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IMAGING OF SINGLE ANTIGENS, ANTIBODIES, AND SPECIFIC IMMUNOCOMPLEX FORMATION BY SCANNING FORCE MICROSCOPY

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Abstract

The most sensitive analytical techniques available today for detecting immuno assay complexes are radio or enzyme immuno analytical techniques, by which quantities of 10^7-10^8 analyte molecules can be detected. With the introduction of scanning force microscopy, a new method for detecting biological processes became available. Here, we examine the feasibility of using scanning force microscopy as a biosensitive tool. We demonstrate that single or multiple rabbit anti-human serum albumin molecules form complexes with preadsorbed single human serum albumin molecules on mica. However, no interaction is observed between human immunoglobulin G molecules and preadsorbed single albumin molecules; only separate antigens and antibodies are observed at random positions on the mica. This shows the ability of scanning force microscopy to act as a biosensor for detection of immunocomplexes, and to act as a very powerful tool to study molecule-surface interactions in general.

Key Words: Scanning force microscopy, antibody-antigen interaction, immuno-complexes, molecular adsorption.

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Introduction

In order to understand the functioning of biological systems, knowledge about the character of specific biomolecular interactions is crucial. In this work, we report about the use of scanning force microscopy (SFM) as a biosensor to detect specific interactions between antigens and antibodies.

Since its introduction [2], SFM has increasingly been exploited in studies of biomolecules adsorbed on surfaces. For example, the supercoiling of DNA [17] and the domain structures of macroglobulins [1] were probed using contact mode SFM. The ability to measure the force acting between two molecules is a step towards the manipulation of individual biomolecules with SFM. Adhesion forces between individual avidin and biotin molecules have been measured and found to be quantified in the 10^{-12} N range [6]. Recently, tapping mode SFM (TM-SFM), a more gentle method, became available for the study of biomolecules [4, 7, 8, 13]. Using TM-SFM, the domain structure of human serum albumin (Fig. 1) [14] can be probed in individual molecules. Finally, recent TM-SFM measurements of the transient enzymatic activity of lysozyme and the inhibitor chitobiose [15] suggest that SFM will be a useful tool in the study of molecular interactions in real time.

Recent work indicates that, in the near future, scanning probe microscopy (SPM) may be employed in ultra-sensitive immunoassay detection without any kind of labelling for both qualitative and quantitative work. For example, scanning tunnelling microscopy (STM) has been employed to study immunocomplexes created in solution and adsorbed as clusters, and a few individual complexes on graphite [16]. Antibody-antigen interactions have been observed by SFM using thick antibody coatings on microtiter wells [3]; also, antibodies were bound to Langmuir Blodgett films containing a minor hapten antigen component [19]. In those experiments, no molecular structure was resolved in the preadsorbed coatings/films, although features interpreted as individual antibodies were observed after the coatings/films had been exposed to antibodies.

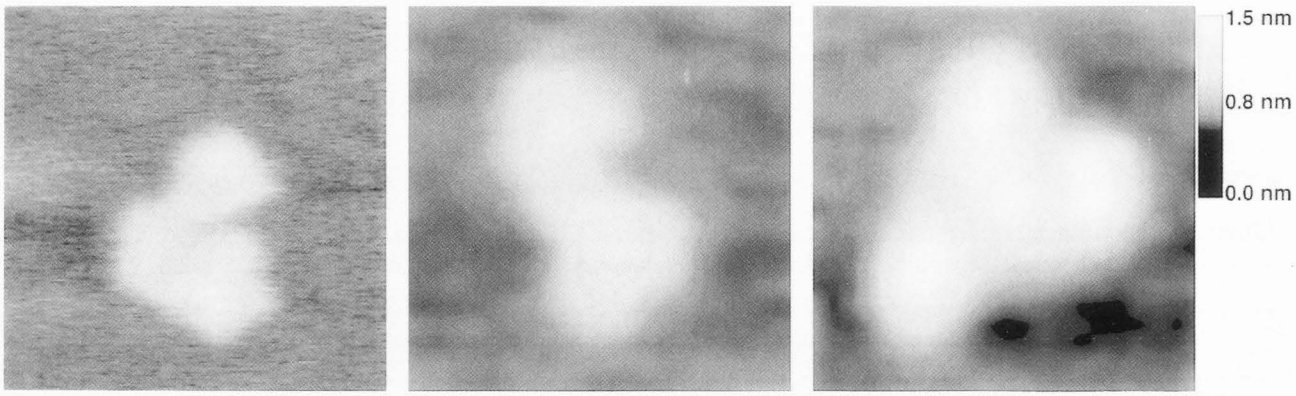

Figure 1. Tapping mode scanning force microscopy (TM-SFM) images of single molecules adsorbed on mica surfaces. **Left**: Human serum albumin molecules (HSA), compact conformation; **middle**: HSA, extended conformation; **right**: human immunoglobulin G (IgG), "Y"-shaped conformation. Each image covers an area of 40 nm x 40 nm.

