Received July 13, 2010; E-mail: mdw3@nyu.edu

Abstract: The attachment of calcium oxalate monohydrate (COM) crystals to renal tubules is thought to be one of the critical steps of kidney stone formation. Patterns of phosphatidylserine (DPPS) bilayers and osteopontin (OPN) were fabricated on silica substrates through the combination of a microcontact printing technique and fusion of lipid vesicles to create spatially organized surfaces of lipids and proteins that may mimic renal tubule surfaces while allowing direct visualization of the competition for COM attachment to compositionally different regions. In the case of DPPS−OPN patterns, micrometer-sized COM crystals dispersed in saturated aqueous calcium oxalate solutions attached preferentially to the OPN regions, in agreement with other in vitro studies that have suggested a binding affinity of OPN to COM crystal surfaces. COM crystals attached with nearly equal coverage to OPN and DPPS surfaces alone, suggesting that the preferential segregation of COM crystals to the OPN regions on the patterned surfaces reflects reversible attachment of micrometer-sized COM crystals capable of Brownian motion. These attached microcrystals then grow larger over time during immersion in the supersaturated calcium oxalate solutions. Free OPN, a major constituent in urine, adsorbs on COM crystals and suppresses attachment to DPPS, suggesting a link between OPN and reduced attachment of COM crystals to renal epithelium. This patterning protocol can be expanded to other urinary molecules, providing a convenient approach for understanding the effects of biomolecules on COM crystal attachment and the pathogenesis of kidney stones.

The primary constituent of kidney stones in patients with urolithiasis is calcium oxalate monohydrate (COM).[1] Previous investigations have suggested that osteopontin (OPN), a urinary protein with substantial anionic character, is a potent inhibitor of COM crystallization.[2] In vitro studies revealed that renal cell membrane and renal brush-border membrane vesicles are involved in crystallization of calcium oxalate, suggesting that lipids also play a critical role in kidney stone formation.[3] Reports of COM nucleation and growth on self-assembled lipid monolayers revealed that the negatively charged phosphatidylserine (PS) interface is a strong promoter of COM crystallization.[4] Understanding the effects of urinary proteins and lipids, among other biomolecules, on COM crystallization is essential for determining their roles in stone formation and unraveling the mechanism of stone formation.

The attachment of COM crystals to renal tubules is one of four critical steps of stone formation (nucleation, attachment, growth, and aggregation). OPN has been suggested to play a role in stimulating adhesion of crystals to cells in the early stages of urolithiasis,[5] but OPN also has been suggested to suppress crystal attachment to cell membranes because of repulsive interactions between the anionic side chains on OPN adsorbed on crystal surfaces and renal tubular cell surface receptors, which contain anionic phospholipids, sialic acid-containing glycoproteins, and hyaluronan.[6] Consequently, the role of OPN in COM crystal attachment to cell membrane remains unclear. Langmuir monolayers, surfactants, and vesicles have been used as model systems to study the formation of calcium oxalate stones.[3,4,7] These systems, however, do not always capture the structure of the fluidic cellular membrane in the presence of other biomolecules, particularly when spatial organization and competitive binding among various binding sites, including protein adsorbed on either crystal or epithelial surfaces, is an important factor. Herein we describe a methodology based on patterned surfaces that enables a direct comparison of the affinity of COM crystals for 1,2-dipalmitoyl-*sn*-glycero-3-phospho-L-serine (DPPS) bilayers and OPN, which has revealed that OPN binds strongly to COM and that the attachment of COM to DPPS is suppressed when free OPN in solution adsorbs on the COM surface.

