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"Ground based program for the physical analysis of macromolecular crystal growth"

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During the past year we have focused on application of *in situ* atomic force microscopy (AFM) for studies of the growth mechanisms and kinetics of crystallization for different macromolecular systems.

Mechanisms of macrostep formation and their decay, which are important in understanding of defect formation, were studied on the surfaces of thaumatin, catalase, canavalin and lysozyme crystals. Experiments revealed that step bunching on crystalline surfaces occurred either due to two- or three-dimensional nucleation on the terraces of vicinal slopes or as a result of uneven step generation by complex dislocation sources. No step bunching arising from interaction of individual steps in the course of the experiment was observed.

The molecular structure of the growth steps for thaumatin and lipase crystals were deduced. It was further shown that growth step advance occurs by incorporation of single protein molecules. In singular directions growth steps move by one-dimensional nucleation on step edges followed by lateral growth. One-dimensional nuclei have different sizes, less than a single unit cell, varying for different directions of step movement. There is no roughness due to thermal fluctuations, and each protein molecule which incorporated into the step remained.

Growth kinetics for catalase crystals was investigated over wide supersaturation ranges. Strong directional kinetic anisotropy in the tangential step growth rates in different directions was seen. Markedly non-linear supersaturation dependencies for tangential step rates in catalase crystallization were observed. The kinetic coefficient of the steps,  $\beta$ , for catalase crystallization was determined to be  $3.2 \times 10^{-5}$  cm/sec. The supersaturation dependence for the rate of two dimensional nucleation was measured, and this yielded the supersaturation dependence of the normal growth rate. From these data the surface free energy of the step edge was calculated to be  $\alpha \approx 3.2 \times 10^{-1}$  erg/cm<sup>2</sup> for  $\sigma > 2.1$ , and  $6.1 \times 10^{-2}$  erg/cm<sup>2</sup> for  $\sigma < 2.1$ . It appeared that at  $\sigma < 2.1$ , two dimensional nucleation occurred at specific surface sites which decreased the nucleation barrier.

The influence of impurities on growth kinetics and cessation of macromolecular crystals was studied. Thus, for catalase, in addition to pronounced impurity effects on the kinetics of crystallization, we were also able to directly observe adsorption of some impurities. At low supersaturation we repeatedly observed filaments which formed from impurity molecules sedimenting on the surfaces. Similar filaments were observed on the surfaces of thaumatin, canavalin and STMV crystals as well, but the frequency was low compared with catalase crystallization. Cessation of growth of xylanase and lysozyme crystals was also observed and appeared to be a consequence of the formation of dense impurity adsorption layers. Crystal growth resumed upon scarring of the impurity

We also applied AFM to image living mammalian cells. This method is shown practical for revealing cytoskeletal features beneath the cell membrane and their restructuring during a variety of cellular activities. Among the processes we have visualized are locomotion, tissue formation, cell division, transformation by viruses, and cell death.

A detailed report on the work performed has now been published or accepted for publication in the *Journal of Structural Biology*, *Surface Science* and *Journal of Crystal Growth*. The abstracts of these papers are below.

These results were also presented at the International NanoScope User's Conference (Santa Barbara, August 1997), seminar at the Marshall Space Flight Center (September 1997), Workshop on Phase Separations (Boston, October 1997), 7th International Conference on Crystallization of Biological Macromolecules (Spain, May 1997), 16th Conference on Crystal Growth and Epitaxy (Fallen Leaf Lake, June 1998), seminar at Schering - Plough Corporation (June 1998), 12th International Conference on Crystal Growth (Israel, July 1998).

There were no patents or inventions related to this research during the reported period.



