Influence of Surface and Protein Modification on Immunoglobulin G Adsorption Observed by Scanning Force Microscropy

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ABSTRACT Scanning force microscopy has been used successfully to produce images of individual protein molecules. However, one of the problems with this approach has been the high mobility of the proteins caused by the interaction between the sample and the scanning tip. To stabilize the proteins we have modified the adsorption properties of immunoglobulin G on graphite and mica surfaces. We have used two approaches: first, we applied glow discharge treatment to the surface to increase the hydrophilicity, favoring adhesion of hydrophilic protein molecules; second, we used the arginine modifying reagent phenylglyoxal to increase the protein hydrophobicity and thus enhance its adherence to hydrophobic surfaces. We used scanning force microscopy to show that the glow discharge treatment favors a more homogeneous distribution and stronger adherence of the protein molecules to the graphite surface. Chemical modification of the immunoglobulin caused increased aggregation of the proteins on the surface but did not improve the adherence to graphite. On mica, clusters of modified immunoglobulins were also observed and their adsorption was reduced. These results underline the importance of the surface hydrophobicity and charge in controlling the distribution of proteins on the surface.

INTRODUCTION

Scanning force and scanning tunneling microscopy (SFM and STM) are useful techniques that allow the observation of the surfaces of adsorbed biomolecules with nanometer resolution (Jahanmir et al., 1992). One advantage of using these techniques with biological systems is their ability to operate either in air or in aqueous buffer, minimizing sample preparation and allowing observation of in vitro processes (Lyubchenko et al., 1993; Lin et al., 1990; Chang et al., 1993). However, the great progress in the immobilization of biomolecules the effort is mainly focused on nucleic acids rather than globular proteins. Thus, the observation of these biomolecules is still developing.

For our studies, we used the protein immunoglobulin G (IgG). The three-dimensional structure of this protein has been solved by x-ray crystallography (Sarma et al., 1971; Silverton et al., 1977), and the protein has been observed by transmission electron microscopy at high resolution (Ryazantsev et al., 1990). It is a relatively large protein, with dimensions of 14 × 10 × 4 nm, and it has already been imaged by STM at moderate resolution (Leatherbarrow et al., 1991; Olk et al., 1991; Müller et al., 1993). However, many groups have reported difficulties with the reproducibility and reliability of the images of such biomolecules obtained by scanning probe methods (Clemmer and Beebe, 1992). We therefore directed our studies to increasing the reproducibility and stability of the images obtained rather than concentrating on the resolution.

Scanning probe microscopy requires atomically flat surfaces. Most studies have used either mica or highly orientated pyrolytic graphite (HOPG). Mica has a higher surface energy and the interactions are often strong enough to immobilize molecules on the surface during the scan, but it cannot be used for STM, as mica is insulating. On the other hand, HOPG is conductive, but the interactions between molecule and surface are very weak and the adsorbates are easily moved by the scanning tip (Salmeron et al., 1990). We therefore attempted to increase the reactivity of the HOPG surface using the glow discharge (Gombotz and Hoffman, 1987). This technique is routinely applied in electron microscopy. We previously used it to stabilize the T4 bacteriophage (Droz et al., 1993), leading to a more homogeneous distribution and increased adherence of viruses on the surface without the need for covalent coupling. Another possible approach is to chemically modify the surface properties of the protein. We used phenylglyoxal, which reacts with the surface arginine residues eliminating their positive charge and enhancing their hydrophobicity (Takahashi, 1968).

In this paper, we show the effect of modification of the substrate and protein on the strength of their mutual interaction. From our results, it is clear that understanding this interaction is of fundamental importance to the application of scanning probe techniques in biology.

Received for publication 3 May 1994 and in final form 20 June 1994.

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Abbreviations used: SFM, scanning force microscopy; HOPG, highly oriented pyrolytic graphite; HEPES, *N*-2-hydroxyethylpiperazine-N′-2-ethane-sulfonic acid; IEF, isoelectric focusing.

0006-3495/94/09/1316/08 $2.00

MATERIALS AND METHODS

Protein purification

Lyophilized polyclonal human IgG (Sigma Chemical Corp., St. Louis, MO) was dissolved in deionized water to a concentration of 500 μg/ml, and 100 μl was desalted using PD10 G25-M (Sephadex) gel filtration column in 20 mM ammonium acetate buffer, pH 7.0. SDS-PAGE was carried out using 8–25% Phast gradient gels (Pharmacia, Uppsala, Sweden) to check the quality

of the IgG samples and the effect of the purification process. The samples are either native IgG or protein reduced with 100 mM β-mercaptoethanol. The gels were stained with 0.2% Coomassie brilliant blue R-250.

