Effects of Relative Humidity and Applied Force on Atomic Force Microscopy Images of the S-form of Phage fd

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Abstract

The filamentous phage fd was converted to spheroidal shaped S-forms and studied by contact-mode atomic force microscopy under conditions of controlled differences in relative humidity coupled with changes in the applied tip force. Stable and reliable sample preparation was achieved by spin-coating freshly cleaved mica with particle containing solutions with very low salt content followed by humidity control. The filaments of fd form a plateau and then drop in height as increased force is applied [15]. The apparent height, however, of the S-forms decreased linearly as force increased, due to their nonrigid side chain packing. The findings that relative humidity plays a role on reliable and reproducible images is consistent with previously published results on other biological specimens.

Introduction

Bacteriophage fd, M13, and f1 are class I filamentous phage of the Ff family [1], which contain a circular, single-strand of DNA. The DNA is enclosed in a flexible tubular protein sheath composed of approximately 2,700 molecules of the major coat protein, also called pVIII because they are encoded by gene 8. These subunits make up about 87% of the phage total mass and are arranged regularly along the length of the filament [2,3]. This highly elongated protein is aligned almost parallel to the filament axis and many copies overlap [1,4], like scales on a fish. Several copies of four different minor coat proteins are in pairs and cap each end of the particle [5]. Interestingly the pVIII sequence in the M13 virion differs in structure by one amino acid at position 12 compared to that of

the others: the carboxyl group of the aspartate in fd and f1 is replaced by the amide group of the asparagine in M13 particles [4].

Extensive structural characterization, including solid state NMR spectroscopy [6,7], polarized Raman spectroscopy on fibers [8], solution CD spectroscopy [9-11], solution Raman spectroscopy [12], and fiber X-ray and neutron diffraction [13,14] has been done on the Ff family. These studies demonstrate that the asymmetric gene 8 subunits are nearly completely α helical and layered about the filament axis with both 5-fold rotational symmetry and an approximate 2-fold screw axis [15]. Thus, the phage capsid can be described by neighboring groups of five gene 8 molecules rotated by 33.23° and shifted by 1.6 nm along the filament axis with respect to one another [5].

These phage are structurally stable, tolerating incubation at 75°C for long periods of time [16], and showing no significant loss of infectivity until approximately 90°C [17]. The phage can also survive exposure to detergents, high salt concentrations, 8M urea[18], and various pH values ranging between 2 and 11.5 [1,19,20].

Very consistent measurements of the phage length can be found in literature, even with different characterization techniques because of the stability of the phage. The phage length has been measured by three methods: atomic force microscopy (AFM) in air gave 883 ± 33 nm [21], scanning transmission electron microscopy in vacuum gave 883 ± 24 nm [22,23], and ordinary transmission electron microscopy in vacuum gave 880 ± 30 nm[24].

Although the lengths of the phage are highly consistent, measurements on the height do not show agreement. Two AFM measurements of heights of fd and M13 phage gave very different results. M13 phage is reported at a height of 5.3 nm [25], while

similar studies on fd report a value of about 0.5 nm [26]. This is more than a 10-fold smaller value. These discrepancies are worrisome because these phage are very structurally similar. Atomic force microscopy is a specific type of scanning probe microscope that is used to study surface properties of materials with atomic to micron resolution [27]. Since this instrument was designed to look at material surfaces not biological particles a new protocol was formed that involved controlling the relative humidity. It has been found that humidity plays a significant role in determining heights and reproducible images by AFM. Approximately a 10-fold change in measured height in AFM studies of phage fd is made at 15% relative humidity (RH) versus 60% RH. The height of phage is extremely sensitive to relative humidity over a range 15-100%, and correct heights of the phage are only obtained by careful extrapolation to zero RH [15].

The basic AFM instrument consists of several components that all play an important role in the instruments functioning properly. The probe (usually referred to as the tip) is the only part of the instrument that makes contact with the sample surface. Tips can be composes of many materials, but usually silicon and silicon nitride are used. The dimensions of tips can also vary from a few μm in length with diameters ranging from a few hundred angstroms to a few dozen nanometers. These small tips are mounted on the end of a holder called the cantilever. The tip/cantilever system can be thought of like a record player head complete with protruding needle. A record player head holds the needle with respect to the record surface, and the cantilever holds the tip with respect to the sample surface. The cantilever, however, also provides flexibility to help minimize surface damage as the tip scans the sample surface.

The atomic force microscope can be operated in three modes: contact mode, noncontact mode, and intermittent contact (tapping) mode [27]. In the contact mode the tip is continually in contact with the sample surface, and is characterized by van der Waals repulsive interactions between the tip and the surface. As the tip scans the sample surface the changes in topography cause the cantilever to deflect. This deflection is converted by a computer to an image. In non-contact mode the tip is not in contact with the sample surface. This mode involves vibrating the cantilever near its resonant frequency just above the sample surface. As the tip nears the surface changes in the cantilever amplitude are detected by the computer and converted into an image. In tapping mode the cantilever is vibrated so the tip just taps the sample at the bottom of it oscillation. The changes in amplitude are monitored by the computer and constructed into an image of the surface [27]. When characterizing a sample the mode of AFM must be selected carefully, especially for biological particles.

