Investigation of the Image Contrast of Tapping-Mode Atomic Force Microscopy Using Protein-Modified Cantilever Tips

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ABSTRACT In this work we have designed a simple system to investigate empirically the image contrast of tapping-mode atomic force microscopy (TMAFM). We modified the cantilever tips with protein molecules (bovine serum albumin or goat anti-biotin antibody) and used these protein-modified cantilevers to scan poly-L-lysine films and antibody layers deposited on mica in air under ambient conditions. We also investigated the effects of manipulating the setpoint voltage in this system. It was found that extra topographic features with a patchlike appearance were introduced into the TMAFM images of both the poly-L-lysine and antibody films when scanned with the protein-modified tips, even at initial preset setpoints, and were superimposed on the topography of the samples. The surface coverage of the patchlike features in the TMAFM images changes significantly with the setpoint voltage in a reversible and nonlinear manner. These are believed to arise from the surface indentation of the sample or from the structural deformation of the proteins at the tip induced in TMAFM imaging. Interestingly, it was observed in the experiment that no structural alteration or damage was discernible on the sample surface, even after continuous scanning with the protein-modified tips for a long period of time, with varying setpoint voltage. This study provides experimental evidence that cantilever tips modified with protein molecules or, under certain circumstances, even unmodified tips introduce extra topographical features (i.e., artifacts) and enhance the image contrast of TMAFM imaging of soft materials, which is dependent on their mechanical properties.

INTRODUCTION

Recent studies have shown that tapping-mode atomic force microscopy (TMAFM) represents a new, promising approach to the study of soft, particularly biological, materials (Henderson, 1994; Hansma and Hoh, 1994; Lal and John, 1994; Hansma et al., 1995; You and Lowe, 1996a). In imaging biological materials, TMAFM offers an important advantage over conventional contact-mode AFM: biological molecules and live cells can be imaged directly without the use of any complicated chemical methods to immobilize biological samples onto the substrate and, thus, the structure and function of biomolecules and cells can be studied at the same high level of resolution. Despite the recent success of TMAFM in imaging biological molecules and cells (Henderson, 1994; Hansma and Hoh, 1994; Lal and John, 1994; Putman et al., 1994; Radmacher et al., 1995; Walivaara et al., 1995; Munoz-Botella et al., 1996; Schabert and Rabe, 1996; You and Lowe, 1996a), there are some basic aspects of TMAFM imaging of biological samples that are still not well understood, for instance, the factors determining the loading force applied to the sample, the deformation of biological samples, and most importantly, the image contrast mechanism(s). Because of the unexpected complexity of TMAFM, there have been few theoretical and experimental studies of the image contrast mechanism(s) (Putman et al., 1994; Hoper et al., 1995; Radmacher et al., 1995; Spatz et al., 1995; Chen et al., 1996; Ho and West, 1996; Howard et al., 1996; Munoz-Botella et al., 1996; Schabert and Rabe, 1996; Tamayo and Garcia, 1996). There is an urgent demand for a detailed explanation and full understanding of the nature of the structure seen in the TMAFM images of biomolecules.

Recently it has been reported that the frequency-dependent viscoelastic properties of the sample play an important role in determining the success and contrast of TMAFM imaging of biological samples (Putman et al., 1994; Radmacher et al., 1995; Munoz-Botella et al., 1996; Schabert and Rabe, 1996). In this work we have designed a simple system for studying the effect of the protein adsorption at the cantilever on the image contrast of TMAFM. We physically attached protein molecules, i.e., bovine serum albumin (BSA) or goat anti-biotin antibody (Ab), to the cantilever tips and used these protein-modified tips to scan poly-L-lysine films deposited on mica (PL/mica) and antibody layers adsorbed onto the poly-L-lysine-deposited mica surface (Ab/PL/mica). We also investigated the effects of the manipulation of the setpoint voltage, which is an important parameter in TMAFM imaging.

The poly-L-lysine (PL) was selected as the substrate for two reasons. PL is commonly used as a coating layer to enhance the adhesion of biological molecules and cells to solid surfaces, such as coverglass and mica. PL also provides a versatile route for covalently attaching protein molecules to solid surfaces, either via coupling of its amine groups to those of the protein or via the modification of some of its lysine residues (Jordan et al., 1994). BSA is often used as a blocking agent for preventing adhesion of bacteria and cells and thrombus formation and for reducing nonspecific binding (Taborelli et al., 1995). Recently BSA