Protein modification

The surface accessible arginine residues were modified using phenylglyoxal 10 mg lyophilyzed IgG were resuspended in buffer and incubated in 50 mM HEPES buffer pH 8.5, containing 25 mM [7-^{14}C]-phenylglyoxal (22.5 mCi/mmol; Amersham Corp., Arlington Heights, IL), at 37°C. At five intervals over a 2-h period, a 50 μl aliquot was removed from the reaction and passed through a PD10G 25-M gel filtration column (Pharmacia) into the same buffer to remove excess reagent and prevent further modification. The double Schiff base formed by the reaction with phenylglyoxal was then reduced by adding $NaBH_4$ (Fig. 1) to a final concentration of 20 mM and incubating for 16 h at 25°C. The final product was dialyzed against 50 mM HEPES buffer pH 8.5 for 24 h. The number of arginine side chains modified was determined from the incorporation of [7-^{14}C]-phenylglyoxal. 50-μl aliquots of the reaction were filtered through pre-wetted nitrocellulose filters (Schleicher and Schuell, Keene, NH). The filters were washed with 10 ml buffer and dried in air before being scintillation counted using 5 ml Optiphase "HiSafe" II scintillant (LKB, Pharmacia). The change in isoelectric point of the Ig upon modification was monitored by isoelectric focusing (IEF; Hoffman, 1977). Polyacrylamide gels (LKB PAGplate) with a pH gradient from 3.5 to 9.5 were used, and the gels were stained with 0.2% Coomassie brilliant blue R-250. Protein concentrations were determined using UV absorption at 280 nm. The proteins were aliquoted in 100 μl fractions and stored at −20°C. The data were fitted to an equation for a first-order exponential approach to equilibrium ($A_t = A_0\, e^{-kt}$) using Grafit 3.01 (Leatherbarrow, 1992).

Surface modification and analysis

Freshly cleaved HOPG (ZYB; Union Carbide, Cleveland, OH) was either used directly or treated by glow discharge (homemade system adapted to a BAF 400 power supply, Balzers Union, Balzers, FL) to increase the hydrophilicity of the surface. A gas discharge was obtained by applying a potential difference of 800 V_{DC} between two electrodes at an air pressure of 10^{-2} mbar and a current of 5 mA. Samples were exposed for 20 s. Mica sheets (Balzers Union, FL) were used freshly cleaved. The increase in macroscopic hydrophilicity was obtained by measuring the static contact angle of a 1-μl water droplet on the surface. The chemical effect of the glow discharge on HOPG was investigated using Auger Electron Spectroscopy (AES; Varian, Lexington, MA). After the glow discharge treatment, the samples were analyzed under ultrahigh vacuum (UHV) at less than 10^{-9} mbar. The spectra were obtained with a cylindrical mirror analyzer in the first derivative mode at a primary beam energy of 2000 eV and 5 V peak-to-peak modulation of the pass energy. The modification induced by the

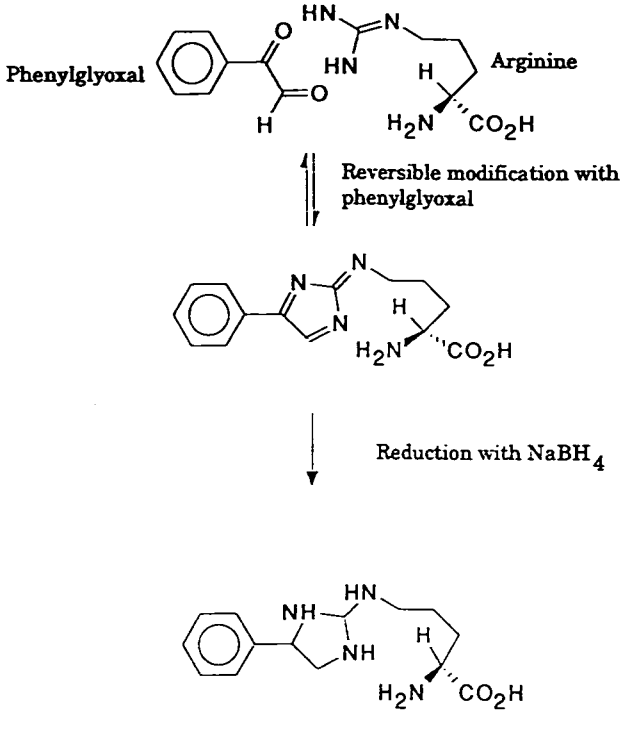

FIGURE 1 Mechanism of arginine amino acid labeling with phenylglyoxal, and reduction with $NaBH_4$.