Patterned surfaces of DPPS bilayers and OPN were created through a combination of microcontact printing[8] and fusion of lipid vesicles,[9a] a protocol that has been used for the fabrication of patterned surfaces of phosphatidylcholine bilayers and bovine serum albumin.[9b] Micrometer-wide grids, squares or stripes of OPN, labeled with Texas Red, were prepared on a silicon wafer using polydimethylsiloxane (PDMS) stamps fabricated by standard photolithography methods. Subsequent exposure of the OPN-patterned surface to unilamellar vesicles comprising DPPS and 2 mol % 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-carboxyfluorescein (DOPE-CF) generated lipid bilayer membranes in corrals surrounded by the existing OPN grids through fusion on the bare regions of the substrate (Figure 1). Excess vesicles and weakly bound protein were then removed by thorough rinsing with water.

The fidelity of the DPPS−OPN pattern formation was confirmed by epifluorescence microscopy, which revealed the carboxyfluorescein chromophore (green) in the lipid regions and the Texas Red chromophore (red) in the OPN regions (Figure 2). Atomic force microscopy (AFM) images of OPN patterns alone on silicon revealed that the height of the OPN gridlines was ∼2 nm with respect to the underlying surface (Figure S1 in the Supporting Information). This measurement, which is consistent with a single layer of immobilized OPN molecules, confirms the persistence and integrity of the printed OPN regions.

COM crystal attachment to DPPS and OPN was investigated by immersion of a patterned silicon wafer in an aqueous solution containing 150 mM NaCl and 1 mM calcium oxalate (relative

[†] New York University.
[‡] Department of Veterans Affairs Medical Center.

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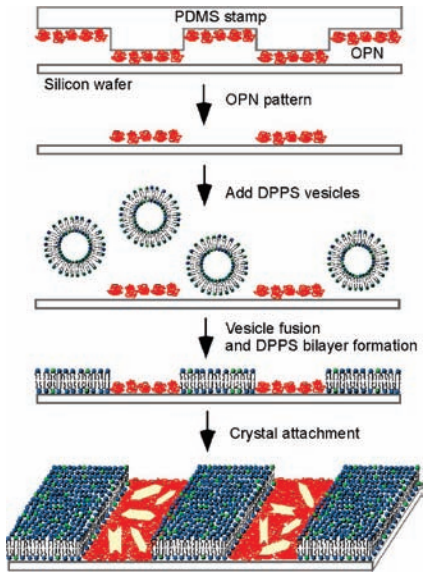

Figure 1. Scheme for attachment of COM crystals to a DPPS−OPN-patterned surface.

supersaturation $\sigma = 5$) at 60 °C contained in a sealed reservoir. This temperature was chosen in order to maintain the DPPS bilayer in a fluid state (the solid−fluid phase transition temperature of DDPS is $T_m = 54$ °C). Circular dichroism of OPN solutions revealed no detectable differences in the protein conformation under these conditions compared with room temperature. The wafer was immersed in a vertical orientation to avoid gravitational settling of crystals. After 24 h the wafer was removed from the reservoir, and optical microscopy revealed selective attachment of COM crystals to the OPN regions (Figure 3), independent of pattern dimensions and configuration (i.e., grids, squares or stripes). Additional investigations revealed that attachment of the COM crystals actually occurred immediately upon immersion, with segregation of COM crystals on the OPN regions becoming clearly distinct after 60 min (Figure S2).

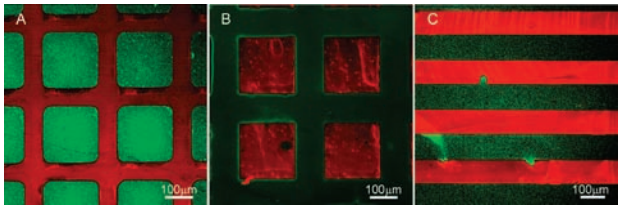

Figure 2. Epifluorescence of DPPS−OPN-patterned surfaces. The DPPS bilayer was labeled with 2% DOPE-CF (green) and OPN with Texas Red. (A) DPPS squares (200 μm) bordered by grids of OPN (70 μm wide). (B) OPN squares (200 μm) bordered by DPPS grids (100 μm wide). (C) Alternating stripes of DPPS and OPN (100 μm wide).