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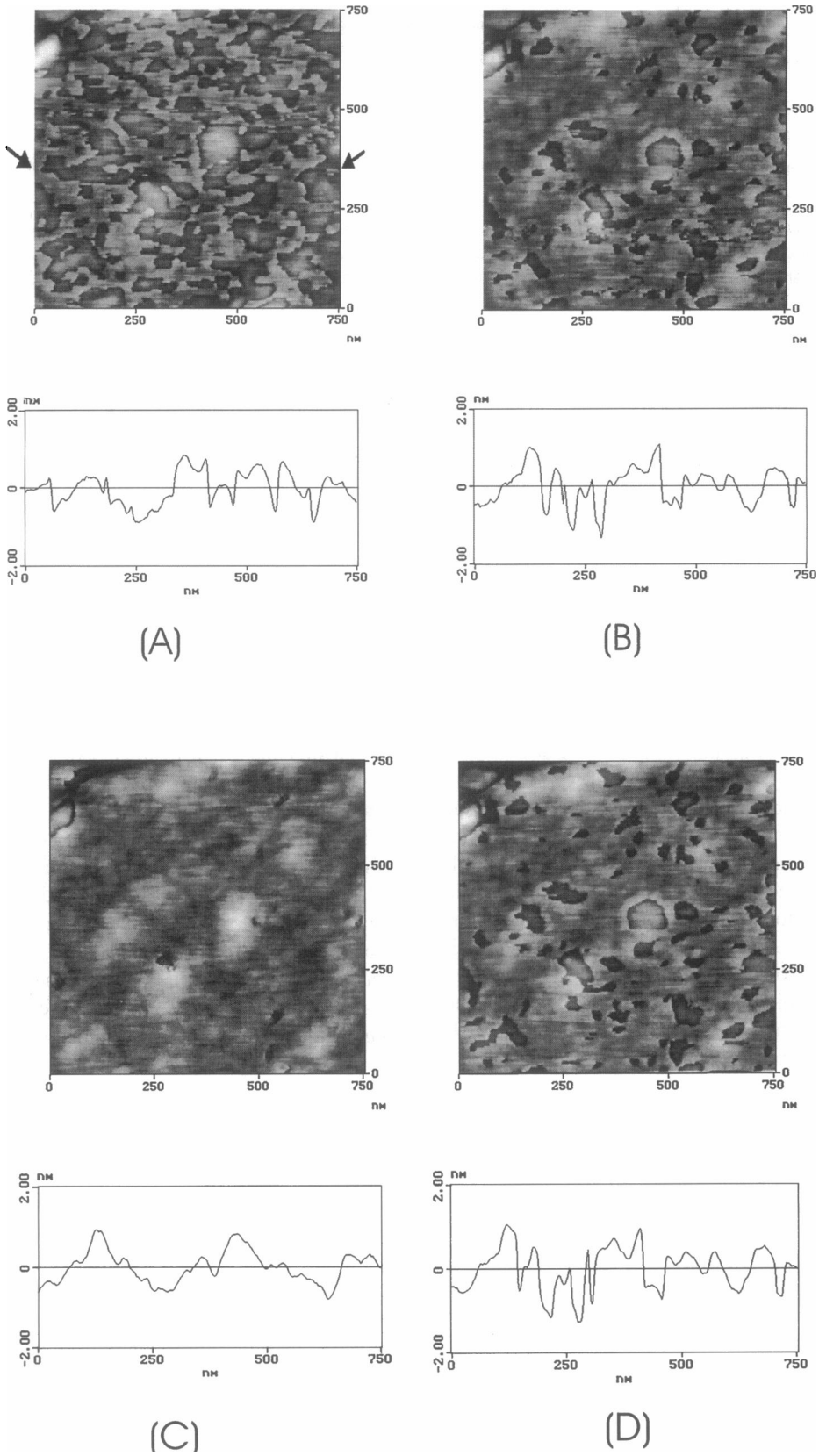

FIGURE 1 Selection of the TMAFM images of poly-L-lysine-deposited mica surface obtained by scanning with BSA-modified tips at the setpoint voltage: (*A*) 4.0 V, (*B*) 2.0 V, (*C*) 1.0 V, (*D*) 2.0 V, (*E*) 4.0 V. Image dimensions: 750 × 750 × 10 nm. The arrows in *A* indicate the position where the line profile is taken from the corresponding image across the *X* direction.

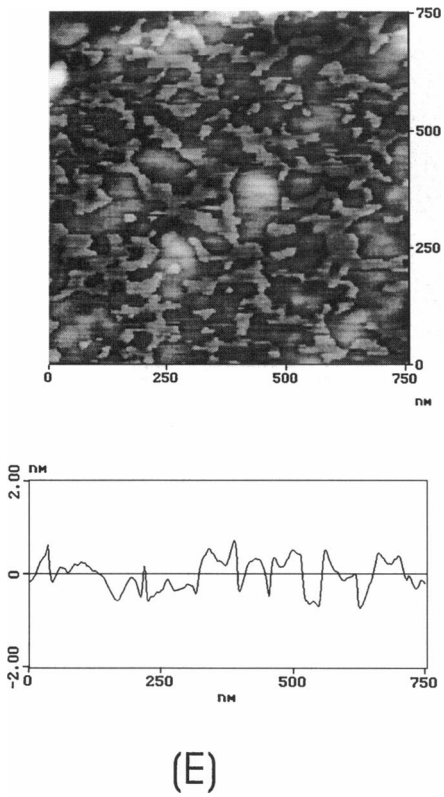

FIGURE 1 *Continued*

has also been used as a coupling reagent for attaching a specific ligand to the AFM tip surface in the measurement of ligand-receptor binding strength (Florin et al., 1994; Dammer et al., 1996). Monolayers of antibody molecules adsorbed on a solid surface are widely used in biosensors or other biochemical analytic devices as a selective binding component (You et al., 1995; You and Lowe, 1996b) and have been used in recent AFM studies of antigen-antibody recognition events (Stuart and Hlady, 1995; Dammer et al., 1996; Hinterdorfer et al., 1996). Furthermore, the system we have designed may represent a real experimental situation, i.e., one or a few biological molecules accidentally attached to the bare tip when biological molecules adsorbed to solid surfaces are imaged with TMAFM. Finally, the protein-modified tip has additional potential for the study of molecular recognition. Much attention has recently been paid to the measurement of adhesion forces between individual ligand-receptor pairs using an AFM tip modified with a specific ligand (Florin et al., 1994; Stuart and Hlady, 1995; Dammer et al., 1996; Hinterdorfer et al., 1996). This concept offers us a prospective method of differentiating and recognizing the specific receptor sites at cell surfaces by mapping both the topography and the force contrast on the basis of the ligand-receptor binding force (Henderson, 1994; Stuart and Hlady, 1995; Hinterdorfer et al., 1996; Ludwig et al., 1997). However, before this goal can be realized, it is important to understand whether the image contrast of TMAFM is affected by these ligand-modified tips. The work presented was our first step toward eventually studying a more relevant biological system.

