Atomic Force Microscopy of Pharmaceutical and Biological Materials

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ABSTRACT

We give four case studies showing how Atomic Force Microscopy is used in the pharmaceutical industry and in medical research. We look at structures on length scales from 1 nm to many μm. Lactose is perfectly stable until you look closely. Molecules diffuse on particle surfaces over time in response to temperature and humidity, causing unanticipated physical changes in bulk materials. Polymer microspheres are not spherical at all. More importantly, the crystalline vs amorphous form of tiny particles on the microsphere surface can affect flowability. Collagen is a monodisperse polymer when first synthesized, but is cross-linked when incorporated into living tissue. When tissue is processed to make an injectable biomaterial, it is helpful to see and measure the now-complex molecular structure. In the hunt for the molecular basis of disease, clinical, cellular, and chemical studies are essential. AFM visualization of a key peptide-DNA complex completed the picture for psoriasis.

Keywords: amorphous and crystalline particles, collagen, DNA, autoimmune diseases, AFM

1 INTRODUCTION

Atomic Force Microscopy (AFM) is a valuable tool for a wide range of materials and device analysis, supporting research, engineering, manufacturing and quality control.[1] Any surface you can touch is a potential candidate for study. In the pharmaceutical industry and in biomedical research, AFM is used to examine particles and biopolymers. AFM images are valued because direct visualization and measurement of individual objects complements the average measures obtained from bulk analysis techniques such as chromatography, spectroscopy and light scattering. For some years now, the FDA has encouraged the use of AFM along with those techniques.

2 IS LACTOSE STABLE?

Lactose is a common inactive ingredient in drug formulations[2]. It is chemically stable under normal storage conditions and it had long been regarded as physically stable also. However, the handling characteristics of lactose particles were found to change over time. We used AFM to investigate changes in surface morphology. Fig.1 shows evidence of molecular diffusion on the surface of a lactose single crystal. Pits 2.1 nm deep, the thickness of a unit cell, changed size and shape as molecules diffused along the surface. Diffusion was faster at higher relative humidity. These results, along with weeks of observations of individual 'real' lactose particles, created a paradigm shift for the drug manufacturer. Diffusion means that surface roughness can change, as well as particle shape and size. Sintering (fusion of touching particles) is just an extension of this process.

3 ARE MICROSPHERES SPHERICAL?

Some drugs are formulated as microspheres composed of the active compound dispersed in a polymer matrix. In one case, the maker found that particle flowability varied from lot to lot and suspected that the microsphere surface characteristics could be important. AFM investigation showed lot to lot variations in crystallinity of the drug particles at the microsphere surface (fig.2). In three different lots, the microspheres had small particles present on the surface, both on the hills and in the valleys. Many of these particles were well-faceted crystallites. We identified them as the drug compound because it can crystallize and the polymer matrix cannot. The crystals ranged in size and shape from needles up to 5 um long to rectangles less than 0.3 um long. Sometimes the drug particles were clustered together. In other cases they were sparse and widely separated. We also found amorphous regions that were distinct from the polymer matrix. By compiling AFM observations of these and other qualitative attributes and considering their possible causes, the maker was able to achieve better process control.

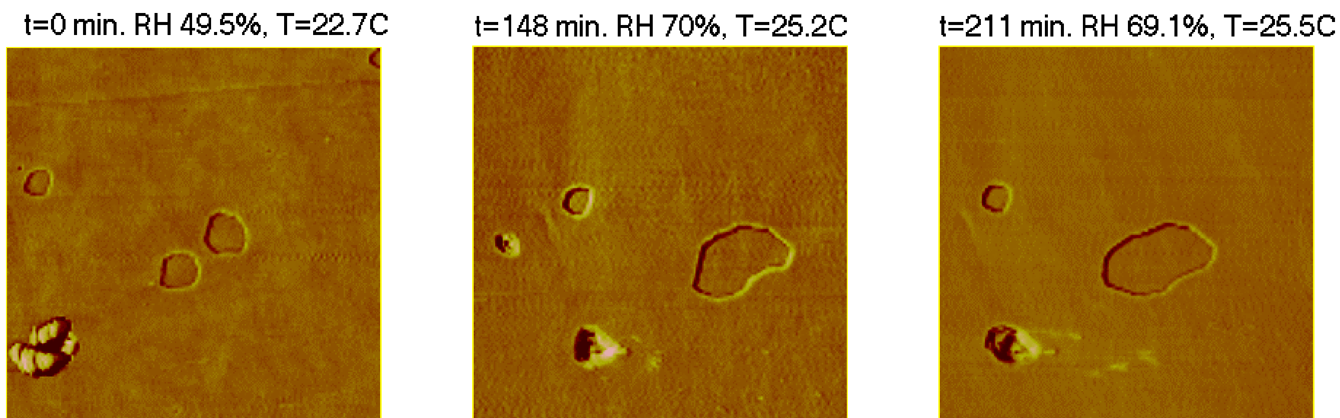

Figure 1. AFM phase images of pits on a lactose crystal. 1 μm scan size.