Here we demonstrate the feasibility of an ultra-sensitive detection scheme for antibody-antigen docking using TM-SFM. We observe the docking reaction, or the appropriate lack thereof, between single human serum albumin molecules (HSA) and single or multiple antibodies, rabbit anti-human serum albumin (a-HSA), and human immunoglobulin G (IgG), adsorbed on mica surfaces. In future work, using longer adsorption times and smaller surface areas, it is feasible to exceed the sensitivity of existing labelling techniques for immuno assay detection.

Materials and Methods

Domain structure studies

HSA and IgG were dissolved in tris buffer {tris-(hydroxymethyl)aminomethane, 0.1 M, pH 7.5} at concentrations of 50 μg/ml and 3 μg/ml, respectively. A 50 μl volume of a protein solution was placed on freshly-cleaved muscovite green mica (Asheville-Schoonmaker Mica Co., Newport News, VA, USA) for 0.5 and 5 minutes, respectively, after which the solution was rinsed away with deionized water. The surfaces were then dried using a flow of nitrogen, and probed with TM-SFM (Nanoscope III®, Digital Instruments Inc., Santa Barbara, CA, USA), using tips with an end-radius of about 10 nm, as specified by the manufacturer.

Antigen-antibody interaction studies

HSA (Sigma Chemical Co., St. Louis, MO, USA), a-HSA (Pharmacia Diagnostics, Uppsala, Sweden) and IgG (Sigma) were dissolved in tris buffer (pH 7.4) at concentrations of 2.5 μg/ml, 13.4 μg/ml and 0.7 μg/ml, respectively, selected to give roughly the same area density of molecules (40-60 per mm^2) adsorbed on the surface. In a given exposure, a 50 μl volume of a protein solution was placed on a freshly-cleaved muscovite green mica surface. The solution, spread out over approximately 1 cm^2, remained on the surface for 5 minutes and was subsequently rinsed away with 1 ml of tris buffer. The surface was then dried using a flow of nitrogen, and probed with TM-SFM.

To study the antigen-antibody interaction, the mica surfaces were first exposed to one protein solution, rinsed with 1 ml tris, dried, and studied by TM-SFM. Thereafter, the surfaces were exposed to the second protein solution (without recleaving), rinsed, dried, and again studied by TM-SFM.

Results and Discussion

Images of the domain structure of HSA and IgG are shown in Figure 1. In the compact albumin conformation, the three domains of HSA [9] may appear as three hillocks in the SFM images. In the extended albumin conformation, a juxtaposition of two pairs of subdomains near the middle of the partly denatured molecule may appear as two hillocks in the images. A more detailed description of HSA adsorption on mica, where 75% of the observed features were attributed to single adsorbed HSA molecules, has been published [14]. The "Y-shaped" conformation of IgG was observed in a few molecules only ($<< 1\%$) and is consistent with the size of an IgG molecule oriented with both the Fab and the Fc subunits bound to the surface. The heights of the molecules appear to be lower than expected from the known structures in all experiments. Height information obtained in TM-SFM appears to be not totally topographical, but may rather be related to the different energy dissipation/damping properties of the molecules and