MATERIALS AND METHODS

Materials

Polyclonal anti-biotin antibody developed in goat, bovine serum albumin, and poly-L-lysine hydrobromide (MW 300,000) were purchased from Sigma (St. Louis, MO). Other reagents used (Sigma) were used without further purification.

Sample preparation

Freshly cleaved mica was incubated in 250 μg/ml poly-L-lysine solution diluted with 10 mM phosphate-buffered saline (PBS), pH 7.4, for 1 h and washed with double-distilled water. The adsorption of the antibody molecules was carried out by incubating the freshly prepared poly-L-lysine-modified mica in the PBS buffer containing 25 μg/ml goat anti-biotin antibody for 1 h (You and Lowe, 1996b). The samples were washed with PBS buffer, followed by double-distilled water. The residual water was removed with filter paper. The samples were mounted and imaged immediately.

Tip modification and characterization

The modification of the cantilever tip was performed as follows. The cantilever tips were exposed to UV light for 5 min; rinsed sequentially with acetone, ethanol, and double-distilled water; and finally, incubated in vials containing either 1 mg/ml bovine serum albumin or 1 mg/ml goat anti-biotin antibody overnight at room temperature (Florin et al., 1994). The cantilevers were washed with PBS buffer and double-distilled water. The water residues on the hold of the cantilever were removed carefully with filter paper. The prepared tips were used immediately.

The tip modification by protein molecules was monitored by a simple and sensitive in situ method, i.e., detecting the displacement of the resonant frequency of the same cantilever before and after the protein adsorption (Chen et al., 1995). In the experiments, the shift of the resonant frequency of a freshly prepared protein-adsorbed cantilever or, as a parallel control, a protein-adsorbed cantilever dried overnight in a desiccator, was measured to qualitatively confirm the protein adsorption. For the experiments in which the setpoint was manipulated, only those protein-adsorbed cantilevers with a free oscillation amplitude in the range of 20–70 nm under a driving amplitude of 35–60 mV were used.

Atomic force microscopy

The atomic force microscope used was a Nanoscope III Multimode microscope (Digital Instruments, Santa Barbara, CA) equipped with a "D"-type piezoscanner with a 13 μm $\times$ 13 μm maximum scan range. Single-beam silicon cantilevers with a length of 125 μm and a nominal tip radius of curvature of 10 nm were used (Digital Instruments).

Image acquisition and processing

The TMAFM was operated under ambient conditions (at a room temperature of 22 $\pm$ 2°C and relative humidity of 45 $\pm$ 5%). The images were collected in height mode in the retrace direction and stored in 256 $\times$ 256 pixel format. A range of scanning rates between 0.6 and 1.55 Hz was used; the rate was typically set at 1.0 Hz.

Before imaging, the resonant frequency and the oscillation amplitude of the protein-modified cantilever tip were allowed to stabilize. The setpoint was initially set at approximately three-fourths of the free oscillation amplitude of the cantilever tip, which was adjusted after the tip was engaged with the surface according to the quality of the initial scan. During

