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*Research Paper*

# Formation of Cholesterol Crystals at a Mucin Coated Substrate

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***Purpose.*** High-resolution, tapping-mode atomic force microscopy (AFM) was used to monitor the early stage of the formation of cholesterol crystals under simulated conditions of the gallbladder environment.
***Methods.*** AFM images of phosphatidylcholine/cholesterol vesicles were obtained using a mucin-coated mica substrate.
***Results.*** The vesicles appeared flattened with diameters from 100 to 300 nm and heights that varied from 10 to 100 nm. Phosphatidylcholine/cholesterol vesicles were mixed with bile salt solutions to yield supersaturated (with respect to cholesterol) dispersions, which were then placed onto mica, silanized mica, and mucin-covered mica substrates. After equilibration, sub-micron sized, plate-like structures were observed at the mica and mucin covered surface, but none were seen at the silanized surface. The morphology was characterized as it pertains to the relative growth rates of the crystal faces.
***Conclusion.*** The comparison of these results with literature reports of cholesterol crystals grown in solution suggests that the physical chemical properties of the surface have an important influence in determining the nucleation and subsequent crystal growth of cholesterol.

**KEY WORDS:** AFM; bile salt; cholesterol; crystallization; gallstone; nucleation; phospholipid; taurocholate.

## INTRODUCTION

Cholesterol crystals have been examined to gain a fundamental understanding of the dissolution process (1–4) as it relates to the pathophysiology of gallbladder stones. Gallstones are organic concrements most often containing crystals of cholesterol (5), which affect 30 million people in the US, and an estimated 750,000 people undergo surgical removal of gallstones each year.

There are three necessary and sufficient conditions for the formation of gallstones (6). The first is secretion of bile supersaturated with cholesterol. The liver secretes cholesterol solubilized in an aqueous dispersion of phosphatidylcholine (PC) vesicles and also secretes bile salts. These separate secretions mix to form bile which flows into the gallbladder. In the gallbladder, bile salts interact with the vesicles to form mixed lipid aggregates. Most importantly, with the formation of these aggregates, the solubility of cholesterol is reduced. When the concentration of cholesterol exceeds its solubility, precipitation can occur. However, bile in normal subjects also may be supersaturated with respect to cholesterol suggesting that the micelles and vesicle remnants stabilize the metastable state (5). This realization led to the second necessary condition that the nucleation of cholesterol is accelerated in those individuals with gallstones. A number of compounds are known to inhibit (inhibitors) or accelerate (i.e., pronucleating agents) the nucleation of cholesterol, but the molecular mechanism(s) by which these agents act remains unknown. The final necessary condition for the formation of gallstones is the agglomeration and growth of nascent cholesterol crystals. Nascent cholesterol crystals are relatively small and pass easily into the intestine. The medical complications arise when the gallbladder retains these crystals, and they undergo further growth and/or agglomeration. As with nucleation, a number of compounds have been empirically related to accelerated/retarded growth of cholesterol crystals.

A material that is abundant in the gallbladder and has been identified as both a pronucleating factor as well as a pro-crystal growth factor is mucus (7,8). In fact, the presence of a mixture of insoluble mucus and small cholesterol crystals is a predisposing factor for the recurrence of gallstones (5). Mucin-type glycoproteins are the major components of mucus. Under normal conditions, mucus is a viscous gel that provides a protective blanket at the surface of the gallbladder. In the human gallbladder, the primary mucin expressed has been designated as MUC5B. MUC5B represents one of 17 mucins that have been identified in the human genome (7). It contains a cysteine knot at the carboxyl terminal, which is connected to a D4 domain that has homology to van Wildebrande factor, a protein involved in blood clotting. The next region is composed of alternating tandem repeats (TR) and cysteine rich domains. The TR domains contain the carbohydrate side chains, and the cysteine rich domains are believed to be involved in forming inter- and intra-molecular disulfide bonds. These features give rise to the formation of a cross linked, hydrated gel.