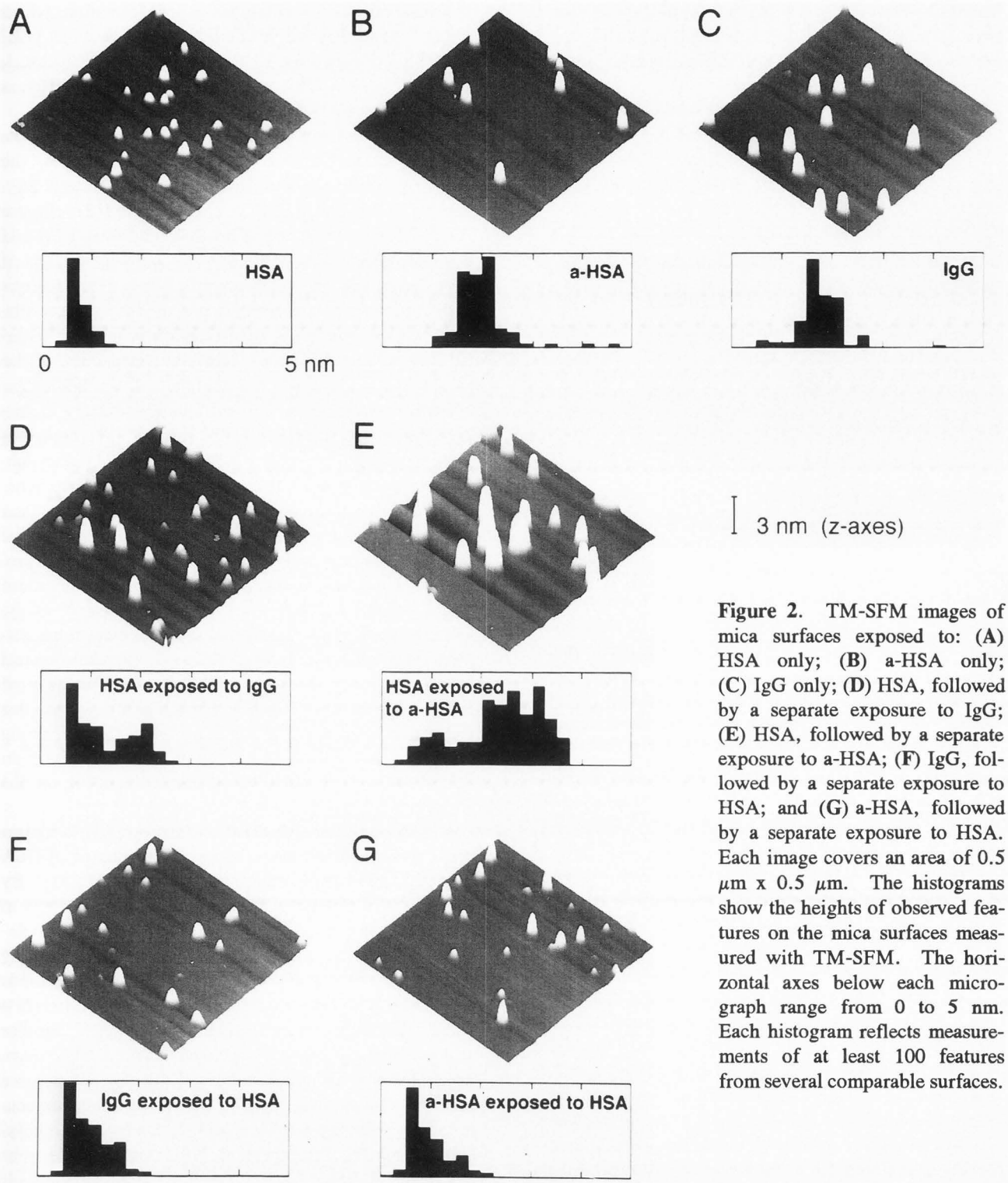

Figure 2. TM-SFM images of mica surfaces exposed to: (**A**) HSA only; (**B**) a-HSA only; (**C**) IgG only; (**D**) HSA, followed by a separate exposure to IgG; (**E**) HSA, followed by a separate exposure to a-HSA; (**F**) IgG, followed by a separate exposure to HSA; and (**G**) a-HSA, followed by a separate exposure to HSA. Each image covers an area of 0.5 μm x 0.5 μm. The histograms show the heights of observed features on the mica surfaces measured with TM-SFM. The horizontal axes below each micrograph range from 0 to 5 nm. Each histogram reflects measurements of at least 100 features from several comparable surfaces.

the mica surfaces [14]. Furthermore, a tip-shape-induced broadening of the imaged molecules is present and is estimated to be about 14 nm.

Figures 2A, 2B, and 2C show images of mica surfaces probed after exposure to HSA, a-HSA, and IgG, respectively. Although height information is not totally topological, the height histograms show large differences which allow one to distinguish quantitatively between the presence of different species on the surfaces (Table 1). For entry A (Table 1), the histogram is parametrized by

Table 1. Peak positions and full widths at half maximum (FWHM) from histograms displayed in Figure 2. For the 2nd peak, the HSA contribution is subtracted. See text for further explanation.