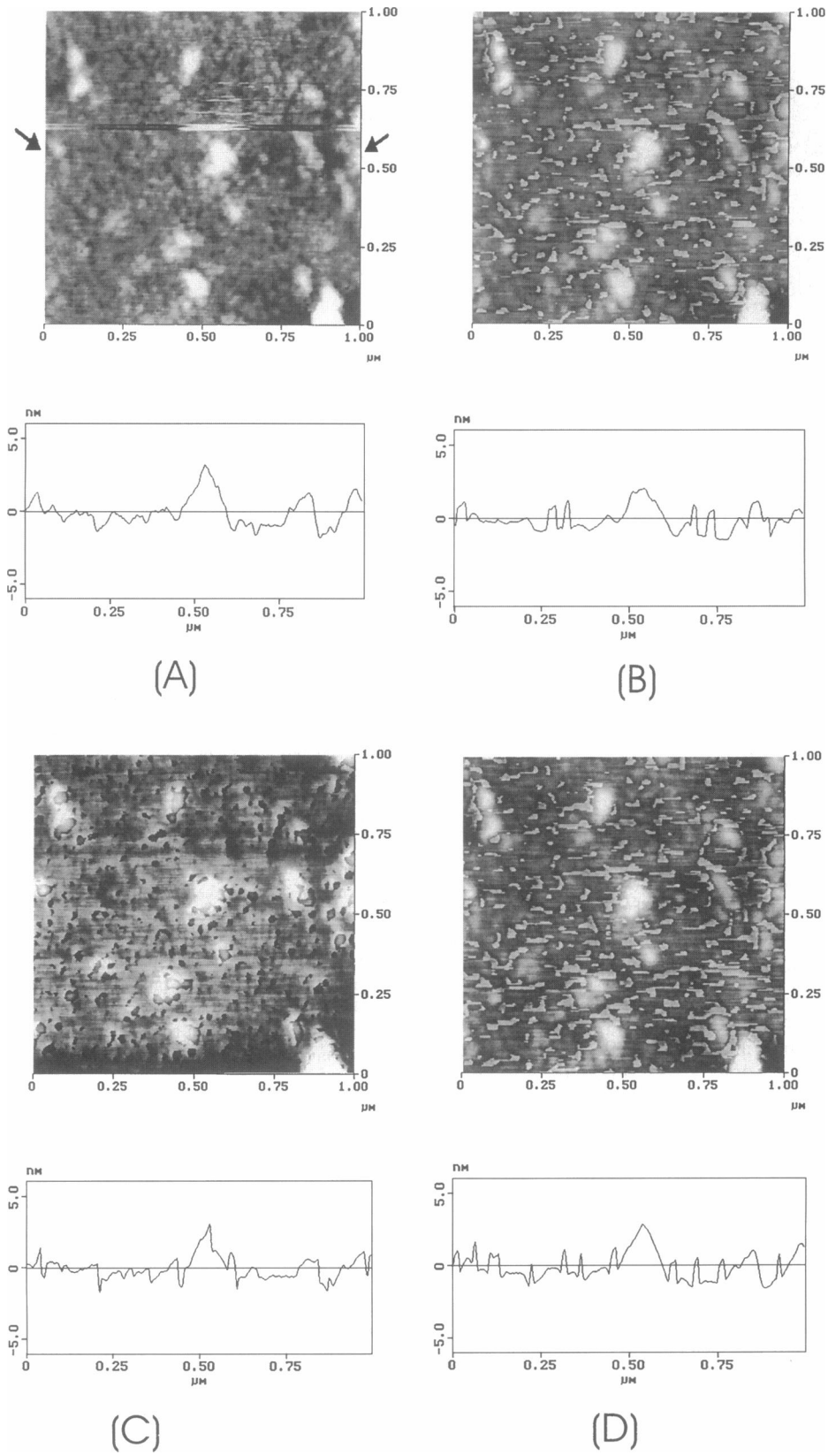

FIGURE 2 Selection of the TMAFM images of poly-L-lysine-deposited mica surface obtained by scanning with an unmodified tip at the setpoint voltage: (*A*) 4.0 V, (*B*) 2.0 V, (*C*) 1.0 V, (*D*) 2.0 V, (*E*) 4.0 V. Image dimensions: 1000 × 1000 × 10 nm. The arrows in *A* indicate the position where the line profile is taken from the corresponding image across the *X* direction.

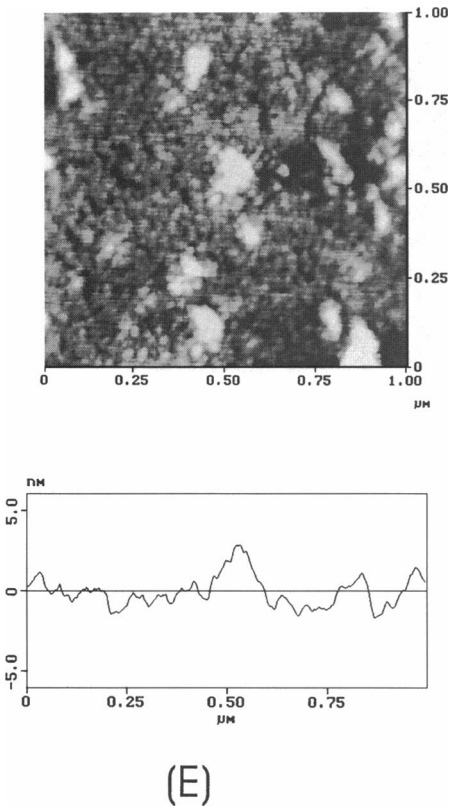

FIGURE 2 *Continued*

the experiment in which the setpoint was manipulated, only the setpoint voltage was varied, while other parameters remained constant. The images shown are the raw experimental data, subjected only to normal imaging processing of plane auto-fitting and gray-level adjusting, unless otherwise specified. Imaging acquisition and data analysis were performed with Nanoscope software (version 4.22).

RESULTS

Poly-L-lysine-modified mica

TMAFM images of the poly-L-lysine-modified mica (PL/mica) surface, obtained by scanning continuously with the BSA-adsorbed tips (BSA-tip) over the same surface area and by decreasing stepwise and subsequently increasing the setpoint voltage with a typical increment of 0.5 V, are selectively shown in Fig. 1, along with line profiles taken across the central position of the images in the *X* direction. During the experiments it was observed that as soon as the initial scan was started, networks of patchlike features appeared in the image. This kind of feature was stable with continuous scanning unless the setpoint drifted. Immediate changes were observed as soon as the setpoint voltage was reduced from the initial value by, for example, as little as 0.1 V. The observation of the patchlike features and its correlation with the setpoint voltage, as illustrated in Fig. 1, are summarized as follows: 1) the surface coverage of the patchlike features in general increases, in a nonlinear manner, with the decrease in the setpoint voltage; 2) changes in the patchlike areas with the setpoint voltage are reversible, e.g., the topographic features seen in Fig. 1 *A* are almost reproduced in Fig. 1 *E*; 3) the patchlike features repeatedly appear in the image when the tip scans other surface areas, unless the tip is worn or in unstable condition; and 4) the patchlike features are superimposed on the topography of the sample. These observations are also clearly demonstrated by the line profiles shown in Fig. 1, in which both the number and the corrugation height of the jagged peaks fluctuate with the setpoint voltage. The thickness of the patchlike features, which is measured as the height difference between the top and the bottom edges of the jagged peaks shown in the line profiles of Fig. 1, is 0.93 ± 0.17 nm, and remains nearly constant when the setpoint is changed.

