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Microcosmic Forests:
An Investigation of Protein Crystallization Using ESEM, Contact AFM and Optical Microscopy
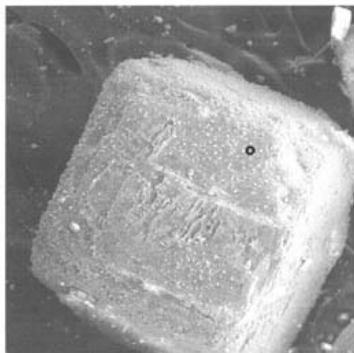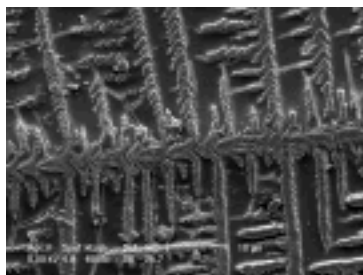

"With every step, I topple microscopic forests."
-Chet Raymo, Professor Emeritus of Physics, Stonehill College, Easton, Massachusetts

SEM view of typical salt crystal – courtesy http://acept.la.asu.edu/PiN/rdg/elmicr/salt.jpg

SEM image of salt & BSA proteins courtesy Dusevich lab, UMKC

Introduction

Proteins, one of the key building blocks of life, are often crystallized as an aid in studying their macromolecular structure via X-ray diffraction and electron microscopy. However, crystallization in general, and protein crystallization in particular, are intriguing phenomena, some details of which—even today—are still not well understood (Kimber 1).

When sodium chloride crystallizes in the presence of a protein, the salt's typical cubic shape is converted into an extensively branched dendritic form (Anderson and Reid).

Furthermore, when such branched or ferned crystals are found occurring naturally in certain biological substances such as blood or saliva, their

appearance indicates the presence of a protein and thus may be used as a diagnostic protocol in medicine (Anderson and Reid). A theoretical understanding of how these protein crystals actually form is still a young and developing area of biological science (Fraden). By investigating the size, shape, and texture of these crystals, scientists hope to gather information about the structure of proteins, as well as the electrochemical interactions and other force interactions which occur between proteins and their solutions during crystal formation.

While building a working theory of protein crystal formation is obviously beyond the scope of our undergraduate group, we were interested in investigating the topography/structure of NaCl crystals formed in the presence of proteins. We wanted to know more about:

* The terrain of the crystals (Are they rough? Are there peaks and valleys in the pattern?)
*How far does the classic fractalline or ferning pattern extend--or in other words--the size of the smallest branch in the crystal's fractal tree.

We hoped (perhaps ambitiously) to find evidence for what causes the crystals to form in a fractalline manner.

Additionally, we wanted to investigate any important differences in structure between bovine serum albumin (BSA) exposed crystals, bovine serum albumin exposed crystals to which urea has been added, and the protein-exposed crystals sometimes found in human saliva. According to prior research, "the more heterogeneous the composition [of the solution], the more elaborate the pattern" of crystallization formed in the dried solution (Anderson and Reid). We wanted to verify the research. We hypothesized that crystals formed in biological environments, as well as crystals formed in the laboratory with more molecules—such as urea—present, would give rise to more complex looking crystal structures.

In order to observe crystals in close detail, microscopes beyond the range of conventional optics were necessary, as optical microscopes cannot be used to scan areas smaller than the wavelength of visible light. The magnified viewing range of optical microscopes is at best 200nm (Flegler 1). An electron microscope or other advanced microscope would thus be necessary for our study.
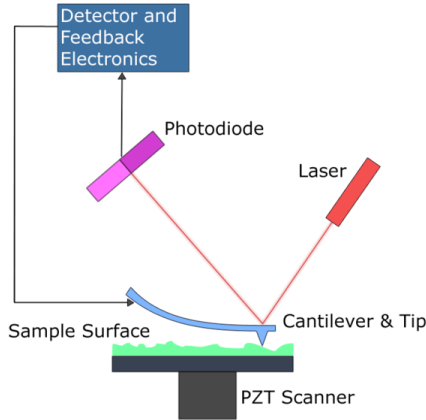
The first electron microscope, the TEM (Transmission Electron Microscope), was developed in Germany during the 1930's by scientists Max Knoll and Ernst Ruska at the High Voltage Laboratory in Berlin. Electrons, a particle much smaller than photons, were directed at a sample to form an image with much greater resolution than a photon based optical instrument could provide.