If an aqueous suspension of phage is mixed with equal amounts of chloroform at 22°C, the filaments contract into hollow spherical particles which have been termed S-forms. These S-forms have been measured under electron microscopy to have a diameter of 40 nm [28]. Similar treatment of the filamentous phage at 4°C results in a contraction to a hollow rod which is flared on one end and rounded on the other. This structure, termed I-form, is 250 nm long and 15 nm wide measured by both mounting onto carbon supports and shadow casting with tungsten, and by negative staining with uranyl acetate. Upon reexposure to chloroform at 22°C I-forms contract into S-forms [18].

I-forms and S-forms are proposed to be molten globules [29]. Molten globules are compact, have nonrigid side chain packing, are non-native protein forms with native-like

secondary structure, and were initially characterized by Tanford et al. [30], but were renamed molten globules by Ohushi and Wada [31]. Properties of these I and S-forms include: they are compact, are rich in α-helix (like native phage), have non-native morphologies, will aggregate with increasing temperature, and have increased accessibility to their hydrophobic regions to the hydrophobic probe 1-anilino-napthanlene-8-sulfonate (ANS). These four properties correspond to characteristic properties of molten globules [29].

Humidity and height studies have been carried out by AFM for filamentous phage fd, but not for the S-forms. The purpose of this study is to determine the effect that relative humidity have on S-forms of phage fd.

Materials and Methods

The filamentous fd phage was originally obtained from Don Marvin, who isolated it from *Escherichia coli* bacteria [32]. In the twenty years since the phage was acquired DNA sequencing of the pVIII gene confirms it has not mutated. The phage was grown on *Escherichia coli* MV 1190 in cloning kits supplied by Biorad.

Methods for growth and purification of phage followed standard procedures, including centrifugation to remove host cells, 5% polyethylene glycol precipitation from 0.1 M NaCl, differential centrifugation, and equilibrium centrifugation on KBr density gradients. To remove the KBr the density gradient bands corresponding to the fd phage were dialyzed at 4°C into a solution of 0.015 M boric acid and 0.1 M NaCl for 48 to 72 hours. The phage was determined to be free of contaminants such as protein or nucleic acid from the absorbence ratio at 268 and 243 of 1.33. This purified phage was then stored in a solution of 0.015 M boric acid and 0.1 M NaCl at pH 8.2 at 4°C.

To avoid the chance of salt crystal formation upon drying samples for AFM, stored fd phage were diluted 1000-fold immediately before use into 1.0 mM ammonium acetate. The filamentous phage was then converted into I-forms by vigorously mixing the phage with equal volumes of chloroform for one minute at 4°C. The phage and chloroform were separated and the phage was kept at room temperature for 10 minutes, before again being mixed with an equal volume of chloroform to convert the I-form to S-forms. The S-forms were then separated from the chloroform and 10µl of the phage solution was placed on a $1cm^2$ surface of freshly cleaved piece of mica. The mica was previously attached to metal sample pucks using super-glue, and then placed on a holder mounted to the shaft of a Dremel Moto-tool and spun dry for ten minutes. The Moto-tool was controlled using a variac to spin the samples at an optimum speed where the excess solution was spun off, but the solid phage samples remain. Dr. Ji and colleagues found that if the variac was set at 40 V, which translates in to a sample rotational velocity of 10,000 rpm, the samples would both remain on the puck and not have excess salt deposits caused by drying of solution layers. Once the sample was spun dry it was immediately placed into a controlled relative humidity (RH) environment. Placement of a sample in the atmosphere above a salt solution resulted in a stable RH. The samples were kept at fixed RH environments for at least 12 hours to assure equilibration. Appropriate salt solutions were chosen for fixed RH values of 15% and 80% [29]. Samples were not removed from these containers until they were mounted in the AFM.

The humidity was controlled within the AFM by the use of a commercial insert, the "Hum Plug" sold by BioForce Laboratory Inc. (Santa Barbara, CA) and a constant flow of nitrogen gas bubbled through the same salt solution that was used to equilibrated the

sample. The RH above the sample was continually monitored by the sensor included with the Hum Plug and are the values reported here.

The AFM used is a Digital Instruments, DI, Nanoscope III with multimode head. Contact mode AFM was only used in this study, and the Si3N4 tips were purchased from Digital Instrument. Only the longest (200μm) and thinnest cantilevers were used, and according to DI literature this cantilever has a normal Hook's law force constant of 0.06N/m and a tip diameter ranging from 10 to 40 nm. Standard DI software was used for imaging and capturing force calibration curves.