The experiments with the antibody-adsorbed tips (Ab-tip) produced similar results, e.g., the patchlike feature, which changed with the setpoint voltage was observed. However, in this system, we observed that occurrence of the patchlike features was not so sensitive to changes in the setpoint voltage, and the patchlike features did not appear in the image scanned at initial setpoint voltages. Furthermore, the Ab-tips were usually worn out after one or two series of scans with setpoint manipulation.

As a control, experiments in which the PL/mica surfaces were scanned with bare, unmodified tips were carried out as well. Surprisingly, patchlike features strikingly similar to those seen in Fig. 1 appeared in the images in the course of setpoint manipulation (Fig. 2). As shown in Fig. 2, the PL/mica surface is generally uneven, consisting of small and large aggregates, formed by local polymerization (Fig. 2 *A*). The PL layers prepared in the experiment have, in most cases, monolayer surface coverage, in agreement with earlier studies (Hartley et al., 1993; Leckband et al., 1993). In Fig. 2, note again that after one cycle of setpoint manipulation, the topographic features seen in Fig. 2 *E* replicate those shown in Fig. 2 *A*, suggesting that the changes in the patchlike feature induced by changes in the setpoint voltage are reversible.

Antibody molecules on poly-L-lysine/mica surfaces

Experiments in which the setpoint was manipulated in the same fashion as described above were conducted on prepared antibody poly-L-lysine/mica (Ab/PL/mica) samples with BSA-tips; selected images are shown in Fig. 3. In Fig. 3, line profiles taken, respectively, across the granule (located at the upper part of the image and with the brightest contrast) in the *X* direction from the corresponding images are included, to illustrate more clearly the variation in the surface topography with the setpoint voltage. As can be seen in Fig. 3, at the initial setpoint voltage, the patchlike features cover the whole sample surface (as revealed by dark pits in a circular shape at the left upper corner of Fig. 3 *A*) and convolute with large granules of antibody molecules, which