In studying the role of mucin in the formation of gallstones, a variety of techniques have been used including

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optical density (9), polarizing light microscopy, video-enhanced microscopy (10), quasi-elastic light scattering (11), and neutron scattering (12,13) as well as negative staining, free fracturing, and vitreous ice cryo-transmission electron microscopy (14,15). A great deal of information has been obtained from these studies; however, there are still several unresolved questions. First, due to the resolution limits of these methods, particles in the 100–1000 nm size range have not been well characterized. While vitreous ice cryo-microscopy is extremely powerful in trapping structures in their original state in solution, it only provides a two-dimensional projection. A possible greater shortcoming has been that investigators have focused on crystallization arising from solutions of putative inhibitors. That is, the role of insoluble components in providing a heterogeneous nucleation site for the formation of cholesterol crystals has received little attention. For such studies, techniques are needed, which are capable of characterizing the structure and morphology of aggregates in an insoluble or adsorbed state (16). The techniques given above either lack resolution or are inappropriate for examining the nucleation and initial crystal growth of crystals in such systems.

In the present work, the nucleation and initial growth of cholesterol crystals were examined on the submicron-scale using atomic force microscopy (AFM). This fundamental approach may have general utility in understanding the crystallization process of colloidal systems (2). AFM has successfully provided detailed molecular information for micelles (17), vesicles (18) and crystal growth (19), although to our knowledge, this report provides the first time application to crystallization of cholesterol from mixed micellar systems. In the present paper, high-resolution tapping mode AFM was used to examine cholesterol crystals grown from phosphatidylcholine/bile salt/cholesterol dispersions at mucin coated substrates. These observations suggest that the physical chemical properties of the surface are important in determining the nucleation and subsequent crystal growth of cholesterol.

## EXPERIMENTAL SECTION

### Materials

Bovine submaxillary mucin (BSM, type I-S, catalog number M-3895), sodium taurocholate and cholesterol were purchased from Sigma (St. Louis, MO) and used as received. Egg yolk phosphatidylcholine (PC) 99% was obtained from Avanti Polar-Lipids Inc. (Pelham, AL). Mica, which can be cleaved to yield a molecularly smooth substrate, was obtained from Ted Pella Inc. (Redding, CA). All other chemicals were reagent grade or better.

### Sample Preparation for AFM

A BSM stock solution was prepared at a concentration of 1 mg/ml in pH = 7.0, 0.15 M NaCl. Cholesterol (20.4 mg) was dissolved in 7 ml egg PC (20 mg/ml) in ethanol. The solution was concentrated by rotary evaporation. Following the addition of cyclohexane, the dispersion was frozen and then freeze-dried under vacuum. The vacuum was released under dried nitrogen, and the lipids were hydrated with 3 ml normal saline solution, pH 7.0. The vesicles were sequentially extruded through filter membranes of pore sizes of 0.8, 0.4 and finally 0.2 μm. The particle size distribution was assessed by dynamic light scattering, and the intensity mean diameter was 290 ± 190 nm. Using the critical tables of cholesterol saturation (20), PC/TC/cholesterol solution (cholesterol saturation index = 1.53) was prepared by adding 160 mg sodium taurocholate to the above PC/cholesterol solution.

Mica (≈1 × 1 cm) was used either freshly cleaved or following treatment with dimethydichlorosilane to render the surface hydrophobic. In addition, the mica substrate was coated with mucin by initially combining 200 μl BSM stock solution with 200 μl 10 mM $CaCl_2$. Of this mixture, 5 μl was placed onto a freshly cleaved mica surface for 5 min. The substrate was then rinsed with excess deionized water and dried with nitrogen gas. For PC/cholesterol vesicle sample, 5 μl of PC/cholesterol dispersion was placed onto the mucin-covered mica substrate and then dried with nitrogen gas. For cholesterol crystal growth experiments, the substrates were immersed in PC/TC/cholesterol dispersion (CSI = 1.53) for 18 h, removed, and then dried with nitrogen gas before imaging. All studies were conducted at room temperature 22 ± 1°C.

All AFM images were recorded in the tapping mode with a Digital Instruments Multimode AFM microscope (Digital Instruments (DI), Santa Barbara, CA). The probe was 125 μM long and had a resonant frequency between 300 and 350 kHz. All measurements made on the images were generated from the DI AFM software.