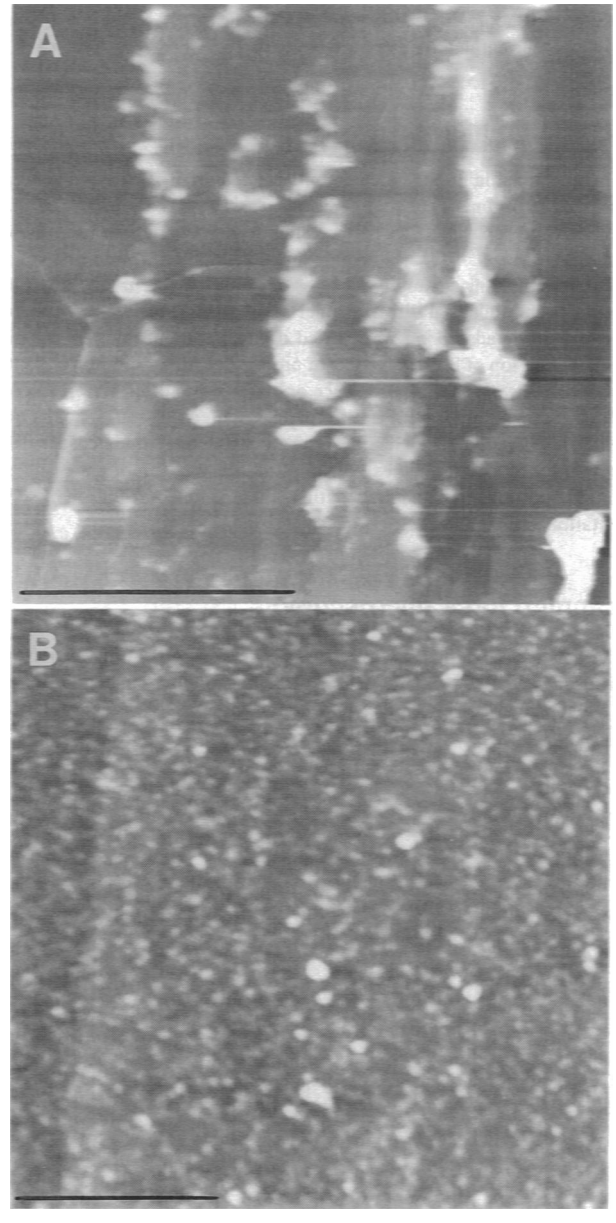

FIGURE 2 (*A*) SFM images of aggregates of IgG adsorbed on freshly cleaved HOPG without glow discharge. (*B*) SFM images of purified IgG adsorbed on cleaved and glow-discharged HOPG. Individual proteins are observed firmly adsorbed on the surface. The scan rate is 0.7 Hz. The scale bar corresponds to 1 μm. The vertical black-to-white scales are 8 nm and 3 nm, respectively.

TABLE 1 Effect of glow discharge on contact angle and surface roughness

Parameter	HOPG		Mica
	− Glow discharge	+ Glow discharge	(− glow discharge)
Contact angle (°)*	61	28	<10
SFM values (nm)			
Peak-to-valley	0.50	3.30	0.36
RMS of surface roughness	0.10	0.30	0.10

* Contact angle was measured on a static droplet of water on the surface.

glow discharge on the topmost atomic crystalline layer was studied using low electron energy diffraction (Omicron, Taunustein, Germany) at 0.5 μA impinging current and 61 eV primary energy.

Deposition method

IgG was diluted in 20 mM ammonium acetate buffer pH 6.8 at a concentration of 10 μg/mL. This volatile buffer was chosen to prevent salt precipitation and deposits on the surface after drying. These have been shown to mask the shape of the adsorbed antibodies. In later experiments where we were comparing adhesion behavior of modified and unmodified IgG, samples were diluted in distilled water at the same protein concentration. In all cases the adsorption was carried out on sheets of mica or graphite deposited on top of 20-μl droplets of protein solution for 5 min at 25°C. The samples were then rinsed twice on drops of distilled water. Excess liquid was withdrawn with a filter paper (Schleicher and Schuell) and the sample allowed to dry in air. We preferred this method because only the affinity of the molecules with the surface was important in determining their coverage and distribution (there is no contribution of sedimentation effects).

Scanning force microscopy

SFM was carried out in ambient conditions on a Park Scientific Instruments universal system (Sunnyvale, CA) with a maximum scan size of 10 μm. Microfabricated Si_3N_4 cantilevers with integrated pyramidal tips and a spring constant of 0.064 N/m were used. The typical loading force was 10^{-8} N in air. The images were recorded at a frequency of 0.3–1.5 Hz/line on 256 × 256 pixels. No image processing was performed except for linear background subtraction.

RESULTS AND DISCUSSION

Modification of HOPG surface by glow discharge

A typical SFM image of the IgG molecules deposited on the freshly cleaved HOPG is presented in Fig. 2 *A*. Aggregates of several molecules are visible aligned along graphite steps or cut by the scanning probe, as indicated by the horizontal white stripes. Most of scanning probe microscopy studies of isolated biomolecules report such difficulties in imaging because of motion of the molecules on the surface caused by the interaction of the scanning tip with the sample (Nawaz et al., 1992).

To improve the adherence of the molecules on HOPG, glow discharge was used. Fig. 2 *B* illustrates the effect of this treatment on the spreading of the IgG. It can be seen that the molecules are homogeneously distributed and almost isolated and not packed at the step on the left side of the image. The protein-surface interactions are clearly stronger than the protein-protein interactions. The antibodies appear as discrete units of about 2.5 nm height and 15–30 nm width. This compares with a molecular size of 14 nm × 10 nm × 4 nm as determined by x-ray crystallography (Silverton et al., 1977). In STM studies the individual domains of the IgG have been resolved; however, the measured dimensions of isolated protein has ranged approximately from 40 nm × 28 nm × 2 nm (Leatherbarrow et al., 1991) to 16 nm × 10 nm × 2 nm (Müller et al., 1993). The variation in the lateral dimensions that we see could be caused by any of three factors. First, all scanning probe microscopies are subject to a combination of the tip shape and sample topography in the resulting images, which limits the resolution, i.e., enlarges

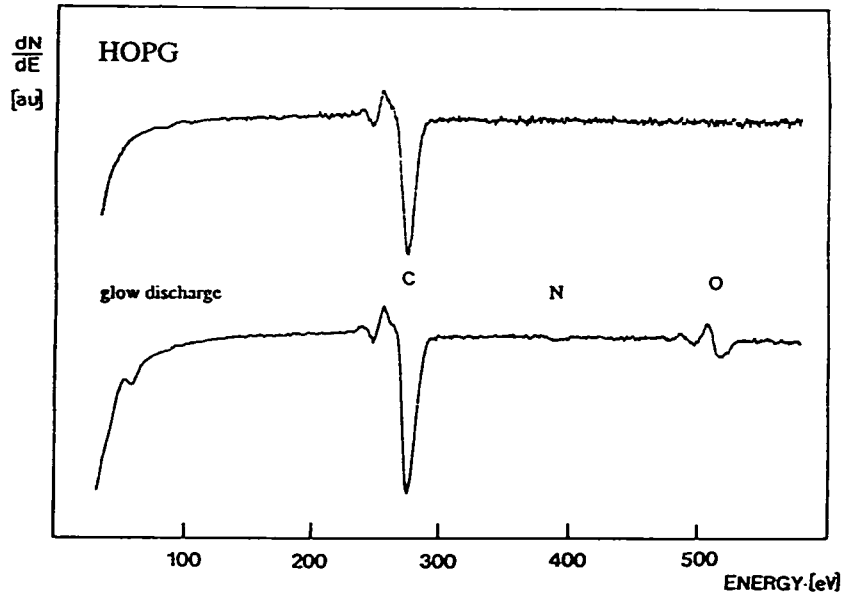

FIGURE 3 Auger spectra of freshly cleaved HOPG before and after the glow discharge.