	Experiment	1st Peak position ± FWHM (nm)	2nd peak position ± FWHM (nm)
A	HSA	0.62±0.28	
B	a-HSA	1.91±0.66	
C	IgG	1.75±0.60	
D	HSA exposed to IgG	≈0.62	≈1.75
E	HSA exposed to a-HSA	≈0.62	3.03±1.60
F	IgG exposed to HSA	≈0.62	1.35±0.72
G	a-HSA exposed to HSA	≈0.62	1.38±0.80

a steeply rising and then exponentially decaying function. For entries B and C (Table 1), the histograms are fitted to gaussian functions. Fluctuations in the data appear to be somewhat outside the limits of Poissonian event counting statistics. IgG and a-HSA single molecules were roughly circular and about 2.5 times higher than HSA, from which we conclude that they are not generally oriented with both the Fab and the Fc subunits bound to the surface.

When we exposed preadsorbed HSA on mica to IgG (Fig. 2D), we observed two distinct populations of molecules within height ranges corresponding to those of separately adsorbed HSA and IgG (Table 1). For entry D (Table 1) the histogram is approximately fitted by a linear combination of the histograms shown in Figures 2A and 2C. No interaction was observed, completely in accordance with the expectation that human IgG should not react with HSA.

When we exposed HSA adsorbed on mica to a-HSA (Fig. 2E), we predominantly observed features larger, in lateral dimensions as well as in height, than either HSA or a-HSA adsorbed separately on mica (Table 1). The area density of features on the surface was comparable to the area density of HSA before the second exposure. The image appears to show complexing, which is expected since the a-HSA is raised in rabbit to specifically interact with HSA. For entry E (Table 1), an appropriate contribution from HSA, the histogram shown in Figure 2A, was noted and subtracted. The remaining contribution in entry E, fitted to a skewed gaussian, is subject to alternative interpretations. We favor the notion that the relatively tall HSA/a-HSA complexes may exist in a wide range of orientations and conformations which are not necessarily height-distributed in a gaussian fashion. This is supported by the a-HSA being polyclonal, so that several a-HSA molecules can bind to different epitopes of one HSA molecule.

To examine how the binding to mica surfaces affects the interaction between antigens and antibodies, the order of adsorption was reversed (Figs. 2F and 2G). When preadsorbed a-HSA was exposed to HSA (Figure 2G), no features taller or wider than individual HSA and a-HSA molecules were observed, showing a lack of complex formation. It is possible that a-HSA adsorbs with the antigen binding epitopes on the surface, disallowing the possibility of a reaction with HSA molecules in a later exposure. However, inspection of the height histograms indicates that not all antibodies (a-HSA or IgG) seem to bind sufficiently strongly to survive the second exposure to the buffer. The contributions remaining for entries F and G (Table 1) after subtracting contributions from HSA are fit to Gaussians. The peak positions correspond to lower heights than those of IgG and a-HSA from Figures 2C and 2B. The origin of this peak shift is subject to alternative interpretations. However, we suggest that taller, possibly more weakly bound antibodies, are more easily rinsed away, leaving lower, more tightly bound antibodies on the surfaces. By contrast, adsorbed HSA is quite stable against repeated rinsing. This indicates that when preadsorbed HSA is exposed to a-HSA and complexing occurs, this is not due to HSA desorbing from the surface, forming complexes in solution, and subsequently readsorbing on the surface. The complexing seems to occur on the surface.

HSA binds spontaneously and irreversibly to hydrophilic mica surfaces, even though the net charge of HSA as well as the surface charge are negative [12, 18]. By contrast, IgG molecules did not seem to bind strongly to the mica. When using contact mode SFM, the molecules were easily swept aside, especially when scanning under buffer in a liquid cell. The reason why IgG binds more weakly to mica than HSA may be related to IgG's smaller capability of conformational changes. Similar relative binding strengths between HSA and IgG have been observed on glass surfaces [10]. A competitive binding process may also be involved; measurements involving thick layers of IgG and HSA adsorbed to polystyrene lattices indicate that HSA can replace preadsorbed IgG [5].