With a resolving power of about 0.2 nm, the TEM produces images which may be magnified 100 to 500,000 times (Flegler 43). TEM makes use of an accelerating voltage to generate an electron beam. The accelerating voltage of a TEM theoretically improves the resolution, and ranges from 20,000 to as high as 1,000,000 volts (Flegler 50). The specimen is inserted into the objective lens, which is the most important, master lens in TEM. After the electron beam is produced, the beam travels in vacuum through a condenser-lens system, which is "used to control electron illumination on the specimen and on the viewing screen for such functions as viewing, focusing, and photography" (Flegler 51). The image is produced by contrast forming specimen-beam interactions. Amplitude, diffraction, and phase contrast are made use of to form and refine the created image. Some electrons pass through the sample without energy loss or change of direction, whereas others are elastically or inelastically scattered. More insight into the sample's structure is possible as the prominence of the diffraction pattern increases. The final image magnification in a TEM is the product of "the objective lens, the diffraction lens, the intermediate lens, and the projector lens...[which] projects the final image onto the viewing screen." (Flegler 59). Vacuums are necessary to produce a coherent electron beam, however the vacuum environment places constraints on what type of samples may be viewed as they must be resistant to the vacuum.

The next electron microscope developed was the Scanning Electron Microscope (SEM). It was first developed and used in 1965. In the SEM, secondary electrons "are produced by interactions between incident electrons and weakly bound conduction band electrons in the atoms of the sample" (Flegler 72). Through the process of absorption and escape, these secondary electrons "produce a predominantly topographical image" (Flegler 73). As these secondary electrons "escape from a small volume of the total specimen-beam interaction volume, the secondary electron image provides the image of highest resolution (73). An Everhart Thornley detector detects nearly all of the secondary electrons that escape from the surface of the specimen, and these electrons are then accelerated and converted into photons by a scintillator. The photons strike an electrode, which then emits electrons. The electrons bounce back and forth in a cascading manner, each bounce causes the electrons to be multiplied in the photomultiplier tube.

The secondary electron image produced by the Everhart Thornley detector "is a complex mixture of electrons of different origins." (Flegler 74). The secondary electrons coming directly from the region of specimen-beam interaction in the sample have the highest resolution capacity because they originate from the smallest volume in the sample. Sample current, voltage contrast, electron-beam induced current, and

magnetic contrast may also be used to create images in SEM.

Detector and Feedback Electronics

Photodiode

Laser

Sample Surface

Cantilever & Tip

PZT Scanner

Two more exciting recent developments in microscopy are the Atomic Force and Scanning Tunneling Microscopes. The AFM, or "Atomic Force Microscope", is not an electron microscope. Instead of using an electron beam to image a sample, a fine mechanical probe scans the sample's surface (Flegler 93). In STM, electrons tunnel between the tip of the probe and the sample, which do not directly touch. In the AFM, a very fine tip is mounted on a triangular piece of metal called the cantilever, and the piezoelectric device moves the sample under the tip. (See figure courtesy Wikipedia.) The variation in attractive forces between the electrons in the orbital shells of the tip and the electrons in the sample cause the cantilever to move. A laser beam hits the foil and is then reflected back onto a photodiode. The current in the photodiode varies with the movement, and this variation is used to create an image. (Flegler 94).

In Atomic Force Microscopy, samples do not need to be conductive, and samples are not required to be in a vacuum. AFM microscopes "have been used to image amino acids, proteins, and macromolecules" but "many difficulties remain to be solved...before these new microscopes can be used fully in biology" (95).

The Investigation

For our investigation, we utilized an ESEM Philips XL30 microscope at the Dusevich lab at UMKC Dental School, and an XE-100 AFM microscope in contact mode at the Zhu lab in the UMKC Physics Department.

ESEM "retains all of the performance advantages of a conventional SEM, but removes the high vacuum constraint on the sample environment" (Kimsen and Meissel 2). While much analysis of SEM samples is done within the magnification range of a light microscope, the total information content of the SEM can be much greater due to its higher

resolution and much greater depth of field.