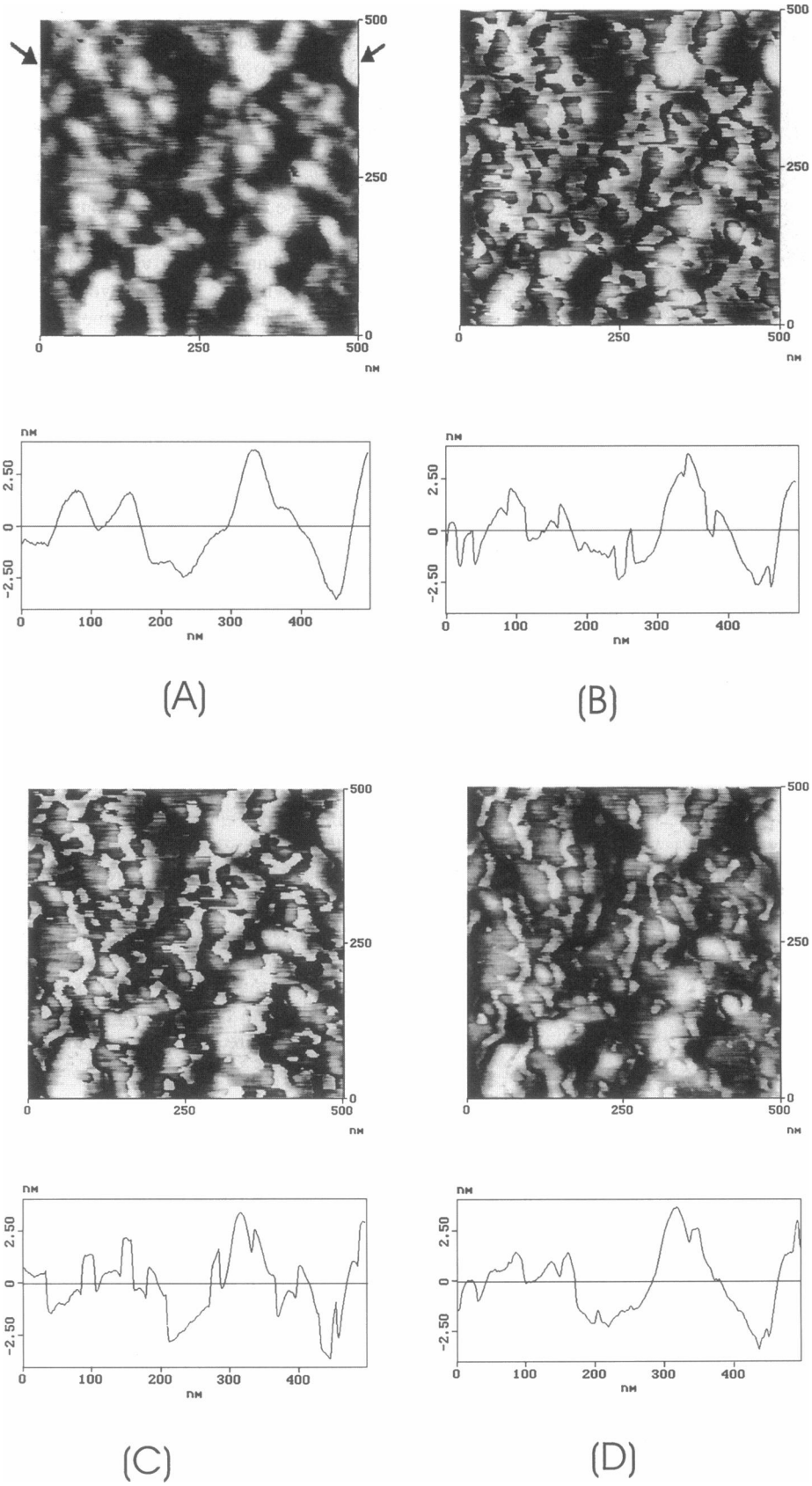

FIGURE 3 Selection of the TMAFM images of antibody layer adsorbed on the poly-L-lysine-deposited mica surface obtained by scanning with BSA-adsorbed tip at the setpoint voltage: (*A*) 2.0 V, (*B*) 1.5 V, (*C*) 1.0 V, (*D*) 0.5 V, (*E*) 1.5 V, and (*F*) 2.0 V. Image dimensions: 500 × 500 × 7 nm. The arrows in *A* indicate the position where the line profile is taken from the corresponding image across the *X* direction.

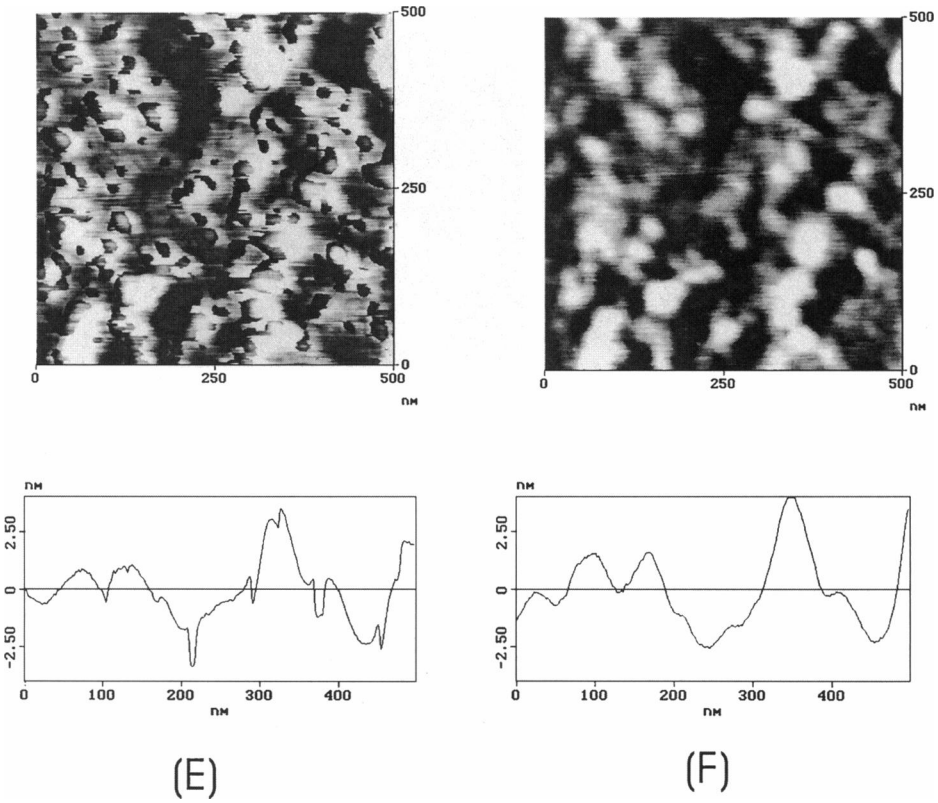

FIGURE 3 *Continued*

are of high corrugation height. These dark circular pits are formed by the incomplete closure of the patchlike features (for example, see Fig. 3 *E*). Soon after the initial setpoint voltage is decreased by 0.5 V, the perceived patchlike features appear in the image (Fig. 3 *B*), and the surface area covered by the patchlike features is decreased when the setpoint is further lowered (Fig. 3, *C* and *D*). As soon as the setpoint voltage is returned to its initial value, the patchlike feature disappears (Fig. 3 *F*), and the original topographical features seen in Fig. 3 *A* reappear. Comparison of Fig. 3 *F* and Fig. 3 *A* (including the respective line profiles) clearly indicates that no obvious structural modification has occurred to the surface as a result of continuous scanning at various setpoint voltages. The patchlike features observed here look very similar, in terms of the pattern and shape, to those described in the previous section, except that the surface coverage of the patchlike feature decreases with the decrease in the setpoint voltage. The thickness of the patchlike features shown in Fig. 3 is measured at 1.55 ± 0.22 nm and remains in this range when the setpoint voltage is changed.

In a parallel experiment, the Ab/PL/mica samples prepared under the same condition were scanned with a bare tip, and the setpoint was manipulated in the same manner as described above. Examples of the images obtained are shown in Fig. 4. Fig. 4 *A*, obtained at a setpoint voltage of 3.0 V, shows a representative granular feature commonly observed by AFM of antibody molecules adsorbed on solid surfaces (Quist et al., 1995; You et al., 1995; Walivaara et al., 1995; You and Lowe, 1996b). Afterward, the setpoint voltage was decreased stepwise and subsequently increased by 0.5 V. From Fig. 4 it is seen that 1) the patchlike feature does not appear in the images in the course of setpoint manipulation; 2) the granular structure becomes geometrically small and part of it gradually turns into somewhat strandlike features when it is scanned continuously while the setpoint voltage is decreased, which indicates that those loosely adsorbed molecules are displaced under the scanning tip (You and Lowe, 1996b); and 3) the strandlike features do not disappear when the setpoint voltage is returned to its initial value (Fig. 4 *D*); therefore plastic modification of the sample surface has occurred as a result of the setpoint manipulation.