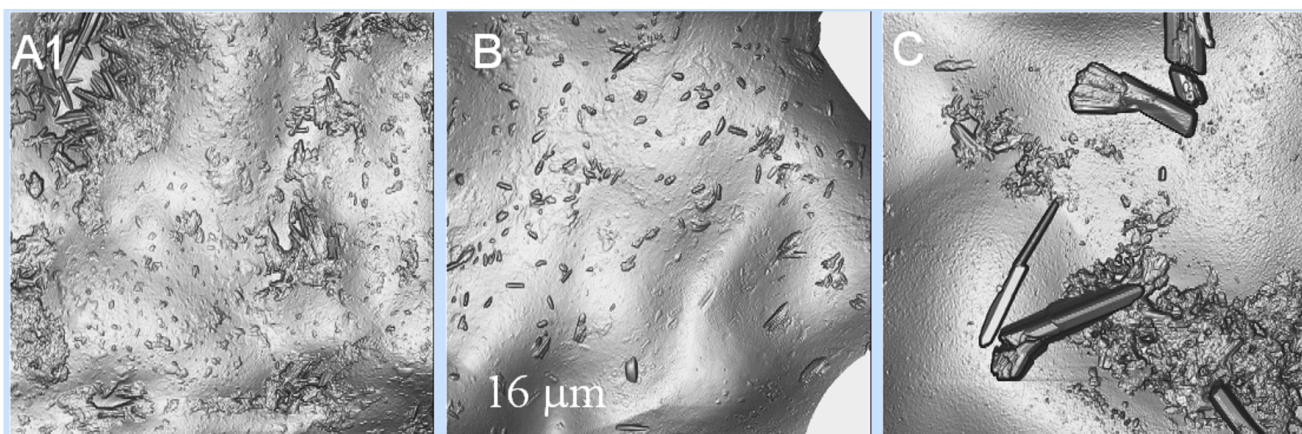

Figure 2. AFM height images of 3 different lots of polymer/drug microspheres. 16 μm scan size. Rendered in slope mode.

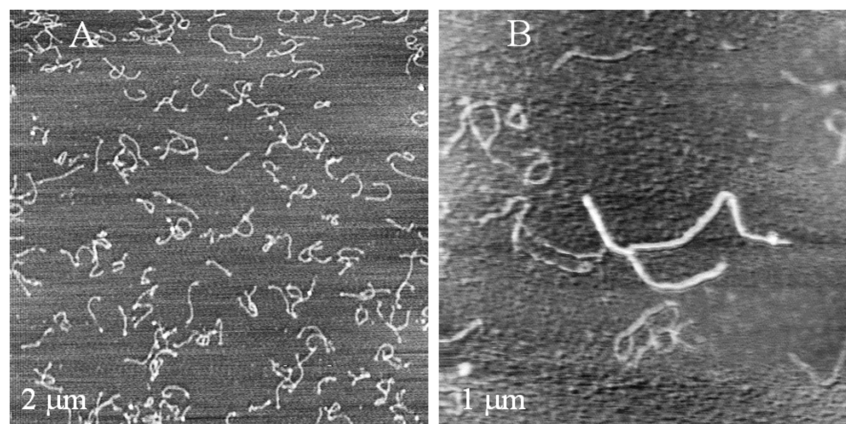

Figure 3. AFM height images of collagen molecules. A) mostly monomers of varying length. B) oligomers. 2 and 1 μm scan size.

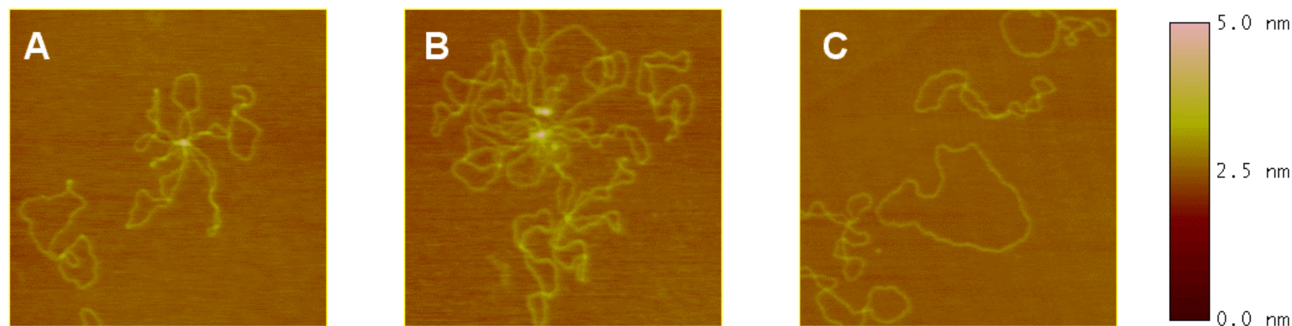

Figure 4. AFM height images of plasmid DNA complexed with LL37 (A and B) and DNA alone (C). 1 μm scan size. 0-5 nm height range.