Our experiments indicate that SFM is a usable detector for observing biological molecular processes like antigen-antibody docking. So far the molecules in our adsorption experiments were not bound sufficiently strongly to the surface to be imaged in the liquid cell in contact mode SFM. The use of a liquid cell for tapping

mode SFM [8, 13] may very well aid in a strong reduction of lateral forces, leading to the possibility to observe the reaction between antigens and antibodies ***in situ*** and **in real time.**

The amount of a-HSA used in our experiments varies from 1-2.5 x 10^{12} molecules; we estimate that about 0.2% of them are consumed in docking to preadsorbed HSA, resulting in a little over 50 detection events per μm^2. It is clearly feasible to exceed the sensitivity of existing techniques, which use of the order of 10^7-10^8 molecules, by employing longer adsorption times on smaller surface areas. Furthermore, classical work [11] examining antibody-antigen docking is performed using thick layers of molecules. SFM, with its ability to study the interaction between single molecules, is likely to provide us with a wealth of new information at the molecular level.

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Discussion with Reviewers

R.J. Warmack: Were the surface densities measured consistent with irreversible binding of HSA in all cases? Have you thought of using a divalent cation or other surface treatments to control the binding of IgG and a-HSA?

Authors: The surface densities of HSA were comparable in all adsorption experiments. We did not study the adsorption and desorption kinetics of HSA. We merely noted that the HSA molecules seemed to remain on the surface after multiple rinsings with water or buffer. Since the HSA adsorbed irreversibly, no cations were required in the buffer. This also reduced the possible level of contamination in the buffer, which is important in our search for individual molecules. To have the same conditions for the experiments in which the antibodies were adsorbed first, the same buffer solution was used. We are thinking, however, of using cations and chemically modified surfaces for the antibody adsorption experiments.

H.G. Hansma: Why did you dry the first protein onto the mica before adding the second protein? Would you not expect this to denature the first protein? Does the assay work if you add the second protein after rinsing off the first protein but without drying it?

Authors: The first protein is dried in order to be able to study the surface with tapping mode SFM before the second exposure. This study was necessary in order to be able to tell the difference between the surface features before and after the second exposure. The SFM studies could only be performed on dried surfaces, since the proteins were not bound sufficiently strongly to image them under liquid in contact mode SFM, and at that time, no liquid cell tapping mode was available to us. Some denaturing of the proteins can be expected upon drying, but we do not know to what extent this occurs. Neither do we know yet if the assay would work in experiments without drying as an intermediate step. We will explore these questions as soon as we are able to work with tapping mode in liquids.

H.G. Hansma: How do you know that the lumps on the molecules are protein domains? Could they be images of the tip shape? What do the other molecules in these fields look like? If less than 1% of the IgG show Y-shapes, maybe these Y's are clusters of 3 IgG molecules, which one would expect to find occasionally. How do you know that the image on the right of Figure 1 is of a single molecule?

Authors: We are convinced that the lumps are domains. These molecules are stable under multiple scanning with the SFM, while the clusters we observed, quite often, break apart under multiple scanning. Also the size, and especially the spacing between the domains, fit very well with the known crystallographic structure of the proteins. There is certainly no tip shape effect involved, since the observed molecules showing domain structures were randomly oriented on the surface. More details about our adsorption experiments of single molecules are given in reference [14].

H.G. Hansma: Why do you think you get the same density of molecules on the surface (40-60 per μm^2) over a 20-fold range of concentrations (0.7 to 13.4 $\mu g/ml$)?

Authors: The fact that albumin adsorbs differently from the antibodies is not surprising, as it is an altogether different molecule with completely different adsorption properties. It may seem strange, however, that IgG adsorbs 20 times more selectively to the surface than a-HSA, because the molecules are essentially similar in their structure. Recently, we performed an isoelectric focusing experiment, which showed that IgG has a broad distribution of isoelectric points. A large fraction of this distribution shows values of the isoelectric points higher than the pH of the buffer we worked with. The a-HSA showed a distribution with lower values for the isoelectric points, indicating that more IgG will be positively charged in the buffer than a-HSA. Furthermore, the intensities of the isoelectric focusing points indicate that the concentration of our a-HSA solution was actually smaller than we thought it was. The a-HSA was supplied in the buffer and we did not measure this concentration ourselves. The difference in adsorption properties may be reduced by using cations in the buffer.

H.G. Hansma: Perhaps the mean antibody height in Figures 2F and 2G is lower than in Figures 2B and 2C because the tallest lumps in Figures 2B and 2C are aggregates of two or more molecules that dissociate with more rinsing. (This is an extension of your washing theory.)

Authors: This is a possibility that we have considered. We would expect, however, that a cluster of two or more antibodies would have a much larger size (lateral and in height) than the sizes observed. Of course, as mentioned in [14], height information has to be treated carefully.