Appearance of the patchlike features was observed at the Ab/PL/mica surface when it was scanned with the Ab tips and the setpoint was changed. Once again, the Ab tips were worn out after one or two series of scans and, as a result, the strandlike features were usually observed.

The occurrence of the patchlike features with the setpoint manipulation is summarized in Table 1 for the experimental system studied. The table is based on reproducible observations from more than four widely separated surface areas in each of at least three independent experiments. The height of the patchlike features measured from one series of the experimental images is also given in the table as supplementary data.

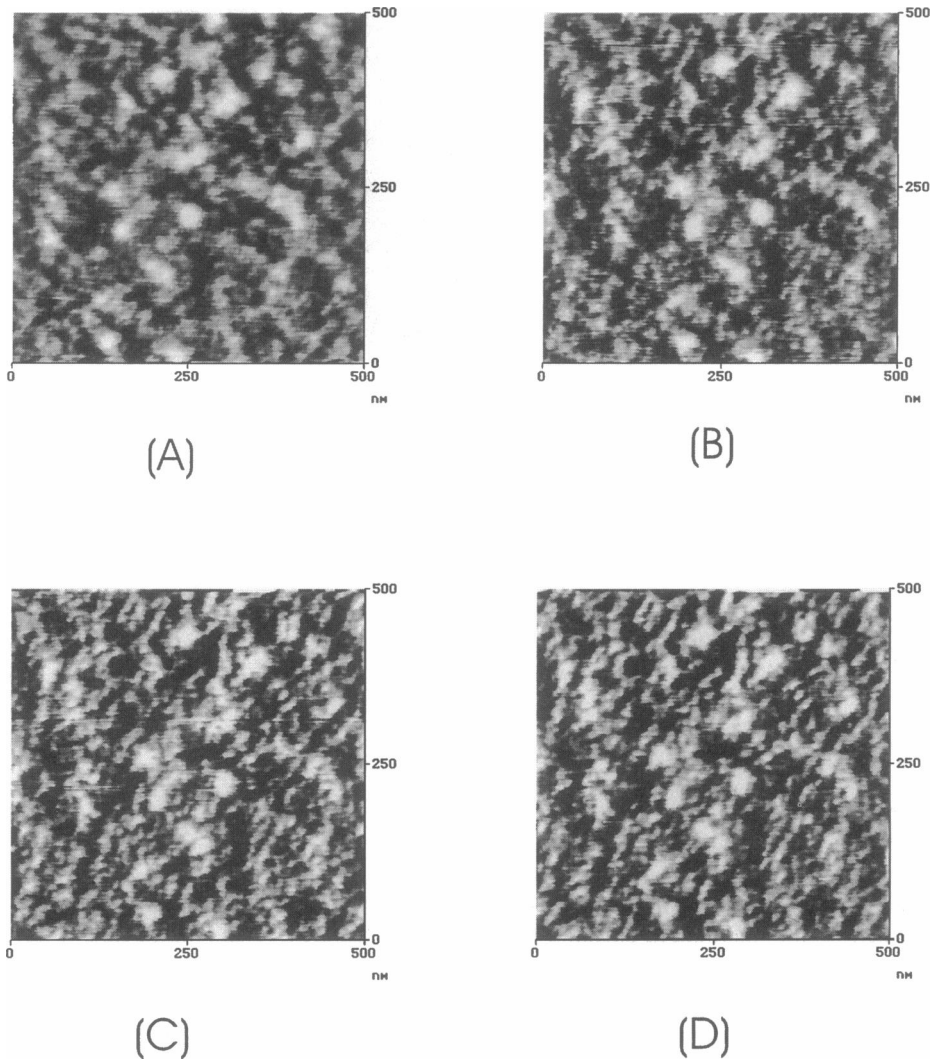

FIGURE 4 Selection of the TMAFM images of antibody layer adsorbed on the PL/mica surface obtained by scanning with an unmodified tip at the setpoint voltage: (*A*) 3.0 V, (*B*) 2.0 V, (*C*) 1.0 V, (*D*) 3.0 V. Image dimensions: 500 × 500 × 10 nm.

DISCUSSION

In TMAFM imaging, the cantilever vibrates near or at its resonant frequency with an oscillation amplitude typically between 20 nm and 100 nm (Zhong et al., 1993). When a soft material is imaged, the oscillating cantilever may cause a surface indentation in local surfaces (the deformation of the proteins at the cantilever tips should be included for our case) during brief encounters, because of the high energy possessed by the TMAFM cantilever. As a result, the damped amplitude of the cantilever at those soft surface areas is always higher than that preset by the setpoint voltage (Tamayo and Garcia, 1996). During TMAFM imaging in the height mode, the increased amount of the damped amplitude will be compensated, via the feedback loop, by applying an extra amount of positive voltage to the Z piezo—the movement of the Z piezo should be approximately equal to the surface indentation. The positive voltage of the Z piezo corresponds to a positive contrast in the TMAFM image, as manifested by the fact that the patchlike features are always superimposed on the topography of the sample. In contrast, the cantilever maintains its oscillation amplitude preset by the setpoint voltage over the hard surface areas, and hence the image obtained at these areas reflects the topography of the sample. For instance, it has observed that the patchlike features do not generally cover the large aggregates of the PL films (Figs. 1 and 2) and the