the observed structures. Second, it can be due to the formation of dimers, which exist chemically (Hoffman, 1977). Third, this increase may be promoted by the remaining solvent (see below).

To explain the improvement in the adsorption of the IgG on glow discharge and native HOPG, we characterized the modified surface by the static contact angle of a drop of water deposited on the surface. This is a measure of hydrophilicity of the surface, which increased with the treatment; after the modification of HOPG the angle decreased from 61 to 28° (Table 1). The glow discharge clearly oxidized the HOPG surface. Before treatment, the Auger spectrum displayed only the peak for carbon (Fig. 3). No contaminants were present on the freshly cleaved surface. After the glow discharge, the main change in the spectrum is an increase of the oxygen signal (Fig. 3) indicating that the major reaction promoted by the treatment is the oxidation of the surface.

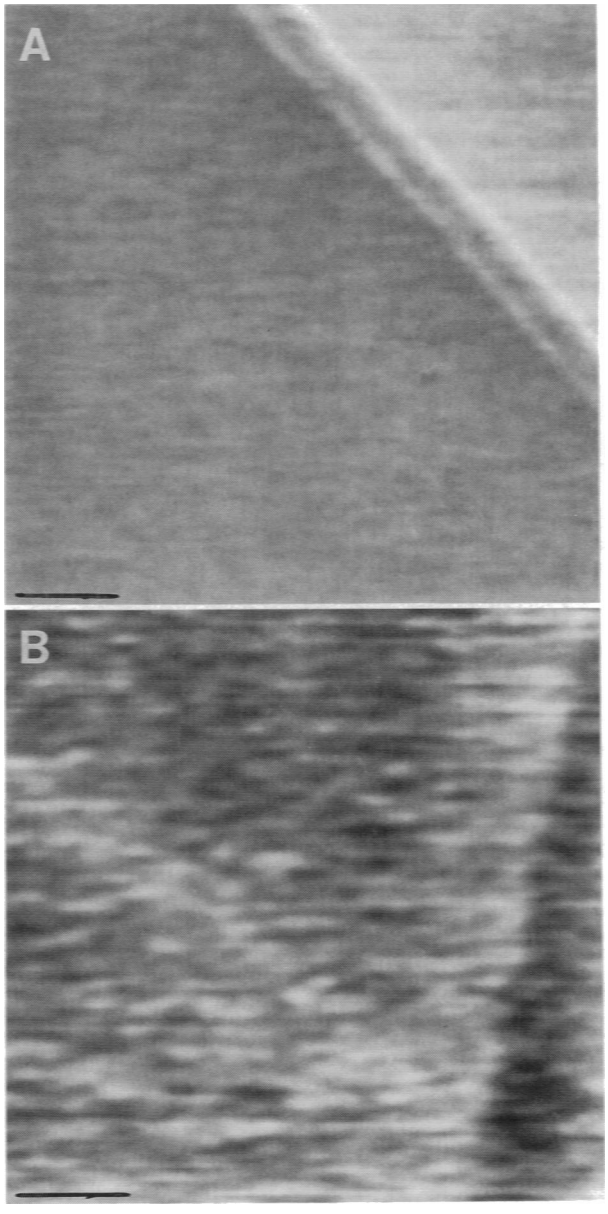

FIGURE 4 SFM images of cleaved HOPG untreated (*A*), and glow discharged in air (*B*). A step is observed on the right part on both images. The scan rate is 1 Hz. The scale bar corresponds to 100 nm, and the vertical black-to-white scale is 1.5 nm.