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# An in situ AFM investigation of catalase crystallization

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## Abstract

Surface morphology, growth and dissolution of crystals of the protein catalase were studied by in situ atomic force microscopy (AFM). Growth of the (001) face of catalase crystals proceeds in alternating patterns by two-dimensional nucleation and successive deposition of two distinctive growth layers, each having a step height equal to half the unit cell parameter. Shapes of two-dimensional nuclei exhibit strong asymmetry due to directional anisotropy in step rates. The shapes of islands on successive layers are related by two-fold rotation axes along the  $\langle 001 \rangle$  direction. Lattice resolution AFM images of the molecular structure of sequential surface layers were recorded. Adsorption of large three-dimensional clusters of molecules was also observed to occur on the surfaces of catalase crystals. These clusters developed into either properly aligned multilayer stacks or misaligned microcrystals. Incorporation of misoriented microcrystals as large as  $50 \times 3 \times 0.1 \mu\text{m}^3$  proceeded without formation of defects. Upon incorporation of microcrystals and subsequent deposition of new layers on the surface of a growing crystal, impressions with depths of up to 0.4 of the growth layer thickness formed due to misfits between the lattices of the microcrystals and those of the growth layers. This produced elastic deformations in growth layers of  $\approx 0.6\%$ . © 1997 Elsevier Science B.V.

**Keywords:** Atomic force microscopy; Biological molecules – proteins; Crystallization; Nucleation; Single crystal surfaces

## 1. Introduction

The systematic manipulation of genes and gene products for understanding biological function, or for the development of new medical, agricultural or biochemical products increasingly depends on the extensive use of three-dimensional macromolecular structure as determined by X-ray crystallography. Currently, the three-dimensional structures are known for only a limited number of proteins. Innovations and novel applications will be dependent in the future upon proteins whose three-dimensional structures have yet to be elucidated.

The objective of physical analyses of macromo-

lecular crystallization, currently the rate-limiting step in X-ray structure analyses, is to correlate growth conditions, mechanisms and kinetics of crystallization with the diffraction properties of protein and virus crystals. In recent years, investigations have been undertaken of the physics of macromolecular crystallization, particularly surface processes amenable to interferometric techniques [1–4], and, more recently, AFM [5–16]. Different growth mechanisms for macromolecular crystallization and the kinetics of crystallization have been described for several macromolecular systems.

Here we present the results of in situ AFM studies of the growth mechanisms for catalase crystallization. Growth of the (001) face of catalase crystals occurs through the nucleation of two-

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# Atomic Force Microscopy Studies of Living Cells: Visualization of Motility, Division, Aggregation, Transformation, and Apoptosis

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**Atomic force microscopy, in contact mode, has been used to image living mammalian cells in culture at both low and high resolutions. The method is shown practical for revealing cytoskeletal features beneath the cell membrane and their restructuring during a variety of cellular activities. Among the processes that we have visualized are locomotion, tissue formation, cell division, transformation by viruses, and cell death. We show that some processes that occur well within cells can, nonetheless, be observed using the atomic force probe. At high resolution, features on the cell surface on the order of 0.5  $\mu\text{m}$ , and their changes with time, can be recorded.** © 1997 Academic Press

**Key Words:** cytoskeleton; scanning probe; cell interactions.

## INTRODUCTION

It has been proposed that morphological and locomotive properties might be used to characterize aberrant or tumor cells and to delineate their biochemical and biological individuality (Folkman and Moscona, 1978; Ambrose, 1967; Vasiliev and Gelfand, 1981). By comparison with corresponding normal cells, such an approach might have applications in diagnosis of disease and possibly also in treatment by providing a means for assessing the efficacy of drugs or other biological effectors.

The success of this approach to correlating cell phenotypes and cell behavior with other, less visible properties, such as genetic faults, will ultimately depend on the range of characteristics that can be observed and the precision with which they can be described, particularly in a quantitative sense. We present here a selection of examples from our own investigations which we believe indicate that the technique of atomic force microscopy (Binning *et al.*,

1986; Morris, 1994; Bustamante and Keller, 1995) can offer certain advantages. AFM, along with other methods, such as light microscopy, enhances our perception of, as well as our ability to characterize individual cells in culture. The particular cell types to which we applied the AFM technique in this study were primary murine osteoblasts, primary chick embryo fibroblasts, and a line of osteosarcoma cells (FAOS-2), but these were rather arbitrary choices, and we see no reason why the techniques used here could not be applied to other cells with equal facility.

The results presented here are not the first examples of the visualization of cells by AFM. Several others exist in the literature, the first being erythrocytes (Hok and Hansma, 1992; Kasas *et al.*, 1993; Butt *et al.*, 1990; Henderson, 1994; Henderson *et al.*, 1992). While these studies demonstrated that it was possible to image cells, and first suggested that AFM might be a useful tool for cell biology, most of the examples were cells that were dehydrated, chemically treated, dead, or somehow fixed for examination. The results we present here extend those investigations to mammalian cells that are alive and which exhibit normal behavior for many hours in culture. We believe that the images presented here, which were chosen from many of similar quality that we have recorded, attain a level of detail that was previously absent. These results further suggest the broad range of possibilities that the AFM technique offers cell biologists.