Results

To study the relative toughness of the S-forms to compression as a function of RH, a series of AFM images were taken at progressively greater force (set point). This series started with the minimum force necessary to acquire a reliable image. Once the series was complete the force was reduced to the initial value and the AFM image rescanned.

In each case an internal control was used by having the initial scan area include three or four S-forms. The image region was then zoomed to include only one or two particles and images of these particles were obtained at progressively higher set points. When a set point of 7 V was reached the set point was reduced to its original value and the larger area containing three or four particles was rescanned.

Figure 1 represents the results of the experiment described above at 15 % RH. The tip forces in nN was computed, using the manufacturer published values for the cantilever force constant and the measured set point-sample displacement curve on a part of the sample which was phage-free. Note that all forces are in Hookian forces, meaning

that no correction for the adhesive contribution was made. The adhesion force, however, was determined from the set point-distance curve to be 19.3 $\pm$ .784 nN for data at both 15% and 80 % RH. In Figure 1(a) a Hookian force of 5.6 nN was used. A smaller scan region was zoomed in on, so only the two particles in the middle of the picture were imaged. As the force was increased images were taken progressively up to a maximum force of 28 nN. The force was then reduced to the original 5.6nN value and the original area was scanned again to produce Figure 1(b). Comparison of these two images shows the S-forms which experienced the high forces appeared as irregular circles, and were reduced in height. In Figure 1(b) a box can be seen around the area that was scanned at higher forces. The tip dragged material off the particle as it scanned, and deposed the material as it reversed directions to scan the other way, forming the box. At 80% RH when the same experiment was carried out a box was also seen around the area scanned at the higher force and particles decreased in height.

The reduction of height occurred uniformly for both S-forms at 15% and 80% RH with increased tip force. Figure 3 provides an evaluation in the measured height of S-form samples at 15% and 80% RH with Hookian forces observed. The height values were averaged from three different cross-sectional measurements taken from several particles. The height of those at 15% RH dropped by 34.4% and the height of those particles at 80% RH height dropped by 35.1% as the force on each progressively increased.

The height of the S-forms at 80% RH were 16.3% lower than the height of particles at 15% RH with minimum force applied. Both of the samples decreased in height almost parallel to each other, with the heights of those at 80% RH being lower than those

at 15% RH. All of the drops in height were irreversible, with the structure of the particle suffering permanent change.

Discussion

In studies to test the hardness of the filamentous form of phage fd as a function of RH, the height reduction does not occur uniformly. At both high and low humidity there is an initial small compression with applied force followed by a plateau (Figure 4) [15]. This plateau is not observed in the S-forms. Rather, the decrease in height occurs very close to linearly at both high and low humidity.

S-forms of phage fd have been characterized as molten globular proteins, one of the cardinal features of the molten globular state is the nonrigid side chain packing. The lack of plateau with an increase in force is due to the less rigid side chain packing in S-forms. Since the particles are softer, as the force increases on them the height decreases in a uniform fashion. The decrease in height of the particles at both humidities are irreversible (data not shown). As the force increases the tip pushes harder as it crosses the particle. The softer nature of the S-forms allows some of it to be scraped off and pushed by the tip until the tip turns around and scans in the other direction. Since material is removed from the particle the decrease in height is irreversible.

When the force is reduced after reaching high forces and the image is enlarged to its original size, a box can be seen around the area scanned at the higher force (Figure 1b and 2b). This box is made of the material the tip gathered off both the particle and the mica surface as it scanned. The enlarged image after some particles are exposed to high forces is of less quality and clarity than the original control image. The tip deposits most

of the material it picks up while scanning when it turns around, but some of the material remains fixed to the tip resulting in a less sharp image.

Both particles at 15% and 80% RH drop in height the same percentage, indicating that a lower RH did not increase the toughness of the S-form. However, those particles at 80% RH were initially 16.3% lower than those particles at 15% RH when the minimum force was applied. It has been proposed that a meniscus-like layer of water containing ions, from the ammonium acetate buffer, potassium and hydroxylions from the mica, and carbonate from the air, surrounds the phage. As the relative humidity increases water would be absorbed into this ionic solution causing the particles to have a lower height as the particle-mica, particle-solution and particle-air interfaces were changed [15]. Thus, particles at higher RH appear shorter than those at lower RH.

S-forms at both 15% and 80% RH exposed to high forces appeared as less regular circles than those not exposed to high forces. Comparing both the particles at 15% and 80% RH after high forces were applied, the samples at 80% RH were considerably less circular than those at 15% RH (Figure 1b and 2b). The particles at high RH may decrease the same percentage in height as those at low RH, but they are softer to start off with causing their edges to disfigure more as force is applied.

The use of AFM to study biological particles is steadily increasing, however, producing reliable reproducible images is often a challenge. It has been know for some time that the relative humidity can alter the appearance of AFM images, but has been poorly appreciated. As the practice of imaging biological molecules by AFM continues to increase, so will the control of RH over these particles. Control of RH is an essential element in achieving both stable and reliable images.