The size of COM crystals attached to the OPN regions increased with substrate immersion time. At the early stages ($\sim$ 2 min), the COM crystals attached to the OPN regions measured 3 μm in length on average. These attached crystals grew to nearly 6 μm after 60 min (Figure S2) and 10−20 μm after 24 h (Figure S3). COM crystals collected from fresh 1 mM calcium oxalate solutions measured $\sim$3 μm in length (Figure S4A), suggesting that the crystals observed on the OPN regions attached as mature crystals, migrating to these regions through Brownian motion[10] rather than growing from nuclei that had formed preferentially on OPN. The increase in size of the attached crystals at longer immersion times was consistent with the sizes of crystals harvested from the solution at periodic intervals (Figures S3 and S4B).

Interestingly, under the same conditions used for the patterned substrates, COM crystals attached with equal coverage to a bare silicon wafer and wafers coated with OPN or DPPS alone (Figure S5). These observations suggest reversible binding of COM microcrystals at the early stages coupled with Brownian motion, resulting in preferred segregation of the crystals on the OPN regions. The unique binding preference of COM for OPN was further revealed by uniformly dispersed COM crystals on patterns of human serum albumin (HSA) and the DDPS bilayer (Figure S6).

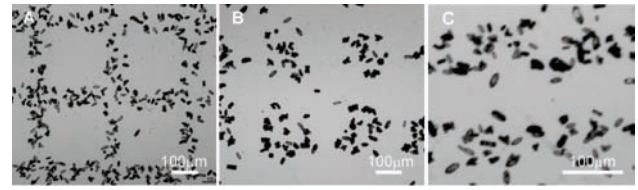

Figure 3. Optical microscopy images of crystals attached to the OPN regions on the DPPS−OPN-patterned surfaces depicted in Figure 2.

Previous reports have revealed that OPN influences the adhesion characteristics of the (100) face,[11a] reduces the growth rate of the (010) face,[12] and alters the crystal habit through binding at edges adjoining the {100} and {121} faces, all consistent with adsorption of OPN on COM.[13] As expected, the immobilized OPN did not affect the crystal habit or growth rate (compared with solution), but the crystals appeared to attach to the OPN regions predominantly through the (100) face, which is consistent with previous observations that this face is the most adhesive toward anionic species.[11b]

In order to examine the role of free OPN (in solution) on COM crystal attachment to DPPS, a silicon wafer coated with a DPPS bilayer alone was immersed in the same growth medium as above but with 5 μg/mL OPN added. This resulted in fewer COM crystals on the DPPS bilayer than in the absence of free OPN (Figure S7), consistent with electrostatic repulsion between anionic OPN adsorbed on COM and the negatively charged DPPS bilayer. As DPPS can be regarded as a mimic of a cell membrane, this observation suggests that the inhibition of COM stone formation by free OPN is associated with suppressed attachment of crystals to renal epithelial cell membranes.[6] The attached COM crystals also were smaller ($\sim$5 μm) in the presence of free OPN, consistent with inhibition of the COM crystal growth by OPN observed in vitro. The attached crystals also exhibited the well-documented dumbbell habit in the presence of free OPN.

The preferential attachment of COM crystals to the OPN regions of DPPS−OPN-patterned surfaces illustrates that OPN is more competitive for crystal attachment than DPPS. Moreover, these experiments demonstrate that free OPN, which is a major constituent in urine, adsorbs on COM crystals and suppresses their attachment to DPPS, suggesting a link between OPN and reduced attachment of COM crystals to renal epithelium. Although the studies presented here are limited to surface-confined DPPS and OPN, this patterning protocol may provide a convenient approach for understanding the effects of other biomolecules, either alone or integrated with DPPS bilayers, on COM crystal attachment and the pathogenesis of kidney stones.

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Supporting Information Available: Experimental details of COM crystallization on hybrid surfaces and characterization of hybrid surfaces

and crystals. This material is available free of charge via the Internet at http://pubs.acs.org.

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