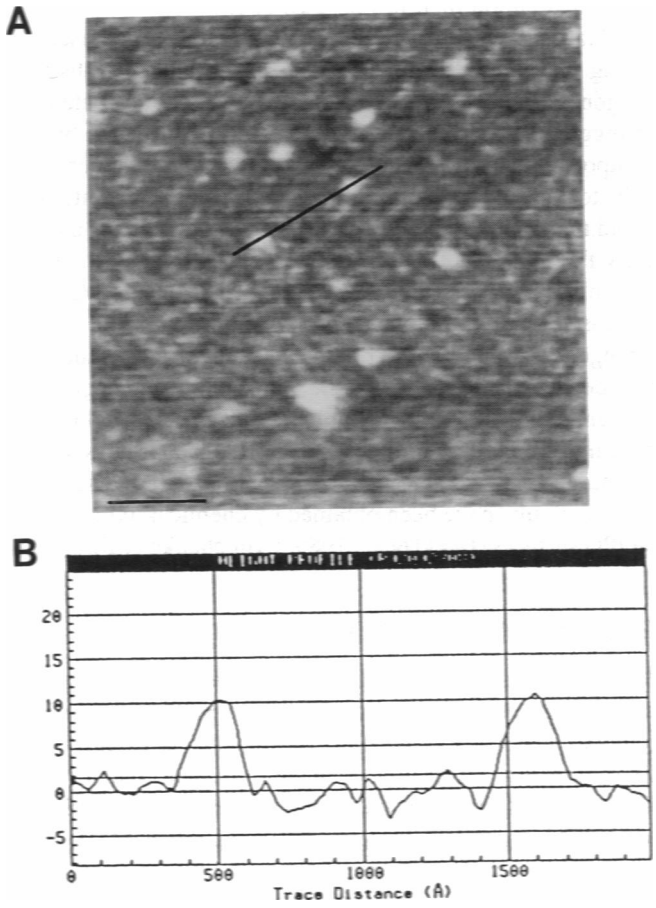

FIGURE 5 (*A*) SFM image of IgG at 6 μg/ml adsorbed on glow-discharged HOPG. The substrate roughness induced by the treatment is visible in the protein-free regions. The scale bar corresponds to 100 nm, and the vertical black-to-white scale is 1.5 nm. (*B*) Profile of (A), across two IgG, which shows the influence of the "trapping effect" mentioned in the text.

Before the treatment, low electron energy diffraction studies of the HOPG surface showed sharp concentric diffraction rings typical of crystallites oriented along the normal surface but randomly oriented in the plane. On the treated surface these sharp rings were no longer observed, implying a disordering of the surface. This was also confirmed by the comparison between SFM images of freshly cleaved (Fig. 4 *A*) and glow-discharged HOPG (Fig. 4 *B*), which showed that the glow discharge increases the roughness of the surface by the creation of defects and reactive sites. The numerical results for the peak-to-valley and root-mean-square roughness obtained by SFM show an increase of 3 nm (Table 1). Previous experiments with STM have shown that electrochemical oxidation of graphite modifies the surface structure in the same way (Gewirth and Bard, 1988).

The increase of roughness promoted by the glow discharge is more visible in Fig. 5 *A* at lower protein concentration and

higher resolution than in Fig. 2 *B*. A profile across two individual molecules in the middle of the image is presented in Fig. 5 *B*. The difference between the apparent substrate roughness with or without the proteins is attributed to changes of the tip shape (e.g., sticking of proteins, buffer components, etc.). The defects created by the treatment can help to trap or anchor the molecules on the substrate and avoid their displacement by the tip compared with the atomically flat native HOPG. Those surface irregularities are not the only parameters affecting the adhesion of the IgG. The increased hydrophilicity observed is also responsible for the stronger adherence of the proteins. The hydrophilic interaction between the protein and the surface is strong enough to prevent the diffusion of the molecules or the removal by the scanning tip and therefore the formation of aggregates. Glow discharge clearly enhances the interaction with the surface. Similar results have been obtained by chemical oxidation of HOPG surfaces (Heckl et al., 1989; Lyubchenko et al., 1991).

Effect of protein purification

Many proteins are stored in the presence of sugars or nonvolatile buffers to enhance their stability (Scopes, 1986). The removal of these nonvolatile components is essential for SFM imaging. These low molecular weight contaminants hinder the adsorption of the proteins on the surface and also lower the image contrast by an embedding effect (Droz et al., 1993). Once these components were removed by gel filtration the IgG molecules appeared clearly as individual objects on the surface as shown in Fig. 2 *B*. In contrast, when nonpurified IgG samples were imaged they showed much more variability in size up to a maximum of 200 nm. In addition the molecules were less well adsorbed and could be easily displaced during the scan, creating the windows seen in Fig. 6 *A*. At higher protein concentration the adsorbates could be deformed by friction phenomena that generated wavelike patterns, which lead to difficulties in the interpretation of the images (Fig. 6 *B*).

Modification of IgG surface by phenylglyoxal

Another method for changing the interactions of proteins with the substrate is modifying the surface of the molecule. Increasing the hydrophobicity of the protein should increase their adsorption to a hydrophobic surface as HOPG. This is effectively a reversed-phase method for adherence of the antibodies. We used phenylglyoxal to increase the hydrophobicity of IgG. This reacted with the side chains of arginine residues in the protein eliminating the charge and increasing its hydrophobicity. Measurement of the incorporation of [^{14}C]-phenylglyoxal showed an exponential approach to an equilibrium value of 8.197 $\pm$ 0.341 residues modified per protein molecule at a rate constant of 0.044 $\pm$ 0.007 min^{-1} at pH 8.5 and 25 mM phenylglyoxal (Fig. 7 *A*). This incorporation caused a change in the isoelectric point of the IgG, as can be seen by the shift of the bands on an IEF gel with respect to the unmodified IgG (Fig. 7 *B*). Samples of protein taken at 120 min, where eight arginine residues were modified, were reduced and used in further experiments.