Figure Legends

Figure 1. Before and after AFM images taken at 15% RH. In the initial (A) scan, the tip force was 5.6 nN. Image B was also taken at 5.6 nN after the S-forms in the middle had been scanned with a tip force of 28nN.

Figure 2. Before and after AFM images taken at 80% RH. Image A the tip force was 4.55nN . Image B was also taken at 4.55nN after the two particles on the right were scanned with a tip force of 27nN.

Figure 3. Apparent height of S-forms as a function of maximum tip force applied. Results obtained from samples prepared and measured at 15% RH and 80% RH.

Figure 4. Apparent height of filamentous phage as a function of maximum tip force applied. Results obtained from samples prepared and measured at 15% RH and at 60% RH by Ji et. al.

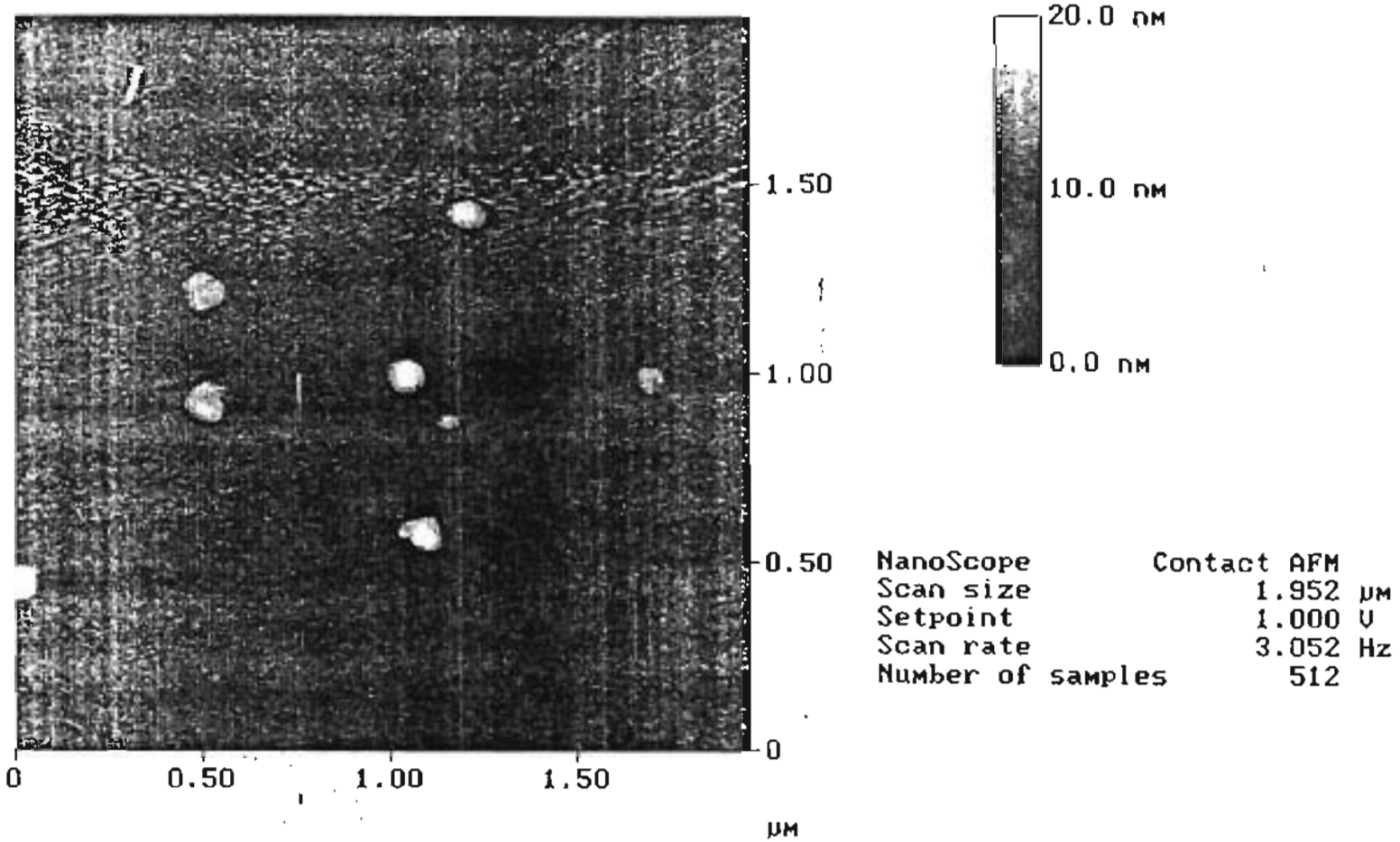
20.0 nm
10.0 nm
0.0 nm
1.50
1.00
0.50
0
0
0.50
1.00
1.50
µm
NanoScope Contact AFM
Scan size 1.952 µm
Setpoint 1.000 V
Scan rate 3.052 Hz
Number of samples 512