For example, here is a 400X magnification of crystallized sodium chloride solution in the presence of bovine serum albumin (.15 M NaCl and 10 mg/mL Bovine Serum Albumin):
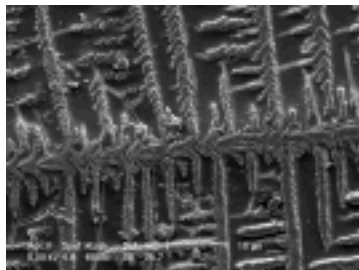

(Taken in the Ferrari lab by Jennifer Nielsen using
Nikon Eclipse TS100 phase contrast inverted light microscope)

Here is the same substance at 500X magnification using the ESEM in the Dusevich lab (after treatment with a 60% gold / 40% palladium alloy):
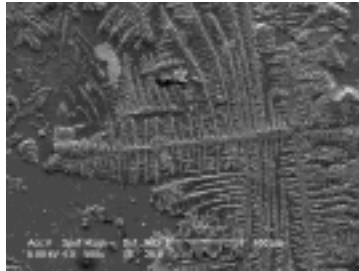
And here is the same substance magnified 4000X using the ESEM:

The enhanced detail resulting from the improved magnification and depth of field is staggering. Whereas it is almost impossible to distinguish the smallest branches in the fractal pattern using optical microscopy alone, at 4000X magnification using ESEM, the fractal pattern is enlarged significantly enough that the smallest branches become discernible.
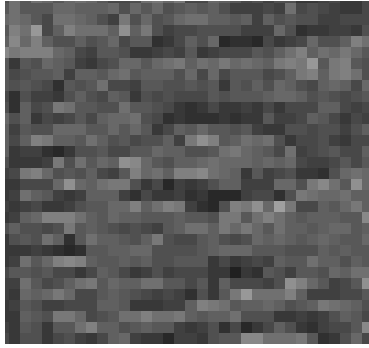
Note the fractures in the underlying film of dried solution on the slide which only come into view under the highest magnification. Which came first, the crack or the crystals, may be an entirely meaningless question, but the crystals appear on first glance to be following the course of the cracks. The cracks bare a resemblance to Lichtenberg figures—lightning-like shapes that are often a sign of a complex process called diffusion-limited aggregation (Hasley). According to the diffusion based theory of crystal formation, "dendrites...can appear in the crystal growth in a diffusion field. When a part of the flat interface grows faster than the other by some fluctuation the advanced part can grow faster owing to a gradient (e.g. of concentration) in the diffusion field, and the deformation is enhanced. Hence, the flat interface becomes unstable, resulting in dendrites"—fernlike branches (Taguchi 1.)

The question now, is why these fluctuations are occurring with the solutions containing proteins, and not the plain salt solutions. It is likely that the fractures and fractal crystals most likely occur simultaneously, due to underlying tensions occurring in the solution as it dries. These tensions are likely occurring due to some type of interaction between the proteins, salts, and the evaporating water, and may be at least partly electrochemical in origin. According to a recent publication, "Water and protein molecules have electrostatic properties...[that] interact and... mutually adjust..." having "a significant effect on...the structural and the dynamical properties of the solvating water in the vicinity of charged residues" (Kim 1). The cracks in the film may thus be a result of the electrochemical effects of hydrophobic and hydrophilic regions of proteins on the local structure of water molecules in the solution. Clearly this is

speculation at best at this point, and more research is necessary.

It is interesting to note that, after we go down to the level of just a few square micrometers, the fractal pattern seems to stop repeating itself. This was further verified using the XE-100 Atomic Force Microscope in the laboratory of Dr. Da-Ming Zhu.

The AFM microscope provided us with an excellent close-up view of both BSA/salt crystals like the ones we viewed under ESEM, as well as biologically occurring saliva crystals.

The saliva crystals, gathered from dried saliva of a female student, are believed to have been caused by levels of proteins which rise during ovulation (Reid). Saliva contains more components than our lab-mixed bovine serrum albumin crystals. As expected, intricate patterns were observed in a more heterogeneous mixture.

(Female student's Saliva crystals; 400X magnification)

Note the unusually "curly" appearance of the saliva crystals. It is noted that human saliva normally contains urea (Dawes). Having observed similar effects in urea exposed crystals before, we hypothesized that the additional presence of urea may account for the "curly" structure of protein crystals found in human saliva. Urea is a denaturant which increases protein solubility. "Despite its widespread use, the molecular basis for urea's ability to denature proteins remains unknown. Urea may exert its effect directly, by binding to the protein, or indirectly, by altering the solvent environment" (Bennion and Daggett).

To check whether urea caused the curling, we mixed a batch of NaCl/BSA protein solution to which urea has been added. Sure enough, the curly structure was again found:

400X magnification using optical microscope, Ferrari lab, UMKC
.15 M NaCl and 10 mg/mL Bovine Serum Albumin +5 mg/mL urea

(The curling structure is even more pronounced here than in the human saliva sample, which may or may not be due to a much higher concentration of urea in our lab mixed sample than in human saliva.)