## RESULTS AND DISCUSSION

Figure 1 is a representative topographic image of a mica substrate covered with mucin. This AFM image is distinct from freshly cleaved mica, which appeared as a molecularly smooth surface with grooves arising from the crystal structure (data not shown). The presence of mucin yielded a uniform film that covered the entire substrate surface. The speckled appearance indicates the presence of aggregated structures with domain sizes on the order of 10 nm. The height of the aggregates was in the 10 nm range, which was estimated by measuring the difference between the minimum and maximum displacements in the image. In doing so, the height reflects the actual physical thickness. The thickness of a single hydrated ocular mucin molecule has been reported to

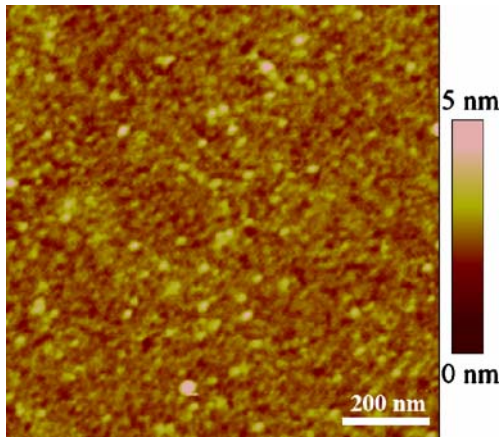


**Fig. 1.** Tapping mode AFM image of BSM-covered mica surface.

be about 1 nm (21), which is considerably less than the observed height. However, the image observed is consistent with that reported for aggregated mucins at a mica surface (21). Thus, BSM also appears to occur as an aggregated, multilayered adsorbed film at the mica surface.

Figure 2 shows the topographical image obtained following deposition of egg PC/cholesterol vesicles on the mucin-covered surface. The use of mucin-covered mica not only provides a more physiologically relevant substrate but also obviates the typically problem encountered with mica. Specifically, vesicles of egg PC/cholesterol have been shown to unfold and form a thin film on the mica surface (17). This result was corroborated in our laboratory where no intact vesicles were observed on a freshly cleaved mica substrate (image not shown). In Fig. 2, about 70 vesicles can be seen, which are randomly distributed over the 2 × 2 μm area. The vesicles range in size from 100 to over 300 nm. Most are round with a height between 10 and 100 nm. There also appears to be aggregated vesicles, which may have been present in solution or arose from the drying process.

Analysis of individual vesicles is facilitated by the use of line-cuts as shown in Fig. 2B. Here, the selected vesicle was measured to have a diameter of 200 nm and height of 40 nm. The diameter is in good agreement with the size distribution measured in solution by dynamic light scattering, but the height is too small for the phospholipid vesicle to have spherical shape. This type of deviation was common to all vesicles examined, and thus may reflect the collapse of the vesicles during drying. Nevertheless, it is clear that phospholipids vesicles can be imaged on a mucin film using AFM.

Following identification of the mucin-covered mica as a suitable surface for imaging aggregates relevant to bile, a supersaturated dispersion of egg PC/cholesterol/bile salt mixture was equilibrated with this substrate for 18 h. The resulting topographical image is given in Fig. 3. Two rectangular projections are seen to emanate from the surface. Each projection has a flat, upper surface, clearly defined edges, and an associated terraced structure. These features are commonly observed in AFM images of systems that undergo crystal growth (19). The projections are about 300 × 400 nm (length × width) and range in height from 150 to 200 nm.

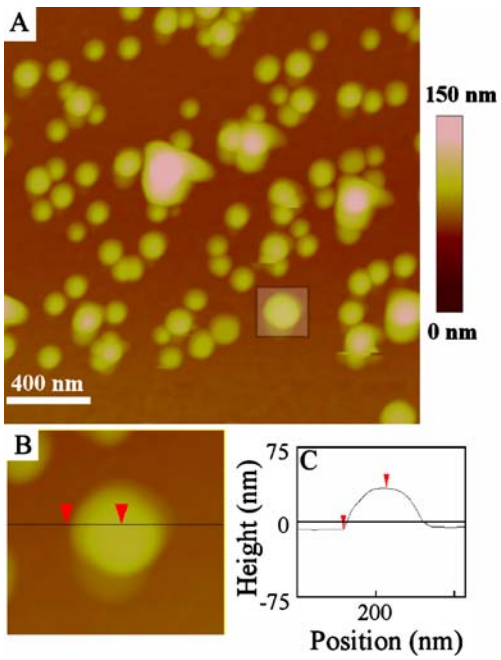


**Fig. 2.** (A) Tapping mode AFM image of egg PC/Cholesterol vesicles on the BSM-covered mica surface. (B) Zoom in area in (A). (C) Line scan across the vesicle in (B).