The adsorption properties of the modified antibodies were compared with the native antibodies by SFM. When HOPG was used as a substrate, the images with modified protein were similar to those obtained with unmodified protein. Only large aggregates were observed and the adsorbates easily removed by the tip. The hydrophobic interaction alone was too weak to stabilize the IgG on the hydrophobic HOPG surface. Clearly, the adsorption of the modified IgG to such surface by a reversed-phase method is not sufficient to increase the adherence. To confirm that the modification of the

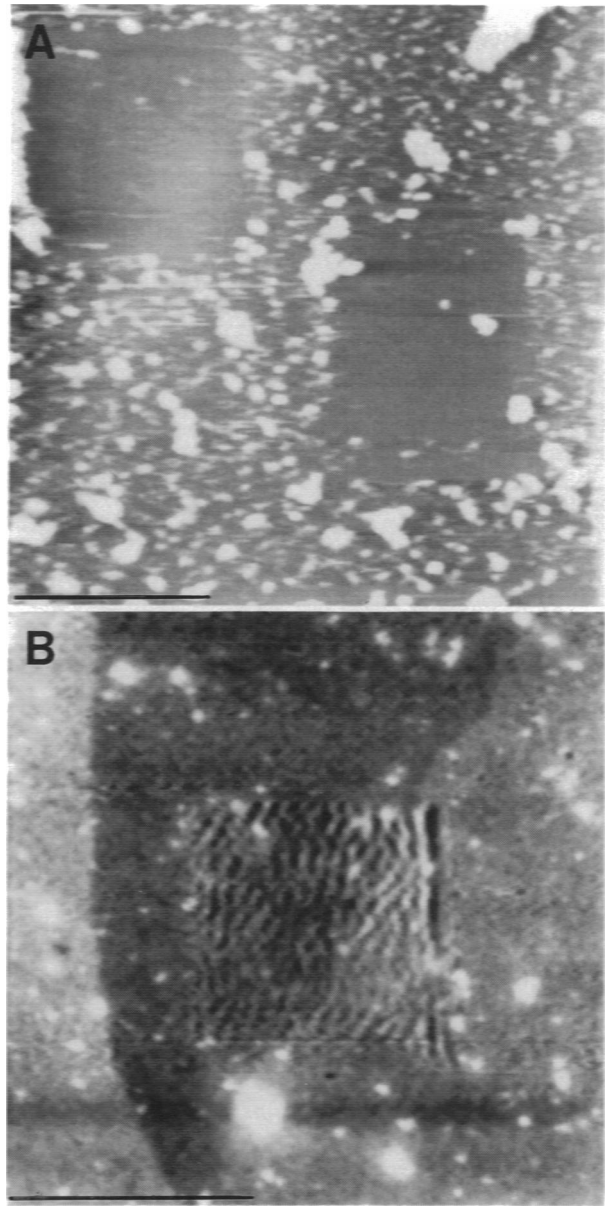

FIGURE 6 SFM images showing typical artifacts observed with unpurified samples of IgG adsorbed on cleaved and glow-discharged HOPG. (*A*) Windows created by molecule displacement. (*B*) Plastic deformation of the surface adsorbates and molecules. The scan rate is 1 Hz. The scale bar corresponds to 1 μm. The vertical black-to-white scale is 8 nm for both images.

IgG can alter its adsorption properties we repeated the experiments using mica as a substrate. On this hydrophilic substrate (Table 1) we expected a weaker adhesion for the phenylglyoxal modified than for the unmodified molecules.

Unmodified IgG can be homogeneously distributed on mica (Fig. 8 *A*). The height of the molecules is about 3.5 nm and the lateral size is between 20 and 35 nm. We found out that the molecules are stable even after more scanning of the same area. When the protein was modified with phenylglyoxal a lower amount of IgG was adsorbed (Fig. 8 *B*). The observed height of the IgG was much less in this case, about 2 nm, and the width ranged from 30 to 40 nm. As predicted, we conclude that the adhesion of the modified molecules on the hydrophilic mica was weaker. This is confirmed by the observation of aggregates with lateral sizes of several hundred nm on other areas of the sample (data not shown), which indicates a more heterogenous distribution of the modified IgG. This phenomenon only takes place with the modified molecules and demonstrates that the molecule-to-molecule interaction is as important as the interaction with the substrate.

From these experiments we have shown the deleterious effect of increasing protein hydrophobicity. A chemical modification to enhance the hydrophilicity of the protein surface (analogous to the glow discharge treatment for the substrate) is not practicable. No mild reagents exist for increasing the hydrophilicity of hydrophobic protein side chains, and in many cases these side chains tend to be inside the protein.