Our AFM images reveal that the biologically derived crystals from saliva were smaller than the BSA derived crystals—in particular, less tall (only about 400 to 700 nanometers in height versus the BSA crystals' maximum height of 900 nm). I think this is because there was most likely less protein and less salt present in the saliva than was present in our laboratory mixed batches.
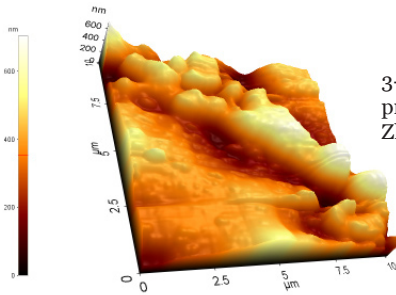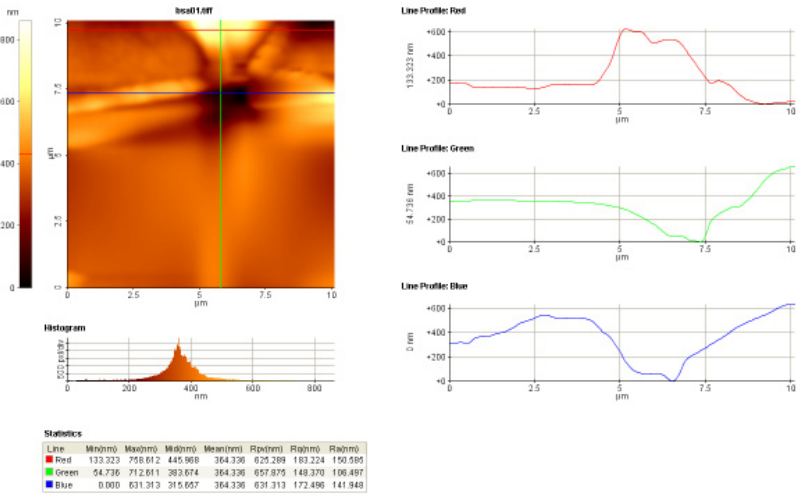

3-D AFM image of naturally occurring protein crystal found in saliva; courtesy Zhu lab; note complex structure

3-D AFM image of lab created bovine serum albumin Crystal; courtesy Zhu Lab
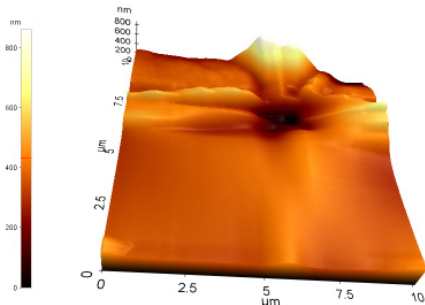
BSA crystal – "Line Profile" – Courtesy Zhu Lab


Total region statistics for BSA crystal:

Statistics

| Region	Min(nm)	Max(nm)	Mid(nm)	Mean(nm)	Rpv(nm)	Rq(nm)	Ra(nm)
■ Red	0.000	862.522	431.261	364.336	862.522	86.403	54.856

From the line profile and region profile of the BSA crystals which we obtained during image processing, we can observe details about the crystals' exact width, height, and slope. For instance, we can see that the smallest BSA (bovine serum albumin) salt crystals observed were about 2.75 micrometers wide, and close to 900 nm tall. It is important to note in these 3-D pictures that each crystal can be seen as a hill rising up from a surrounding valley or gorge. I believe that the dark crevice around the crystal segments as seen in the AFM images are actually the same as the cracks in the surface film like those noted in the ESEM pictures.
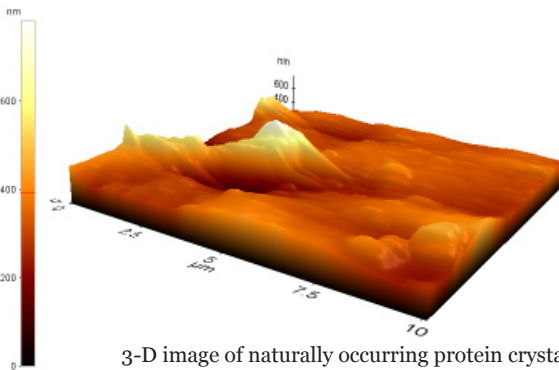
3-D image of naturally occurring protein crystal found in saliva