Figure 1(a)

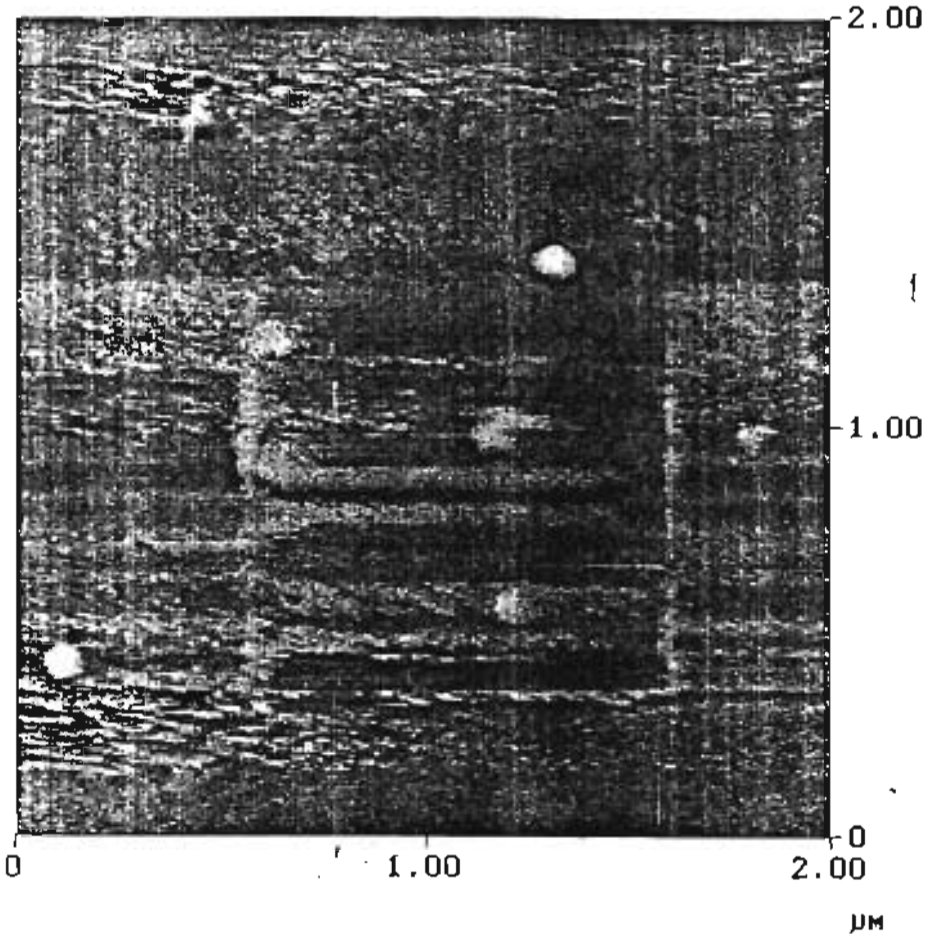
2.00
1.00
0
0
1.00
2.00
µm
20.0 nm
10.0 nm
0.0 nm
NanoScope Contact AFM
Scan size 2.000 µm
Setpoint 1.000 V
Scan rate 3.052 Hz
Number of samples 512

Figure 1(b)