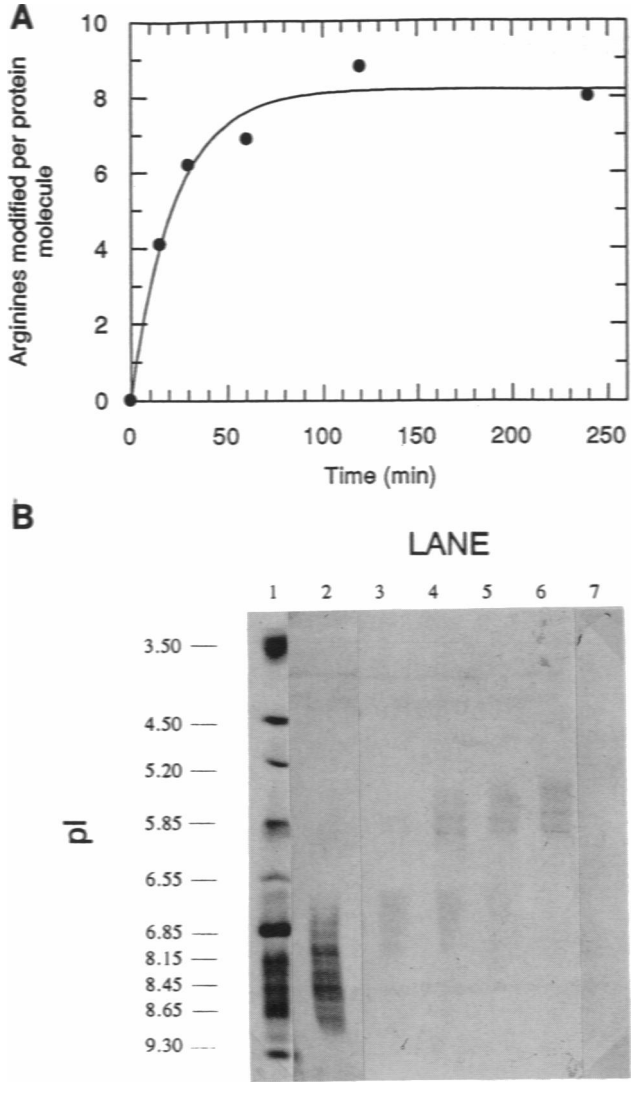

FIGURE 7 (*A*) Number of arginine residues modified during the modification reaction with phenylglyoxal. (*B*) IEF gel of the IgG modification with phenylglyoxal. Lane 1 shows marker proteins of known isoelectric point; lane 2 shows polyclonal IgG unmodified; and lanes 3–7 show the time course reaction of modification after 15, 30, 60, 120, and 240 nm respectively.

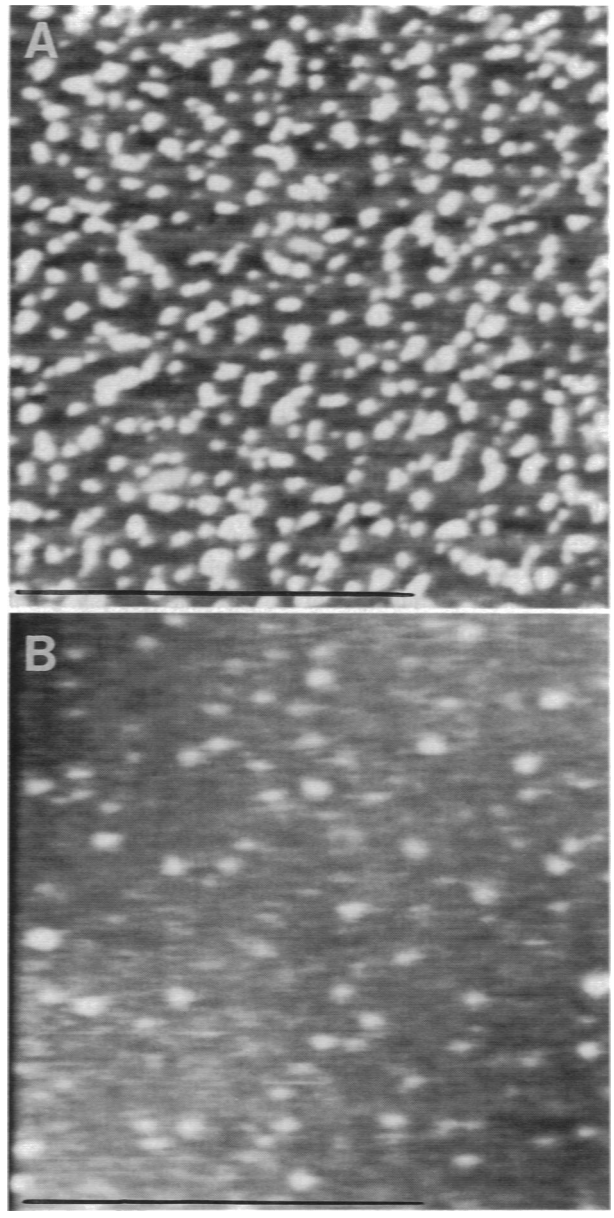

FIGURE 8 SFM images of (*A*) native, unmodified IgG adsorbed on cleaved mica, and phenylglyoxal modified IgG (*B*). The scale bar corresponds to 1 μm, and the vertical black-to-white scale is 4 nm.

Observed molecular dimensions

To describe the various observed dimensions of the IgG we made a statistic of height and width of the proteins on HOPG and mica (Fig. 9 *A,B*, respectively). There was a wide scattering in the size of the molecules adsorbed on native HOPG. Large aggregates of several hundred nanometers as well as a few individual IgGs were observed around 30 nm in width (Fig. 9 *A, filled triangles*). When the graphite was glow-discharged in air more isolated molecules were obtained (Fig. 9 *A, empty squares*). This effect is illustrated by a smaller dispersion in the dimensions of the measured molecules. The dimensions of an individual molecule are not correct when compared with the x-ray data (Sarma et al., 1971). The apparent width of the IgG is enlarged because of the geometry of the tip and also because of the applied force on the sample, which compresses the molecules as displayed by the low height values. However, this can be an artifact caused by the defects of the glow discharge, where the molecules can be "trapped" in the irregularities of the substrate (see Fig. 5 *B*).

The dimensions of the native IgG adsorbed on mica can be classified into two populations (Fig. 9 *B, empty squares*). Isolated proteins had dimensions from 22 to 35 nm in width and 1.5 and 2.5 nm in height; in the same observed area were aggregates of higher dimensions, probably dimers or trimers (see Fig. 8 *A*). The phenylglyoxal-modified IgG can also be classified into two groups, the isolated molecules that are larger than the native individual IgG, and very large aggregates (Fig. 9 *B, filled triangles*). The differences in height of the isolated molecules were due to the applied force on the sample, which changed from one experiment to another because the adhesion force cannot be controlled.

Comparison of the lowest values of height obtained on mica (0.7 nm) with those obtained on glow-discharged HOPG (0.3 nm) points toward the "trapping effect" of this treatment. Thus, molecules in the size range of IgG seem to be the smallest proteins that can be identified from the surface on glow-discharged HOPG.