4 CONTROLLING NATURAL PRODUCTS

Collagen is a natural product which is used in aesthetic surgery, cosmetics, drug delivery systems and biocompatible coatings. Commercial preparation of injectable collagen starts with bovine skin, which is treated with chemical and enzymatic digestion steps, followed by purification. In order to control the product, one must know the molecular structures produced by this process.

As with other polymers, molecular weight and conformation of a given collagen sample are two of the most fundamental characteristics. AFM is important because rodlike molecules such as collagen[3, 4] or DNA are the easiest to image. Collagen is a structural protein consisting of three polypeptide chains, which associate to form a triple helix ("monomer") over most of their length. In type I collagen, a monomer is nominally 300 nm long x 1.5 nm diameter. In a commercial preparation of injectable collagen, we found many individual collagen monomers, of varying lengths, as well as small fragments and a variety of oligomeric structures (fig.3). Based on such images, histograms reflecting variations of the monomer size (contour length) can be constructed. Such quantitative evaluation is an important tool for optimizing the digestion and purification of collagen from animal tissue for subsequent use as a medical biomaterial.

5 CAN PSORIASIS BE CURED?

Psoriasis is a chronic disease characterized by the appearance of raised, red patches of skin with a silvery scale. The skin is often tender and itchy, and scratching makes it worse. It is caused by an autoimmune response and there is no known cure.[5]

Atomic force microscopy was an important part of recent multi-disciplinary research that gives new hope for rational treatment of psoriasis.[6] This research shows that a specific polypeptide-DNA complex triggers the characteristic inflammation of psoriasis. One type of skin cell (plasmacytoid dendritic cells, pDC) is specialized for sensing infection. When pDCs recognize viral or microbial nucleic acids, they initiate a particular inflammatory response to fight the infection. In psoriasis, pDCs apparently respond to self-DNA. The question was how.

The skin normally produces an antimicrobial peptide, LL37 (mass 4493 Da). Among extracts of psoriatic skin, only those containing LL37 were able to trigger pDCs in cell culture to produce interferon. Extracted LL37 was characterized by mass spectroscopy, sequenced, and synthesized. The synthetic LL37 had the same effect. Using histochemistry and RNA analysis (PCR), LL37 and its messenger RNA were found in much higher concentrations in psoriatic than in healthy skin.

In cell culture as in normal tissue, extracellular DNA is always present due to dead and dying cells. When DNA was removed (using DNase), LL37 alone could not produce a strong pDC response. Therefore, both DNA and LL37 are required. Chromatography showed that LL37 and DNA formed a complex, apparently involving negatively-charged phosphate groups on the DNA and positively-charged residues on LL37.

AFM visualized the complexes of LL37 and plasmid DNA. Figure 4 shows DNA-LL37 complexes as well as DNA alone. Panel A shows several DNA molecules gathered together at a single node, like pieces of string in a mop head. Panel B shows additional complexes. Panel C shows plasmid DNA without LL37. In the complexes, the nodes were more than twice as tall as ordinary high spots produced when DNA alone coils or overlaps (3.0 vs. 1.3 nm).

Confocal fluorescence microscopy (using fluorescent-tagged DNA) and other experiments showed that DNA entered pDCs only when LL37 was present and then the

MopHead DNA™ complexes triggered a specific receptor (TLR9).

We have the following molecular mechanism. LL37 binds extracellular DNA. The complexes enter pDCs, defeating a safety mechanism that normally prevents pDCs from responding to extracellular self-DNA (normally present from dying cells). Once inside the cells, the complexes trigger interferon production, leading to inflammation. Since LL37 is produced normally in response to skin injury, it appears that its over-production is the key factor in psoriasis.

Until now, treatments for autoimmune diseases have relied on drugs which have broad effects on the immune system. The unwanted side effects can be severe. The comprehensive cellular, biochemical and molecular research summarized here has identified a specific molecular mechanism in one autoimmune disease, psoriasis. The researchers suggest that LL37 may also be involved in other autoimmune diseases and that LL37 antagonists might be a potential therapy. Such a ‘magic bullet’ would target only the dysfunctional part of the immune system, resulting in effective treatment with few side effects. This gives hope for sufferers of all autoimmune diseases.

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