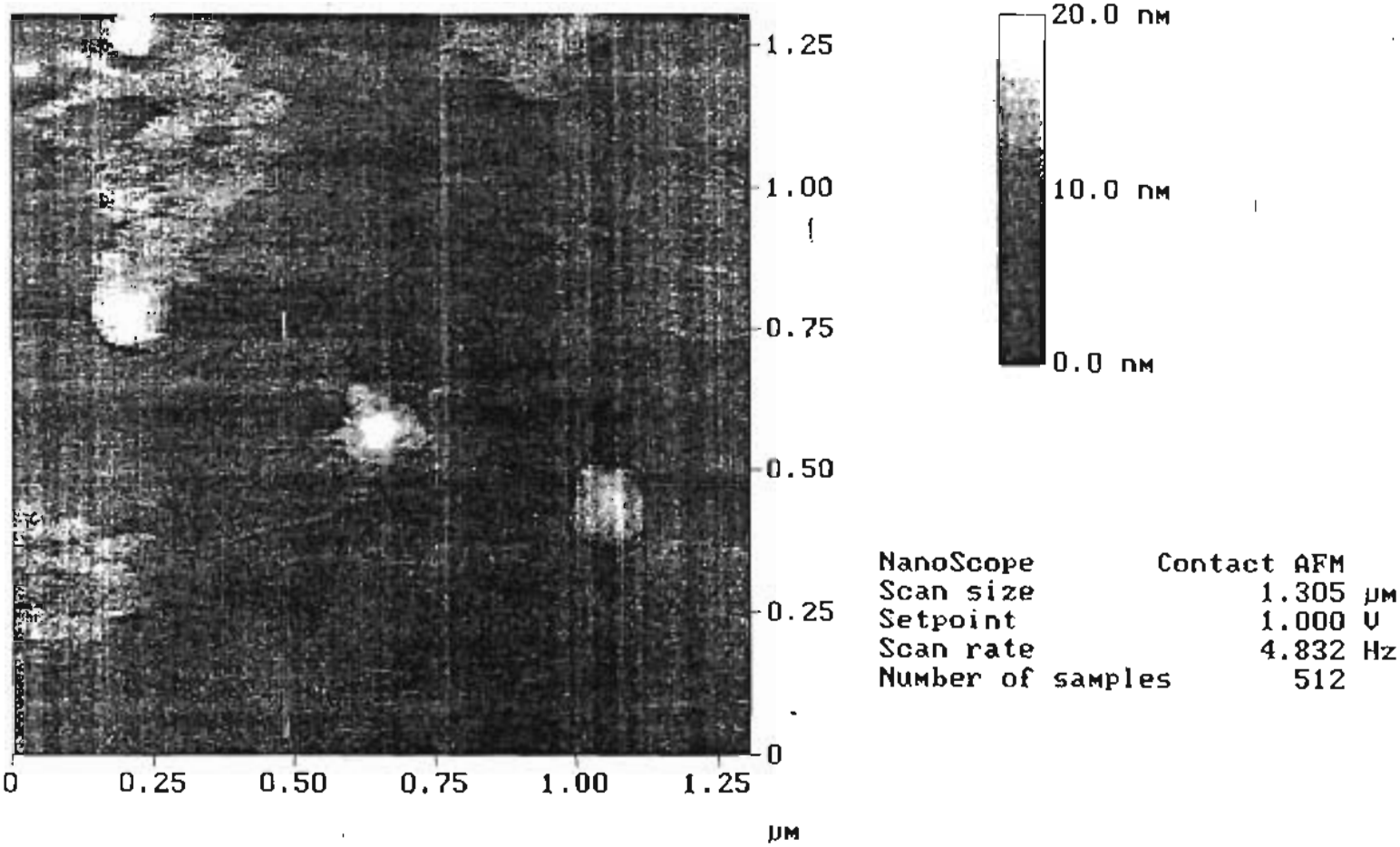
1.25
1.00
0.75
0.50
0.25
0
0
0.25
0.50
0.75
1.00
1.25
µm
20.0 nm
10.0 nm
0.0 nm
NanoScope Contact AFM
Scan size 1.305 µm
Setpoint 1.000 V
Scan rate 4.832 Hz
Number of samples 512

Figure 2(a)

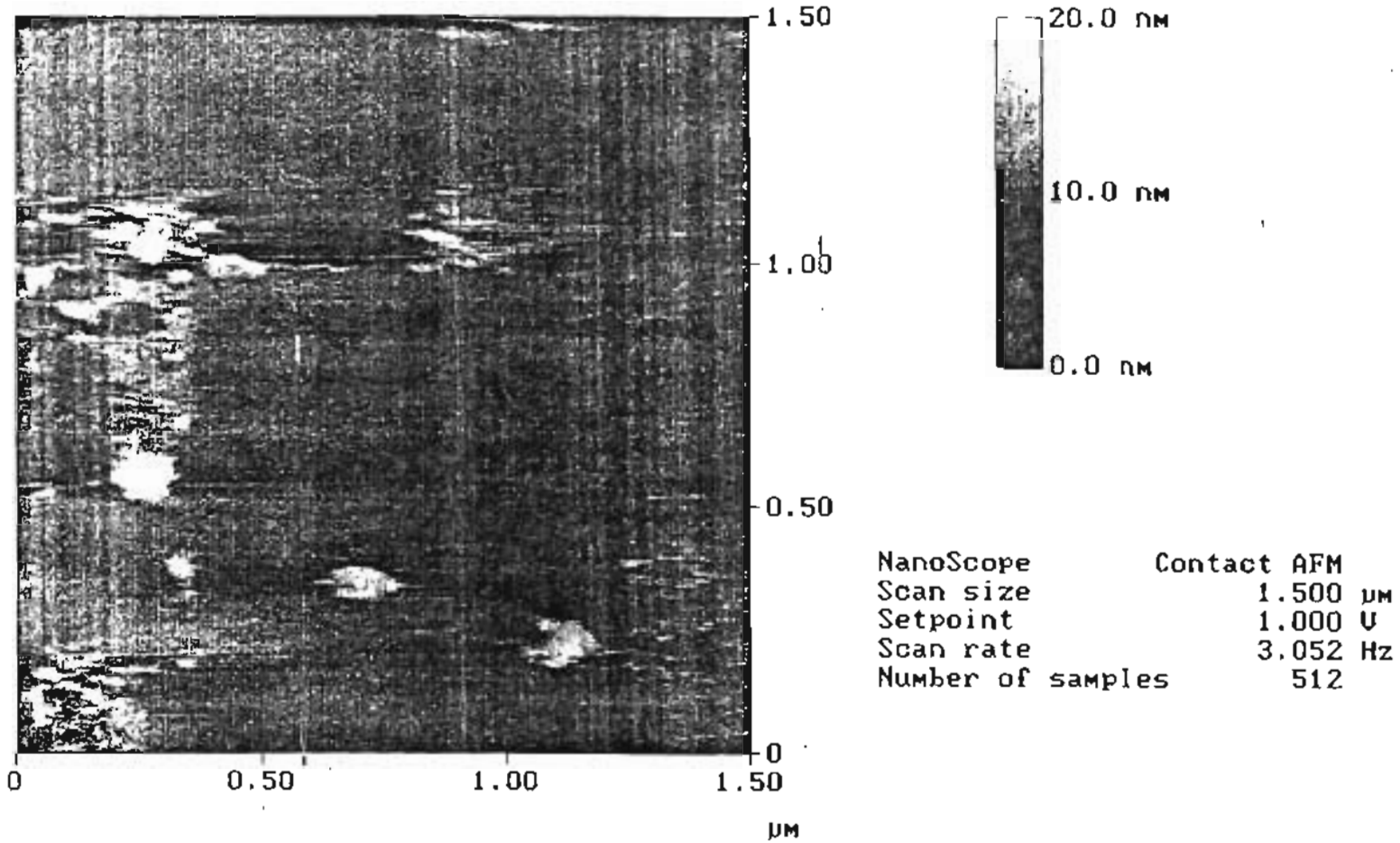
1.50
1.00
0.50
0
0
0.50
1.00
1.50
µm
20.0 nm
10.0 nm
0.0 nm
NanoScope Contact AFM
Scan size 1.500 µm
Setpoint 1.000 V
Scan rate 3.052 Hz
Number of samples 512