CONCLUSION

We have shown that SFM is a suitable technique for investigation of protein adsorption. We adopted two approaches

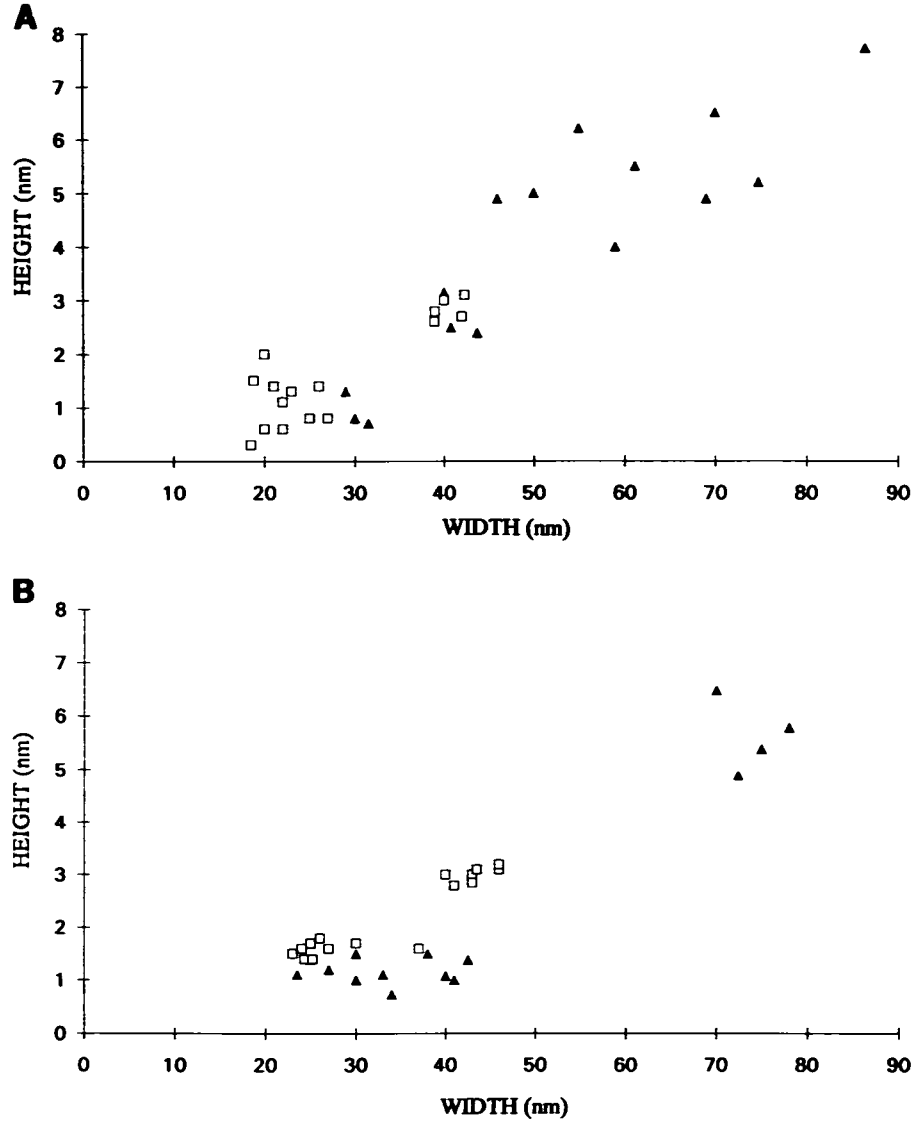

FIGURE 9 Graphs of IgG dimensions measured on various samples, on different areas. The total width of the isolated or aggregated proteins is measured. (*A*) Dimensions of native IgG adsorbed on cleaved, native (*empty squares*), or glow-discharged (*filled triangles*) HOPG. (*B*) Dimensions of native (*empty squares*), and phenylglyoxal modified (*filled triangles*) IgG adsorbed on cleaved mica.

to find out some of the interactions influencing the adsorption: first, the modification of the hydrophobic HOPG substrate by glow discharge and second, the modification of the molecules by phenylglyoxal. After glow discharge treatment of the HOPG, thanks to the increase of surface roughness and hydrophilicity, the IgG molecules exhibited a better adhesion to the substrate and were not swept away by the scanning tip. Moreover, the distribution was more homogeneous and no aggregates were observed.

The phenylglyoxal modification of the IgG influences the hydrophobic-hydrophilic interaction: the modified molecules are more hydrophobic; thus, their adsorption on hydrophilic mica is decreased, and more aggregates are observed than in the unmodified proteins. The modification of IgG to increase their adsorption has already been performed by protein engineering (Ill et al., 1993), a technique that is very specific but more fastidious for immobilizing protein on surface.

One other way to immobilize the protein is to functionalize the surface to enable a covalent coupling of the molecules. This is restricted to a few surfaces such as gold. Self-assembly monolayer or thiol group reagents have been used successfully to immobilize protein (Leggett et al., 1993). This method, which enables a stable adsorption of molecules by preventing their movement, is now under investigation in our laboratory.

We would like to thank Paul Scully for help in protein modification, Florence Coulin, Nicole Aubonney for help in protein purification and analysis, and C. Lüthi for technical assistance.

This work was supported by CERS (grant N 2256.1), Glaxo Institute for Molecular Biology Geneva, and Park Scientific Instruments Geneva.

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