Conventional optical approaches to visualizing living matter rely on variations within the cell of light, absorption, or refractive index, and these depend in turn on the material constitution of the cell's components. Generally, organelles and substructures are rather uniform in this regard and it is necessary to take advantage of specific chemical or biochemical properties of the individual components to enhance structural features, e.g., stains, labels, or antibodies.

Atomic force microscopy senses two somewhat different but interrelated properties. First it records

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## **AFM Studies on the Mechanisms of Nucleation and Growth of Macromolecular Crystals**

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### **Abstract**

Atomic force microscopy (AFM) has been used to visualize events arising from the formation of intervening metastable phases at the surfaces of macromolecular crystals growing from solution. Crystals investigated were of the proteins canavalin, thaumatin, lipase, xylanase, and catalase, crystals of transfer RNA, and crystals of satellite tobacco mosaic virus. The appearance of aggregates on crystal surfaces was observed. The aggregates we infer to originate from liquid-protein droplets. These were particularly evident in freshly mixed mother liquor solutions. Droplets, upon sedimentation, have two possible fates. In some cases they immediately restructured as crystalline, multilayer stacks whose development was guided by, and contiguous with the underlying lattice. These contributed to the ordered growth of the crystal by serving as sources of growth steps. In other cases, liquid-protein droplets formed distinct microcrystals, somehow discontinuous with the underlying lattice, and these were subsequently incorporated into the growing substrate crystal. Scarring experiments with the AFM tip indicated that, detached from the crystal, molecules do not dissolve in the fluid phase but form metastable liquid-protein droplets with a potential to rapidly crystallize on the crystal surface. The molecular structures of growth steps for thaumatin and lipase protein crystals were delineated. There was no step roughness due to thermal fluctuations, and each protein molecule which incorporated into the step edge remained. Growth steps propagated by addition of individual molecules which formed subkinks of different size on the step edge.

**In Situ Atomic Force Microscopy Studies of Surface Morphology, Growth Kinetics, Defect Structure and Dissolution in Macromolecular Crystallization**

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**Abstract**

Surface morphologies of thaumatin, catalase, lysozyme and xylanase crystals were investigated using *in situ* atomic force microscopy. For thaumatin, lysozyme and xylanase crystals, growth steps having a height equal to the unit cell parameter were produced both by screw dislocations and two dimensional nuclei. Growth of catalase crystals proceeded in alternating patterns exclusively by two - dimensional nucleation and the successive deposition of distinctive growth layers, each having a step height equal to half the unit cell parameter. The shapes of islands on successive layers were related by 2-fold rotation axes along the  $\langle 001 \rangle$  direction. Experiments revealed that step bunching on crystalline surfaces occurred either due to two- or three-dimensional nucleation on the terraces of vicinal slopes or as a result of uneven step generation by complex dislocation sources. Growth kinetics for thaumatin and catalase crystals were investigated over wide supersaturation ranges. Strong directional kinetic anisotropy in the tangential step growth rates in different directions was seen. From the supersaturation dependencies of tangential step rates and the rates of two dimensional nucleation, the kinetic coefficients of the steps and the surface free energy of the step edge were calculated. Adsorption of impurities which formed filaments on the surfaces of catalase and thaumatin crystals was recorded. Cessation of growth of xylanase and lysozyme crystals was also observed and appeared to be a consequence of the formation of dense impurity adsorption layers. Crystal growth resumed upon scarring of the impurity adsorption layer and clearing of the crystal surface with the AFM tip. Adsorption of three - dimensional clusters, which consequently developed into either properly aligned multilayer stacks or misaligned microcrystals was recorded. For catalase crystals, incorporation of misoriented microcrystals as large as  $50 \times 3 \times 0.1 \mu\text{m}^3$  produced elastic deformations in growth layers of  $\approx 0.6\%$ , but did not result in the defect formation. Etching experiments on catalase crystals revealed high defect densities.