Line Profile: Red
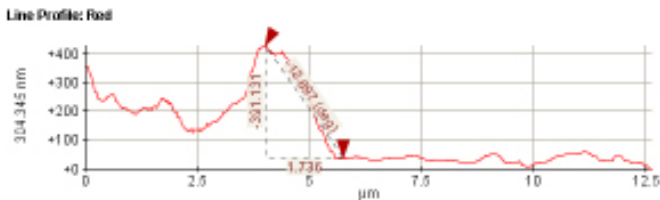
As demonstrated here, AFM can be used to provide a 3-D images of samples, unlike the electron microscopes, which provide images in 2-D. Additionally, samples viewed using AFM do not require special metallic treatments that may irreversibly change or damage the sample. Disadvantages of AFM are that the images size is limited and that at high resolution, image quality may be affected by the radius of curvature of the probe tip. An incorrect choice of tip for the required resolution may lead to image artifacts. AFM is not an unusual choice for viewing protein crystals. "The mechanisms by which crystals of [biological] macromolecules grow are quite varied and complicated. The AFM, by probing the crystal surface in situ with a sharp tip, allows direct observation of these mechanisms" (Lonnert). Using some AFM's with even higher magnification than we made use of in our project, it is even possible to actually reveal the molecular structure of a protein, as in this picture from a recent paper on protein structure, reproduced here (Lonnert 1).
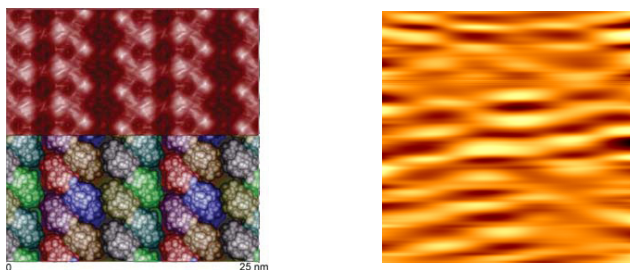
After observing biological crystals and plain BSA crystals, we wanted to view our urea exposed BSA/salt crystals under AFM. We unfortunately ran into some difficulty. This is the image that AFM took of BSA and urea. We concluded with our graduate student supervisor O-Sung Kwon that there are most likely a number of artifacts in this image. We hypothesize that either the probe tip was scratching the surface of the crystal and causing the lines, or the lines were caused by scanning errors due to abrupt shifts in the depth of the terrain.

We encountered similar difficulty—albeit to a lesser extent—in one of our shots of the plain BSA crystals.
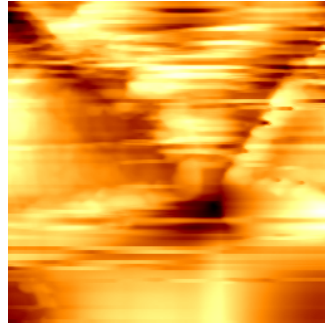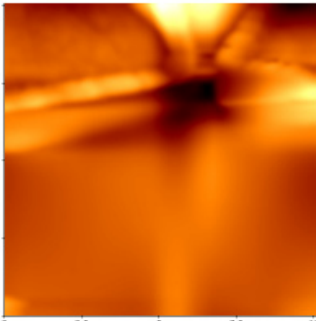
      In our first shot of the BSA salt crystals, the image came through beautifully.

      In our second shot, the errors and artifacts were compounded. Note that there is a good deal more terrain to cover in the second shot. It is possible that the abrupt changes in the terrain are what caused the errors; I ruled out actual physical scratching of the terrain, as the first shot is beautifully captured with no scratching and the surface's texture should not have significantly changed in the time between the shots.

There are a number of possibilities for the exact cause of the artifacts. These artifacts may have been caused by constructive interference between the reflection of the laser from the tip of the probe, and the reflection from the sample. This effect often registers as broad stripes in the AFM images (Eaton). Also, a common cause of artifacts is sample height versus size of the tip (Velegol). Sound waves in the room are another possible cause of image artifacts.

If abrupt terrain shifts are what is causing the line errors, this may explain why the BSA and urea shots, which had the most variable terrain, came up with the highest amount of error.

Conclusion

Our group concluded that AFM and ESEM are an excellent combination for studying the topography of protein crystals. AFM provide clear and crisp 3-D closeup images, while ESEM provides the best overall aerial view of the overall crystal topography. With more time and effort, we could have perfected our methods of taking protein images and learned much more about their structure. We would furthermore have been able to precisely determine the cause of the artifacts and eliminate them from future images. However, this was beyond the scope of our undergraduate level class.

 Our findings seem to support the Reid hypothesis that crystals formed in the presence of more diverse solutions are more variable in shape. Our evidence furthermore seems to support the diffusion-limited aggregation

model of protein crystal formation.

Electron microscopes, as well as STM and AFM microscopes, reveal vast quantities of information that are unavailable using conventional optical microscopy. Through ESEM and AFM, new worlds of information about protein crystals and other microcosmic forests can be revealed.

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