Figure 2(b)

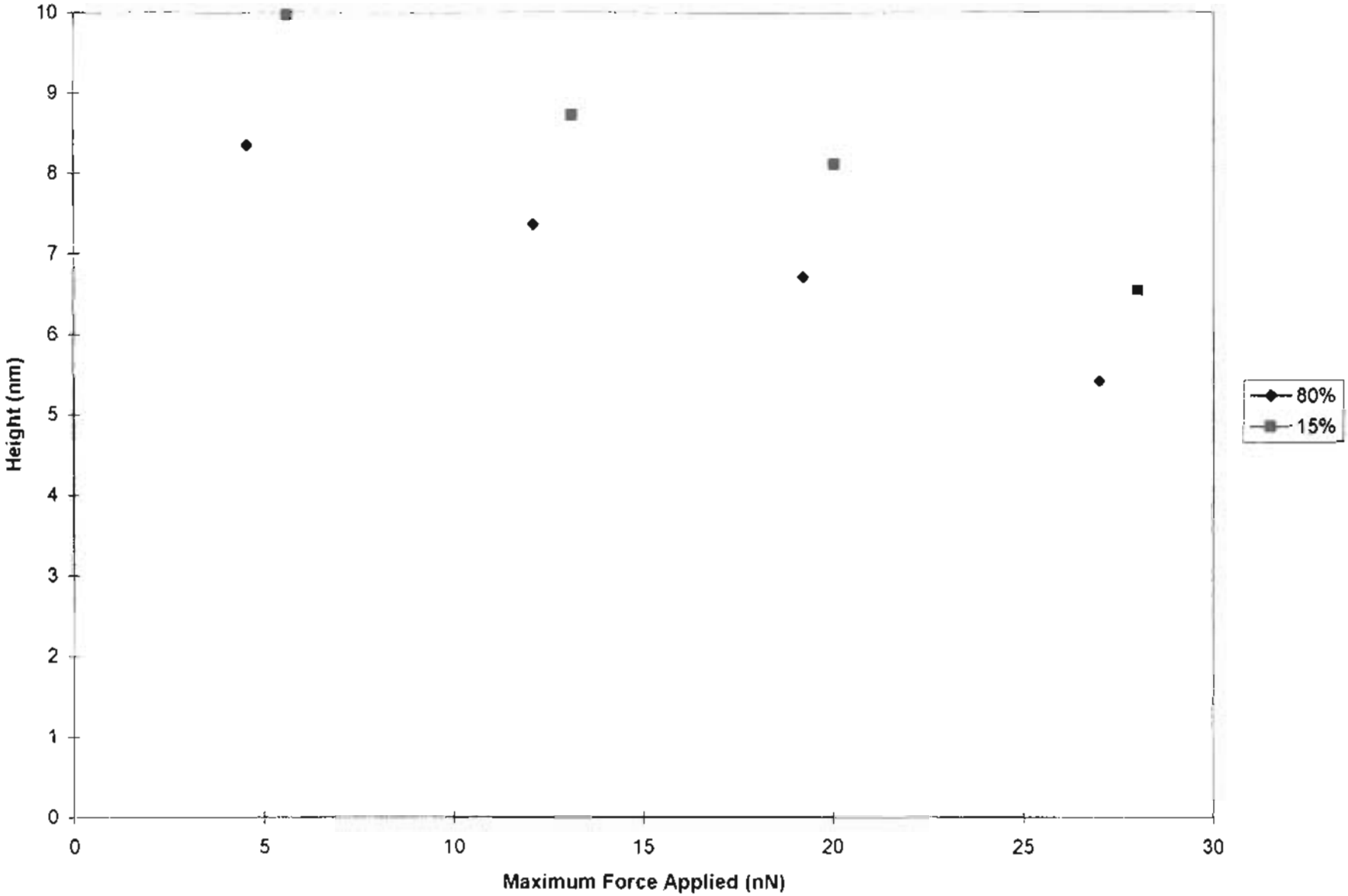
Height (nm)
Maximum Force Applied (nN)
0
1
2
3
4
5
6
7
8
9
10
0
5
10
15
20
25
30
80%
15%