TABLE 1 The occurrence of the patchlike features with correlation of the setpoint manipulation for the experimental system studied

Samples	Bare tips	BSA tips	Ab tips
Mica	−	−	−
PL/mica	+	+	+
	(1.39 ± 0.19 nm)	(0.93 ± 0.17 nm)	(1.61 ± 0.41 nm)
Ab/PL/mica	−	+	+
		(1.54 ± 0.22 nm)	(1.28 ± 0.24 nm)

The figure in parentheses (mean ± standard deviation) is the height of the patchlike feature measured from one series of TMAFM images. The observation is based on triplicate experiments.
+, Observed; −, not observed.

large Ab granules (Fig. 3), because these large molecular aggregates are believed to be structurally more rigid than individual molecules (Radmacher et al., 1994).

Theoretically, the setpoint voltage, which defines the amplitude of the cantilever oscillation to be maintained by the feedback loop, determines the loading force exerted by the cantilever to the sample indentation (Spatz et al., 1995; Tamayo and Garcia, 1996). The decrease in the setpoint voltage means the increase in the loading force and, in turn, the increase in the possibility of the surface indentation. Therefore, it is expected that the surface coverage of the patchlike features increases with the decrease in the setpoint voltage, as has been observed in the experiments with the PL/mica surfaces. The patchlike features observed at the surfaces of the Ab layers behave differently, i.e., the surface coverage of the patchlike features decreases with the decrease of the setpoint voltage. It is believed that the deformation of the protein molecules at the cantilever contributes to the formation of the patchlike features at the initial setpoint, and as the setpoint is lowered, the deformation of the sample surface plays a major part in the formation of the patchlike features. As can be seen in Fig. 3, *C* and *D*, the patchlike features appear predominantly at the boundary areas between the Ab granules, where the lateral interaction is weak and structural deformation is more likely (You and Lowe, 1996b).

Based on the above discussion, the appearance of the patchlike features is dependent on the indentation of the local sample surface and the deformation of the proteins at the tip induced in TMAFM imaging. In the experiments, the indentation of the samples and the deformation of the protein-modified tips were monitored indirectly from the measurement of the sensitivity in the amplitude calibration curves (Hoper et al., 1995). The sensitivity, which is measured as the slope of the cantilever amplitude versus the Z voltage plot, is listed in Table 2 for the experimental system studied. As can be seen in Table 2, for solid and incompressible mica surfaces, the damping of the cantilever amplitude with the decrease in the tip-sample separation is fast, as indicated by a high sensitivity value; whereas for the Ab/PL/mica surface, the amplitude of the cantilever is attenuated slowly, owing to the occurrence of sample deformation, giving rise to a low sensitivity value. The sensitivity measurement does not reflect the effect of attenuation of the thin PL layer on the cantilever oscillation amplitude unless the antibody molecules of large structural dimension ($14.0 \times 10.0 \times 4.5$ nm) are involved (Table 2). However, at this stage, it is not fully understood why no patchlike features were observed, either on the bare mica surface with the protein-adsorbed tips or on the Ab/PL/mica surface with the bare or unmodified tips (see Table 1), assuming that the proteins at the tips suffer structural deformation and the Ab layers are indented under the scanning tip. Here it is hypothesized that the appearance of the patchlike features depends on whether the surface indentation of the sample is elastic or plastic and on how the feedback loop responds to these elastic or plastic indentations. Further experiments using the phase imaging mode may provide additional data to prove this hypothesis (Leclere et al., 1996; Tamayo and Garcia, 1996).

TABLE 2 Sensitivity measured as the slope of the cantilever amplitude versus the Z voltage curves for the experimental system studied

Samples	Sensitivity (V/nm)		
	Bare tips	BSA tips	Ab tips
Mica	0.078 ± 0.015	0.083 ± 0.016	0.071 ± 0.019
PL/mica	0.077 ± 0.017	0.074 ± 0.023	0.049 ± 0.016
Ab/PL/mica	0.033 ± 0.014	0.042 ± 0.015	0.041 ± 0.018

The data were obtained from duplicate experiments under normal tapping mode conditions.

CONCLUSIONS

In this work we report an empirical investigation of the image contrast of TMAFM, using cantilever tips that have been modified with bovine serum albumin or goat anti-biotin antibody. During scanning with these protein-adsorbed tips at initial setpoints, additional topographical features, namely the patchlike features, are induced in the TMAFM image of the surfaces of poly-L-lysine films and antibody layers deposited on mica. The observed patchlike features vary significantly with the setpoint voltage in a reversible and nonlinear manner. The occurrence of the patchlike features is believed to arise from the surface indentation of the sample and from the deformation of the proteins at the cantilever induced in TMAFM imaging. The experimental results obtained show that the cantilever tips modified with or without the protein molecules introduce, under certain circumstances, extra topographical features into those of the sample, and enhance the image contrast of TMAFM of the sample.

Furthermore, the experimental results show that the sample surfaces suffer remarkably little structural alteration or damage after being scanned with the protein-modified tips for a long period of time and under high loading forces. It is clear that surface damage in TMAFM imaging can be significantly reduced by taking advantage of the mechanical nature of the protein molecules adsorbed at the tip.

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