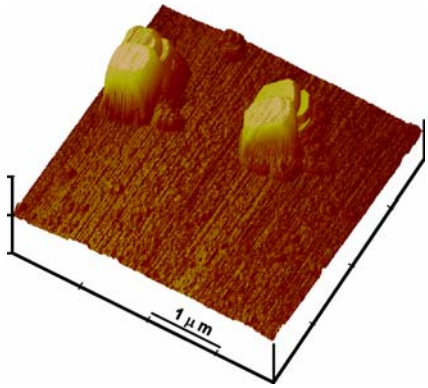


**Fig. 3.** Tapping mode AFM image of Cholesterol crystals after Egg PC/Cholesterol/Bile salts deposited on BSM-covered mica surface for 18 h.

Figure 4 is another image obtained with the same sample but from a different perspective. As above, rectangular shaped images are observed. The length of each side and both diagonals of the crystal were measured from which the angles were deduced. The top of the crystals represented a parallelogram, since the diagonal angles were similar and had values of 82° and 98°. These values are in good agreement with published results (79° and 101°) of cholesterol hydrate crystals grown in solution (22). Since these images are separated in space, it is likely that each structure represents a unique site of crystal nucleation. AFM does not provide direct chemical information, and thus proof that these images are cholesterol crystals requires corroboration. Samples were submitted for analysis by X-ray photoelectron spectroscopy (University of Minnesota, Chemical Analysis Facility), and high levels of carbon were detected but no phosphorus or sulfur was found. This suggests the presence of cholesterol and the absence of phospholipids and bile salts at the surface.

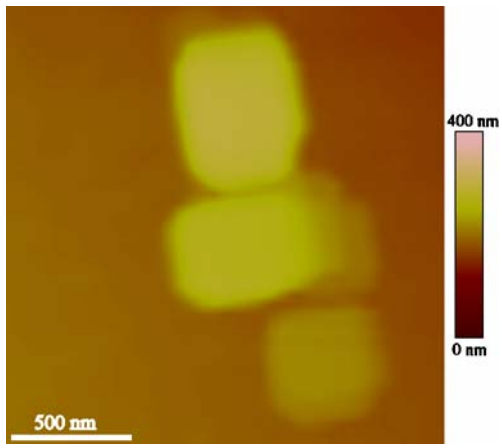


**Fig. 4.** Tapping mode AFM image of Cholesterol crystals after Egg PC/Cholesterol/Bile salts deposited on BSM-covered mica surface for 18 h.

Additional information of the initial growth process may be garnered by a more detailed analysis of the AFM topographic images. From the observed dimensions, the growth rates of cholesterol crystals are different in three dimensions, since the observed crystals did not have a cubical shape. With analysis of the crystals in Fig. 3, the growth rate parallel to the substrate was found to be twice that observed in the direction normal to the surface as inferred from the crystal dimensions. It is interesting to note that while the cholesterol crystals have a plate-like morphology; they are distinct from the larger plate crystals grown in an ethanol solution where the width and length is typically ten times that of the height (4). Such a difference in growth rates may be related to the specific interaction of the cholesterol supersaturated vesicles with mucin in the nucleation or initial stage of crystal growth.

Using the same cholesterol/phospholipid/taurocholate composition, cholesterol crystals with similar morphology were observed to nucleate and grow at a freshly cleaved mica surface. However, in rendering the mica surface hydrophobic by silanization, no crystals were seen. Perhaps not unexpected, the nature of the surface appears to have had a profound effect on the nucleation process of cholesterol. In particular, a surface consisting of a hydrogen-bonded network was suggested to provide a favorable template for the nucleation of cholesterol, since the crystal structure of cholesterol hydrate also contains a network of hydrogen-bonds in the plane (22, 23). Muscovite mica and mucin-type glycoproteins have extensive hydrogen bonding capability and thus the results given here support the suggestion that this surface feature is favorable for cholesterol nucleation. The alternative possibility for inducing nucleation is that mica induces vesicle instability. However, crystals are observed both with mica and mucin, the latter of which does not induce vesicle instability, indicates the properties of the surface are more critical.

In summary, high-resolution tapping-mode AFM topographic imaging has provided visualization of the initial stages of growth of cholesterol crystals. In addition, mucin and mica substrates have been shown to promote the nucleation and growth process of cholesterol hydrate. These results suggest that the surface properties of compounds that affect the formation and growth of gallstones deserve greater scrutiny.

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