Figure 3

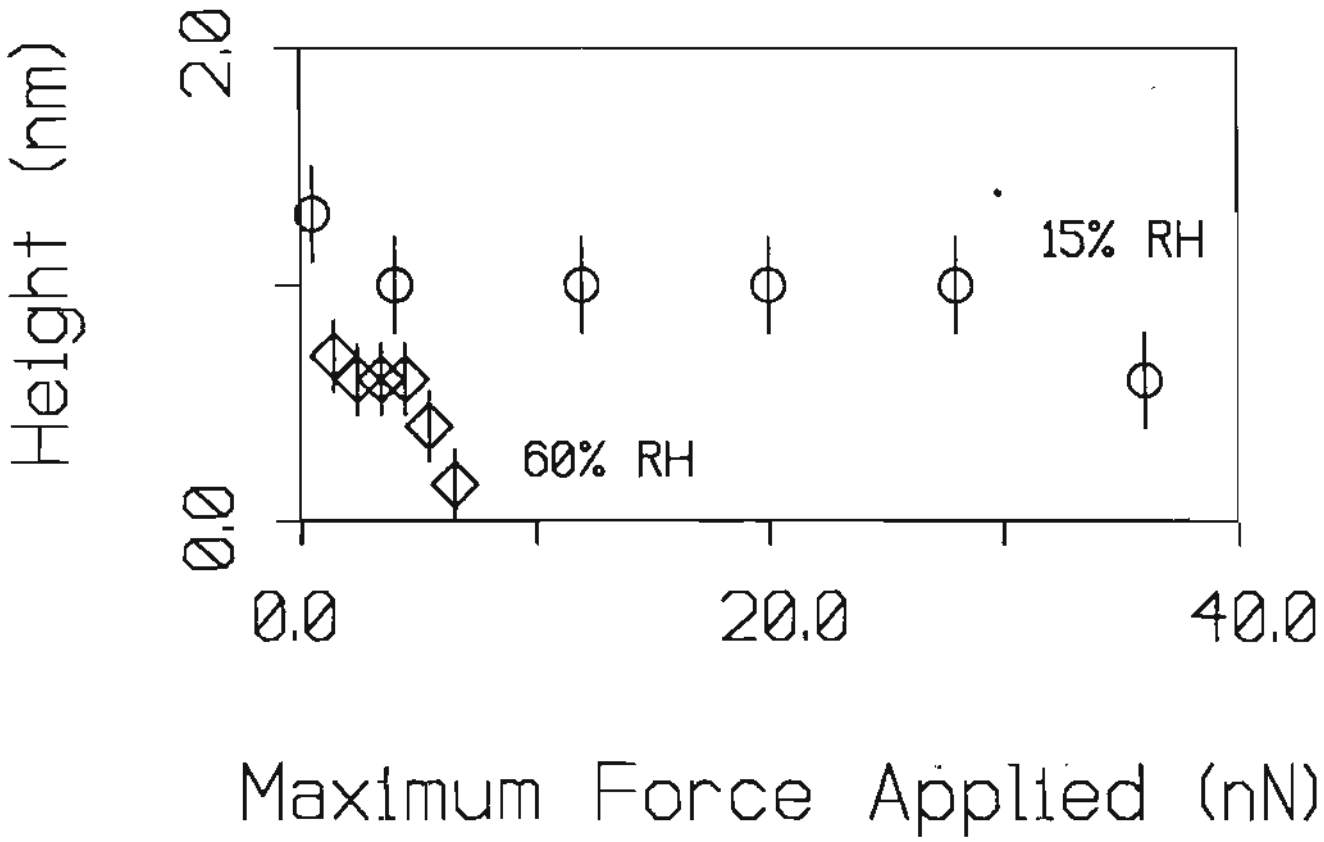

Figure 4

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