

## Specific Antigen / Antibody Interactions Measured by Force Microscopy

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**ABSTRACT** Molecular recognition between biotinylated bovine serum albumin and polyclonal, biotin-directed IgG antibodies has been measured directly under various buffer conditions using an atomic force microscope (AFM). It was found that even highly structured molecules such as IgG antibodies preserve their specific affinity to their antigens when probed with an AFM in the force mode. Evidence that only a very limited number (less than 10) antibody/antigen pairs contribute to the measured interaction force is given. The potential and limitations of this new approach for the measurement of individual antigen/antibody interactions, and some possible applications are discussed.

### INTRODUCTION

A fundamental prerequisite for the amazing complexity of life is specific recognition on the molecular level. The interplay of multiple non-covalent bonds, e.g. electrostatic, electrodynamic (van der Waals), hydrogen bonds or hydrophobic interactions, is the basis for highly specific interactions. Various assays and biosensors have been successfully developed over the last few decades (Chaiken *et al.*, 1990). Recently, a new approach has been explored, *i.e.* the direct measurement of the intermolecular interaction by suitable "force measuring devices". It turns out that the atomic force microscope (AFM) (Binnig *et al.*, 1986; reviews by Hansma and Hoh, 1994 and by Morris, 1994) fulfills important requirements for such a device since measurements in the piconewton range are readily achievable under physiological conditions. Promising results were obtained from the biotin/streptavidin model system (Lee *et al.*, 1994a; Moy *et al.*, 1994; Florin *et al.*, 1994; Dammer *et al.*, 1995a), from complementary DNA strands (Lee *et al.* 1994b), and from adhesion proteoglycans (Dammer *et al.*, 1995b). In addition, adhesion forces and the elasticity of proteins have also been mapped (Radmacher *et al.*, 1994a,b). However, one of the biggest scientific challenges in this field, and potentially the most rewarding applications for the new technique, are related to the interaction between antibodies and antigens.

Although the antibody/antigen interaction is of great practical and theoretical relevance, its direct study by force measurement is rendered more difficult by several factors. Both antibodies and antigens usually have complicated tertiary

structures and need firm attachment to the force measuring device. Their interactions (Davis, 1990; van Oss, 1994; Webster 1994) depend on geometric factors, conformational state and environment, and steric hindrance has to be avoided. The expected forces are in the piconewton range and the active sites of the antibodies are small compared with the antibody itself, so that non-specific interactions need to be considered.

In this study, immunopurified polyclonal goat IgG antibodies directed against biotin were used. In our case, the biotin is coupled via long (2.24 nm) spacer arms to bovine serum albumin (BSA) and together with the BSA forms an artificial antigen (biotinylated BSA or BBSA). The advantage of this is that the antigenic effect of the BBSA molecule containing 8-12 biotin moieties should be less sensitive to conformational changes, steric hindrance and orientation. In our experiment, we have covalently immobilized the two components on the AFM sensor tip and on a flat gold surface via a newly synthesized  $\omega$ -functionalized (NHS) dialkyldisulfid crosslinker in order to guarantee stable and reproducible conditions. The chemisorption of the long-chain crosslinker in a self-assembly process provides a highly reactive monolayer surface for coupling primary amines under mild conditions. Furthermore, we have used ultrasoft cantilevers for high force sensitivity and blocking molecules (BSA) to reduce non-specific background. Several control experiments were carried out in order to check the validity of the force measurements.

## EXPERIMENTAL

All proteins and immunocompounds including the biotinylized BSA were purchased from PIERCE (PIERCE Europe, Oud Beijerland, The Netherlands) and of highest available quality. Dithiobis(succinimidylundecanoate) (DSU) crosslinker was synthesized according to Wagner *et al.* (1994; 1995 submitted for publication). Solvents of highest purity were from FLUKA (Buchs, Switzerland), gold (99.99 %) from BALZERS (Zürich, Switzerland). Flat gold surfaces were either produced conventionally by thermal evaporation on silicon wafers at room temperature (Dammer *et al.*, 1995b) or as ultraflat template-stripped gold as previously described (Hegner *et al.*, 1993; Wagner *et al.*, 1995; Hegner and Wagner, 1996). The two methods showed no effect on the results. The two gold surfaces, i.e. the tip and the substrate, were treated basically parallel. Amino-reactive self-assembled monolayers of DSU on gold surfaces were prepared by immersion of the fresh gold in a 0.5 mM solution of DSU in 1,4-dioxane over night at room temperature. After careful rinsing with 1,4-dioxane and air drying, the surfaces were immediately subjected to the immobilization of antibodies and derivatized albumin (incubation 90-180 min, four times washing with 3 ml buffer). The washing steps guarantees that no multilayers of protein absorb as they show no tendency to aggregate in solution. On the other hand, there might be some unspecifically bound molecules that are washed away and leaving some uncoated surface. AFM imaging always shows a coverage close to one monolayer (data not shown) proving that this effect only plays a minor role. As standard buffer, degassed phosphate buffer (165 mM NaCl, 2.7 mM KCl, 10.1 mM Na<sub>2</sub>HPO<sub>4</sub>·7 H<sub>2</sub>O, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.3) was used. All measurements were performed in this standard buffer using a commercial fluid cell (Digital Instruments, Santa Barbara, CA). Biotinylated bovine serum albumin (BBSA) was used at 0.6 mg/ml buffer (0.2 mg/ml for surface plasmon resonance (SPR) measurements), bovine serum albumin (BSA) at 1 mg/ml (SPR: 0.2 mg/ml), immunopurified polyclonal goat IgG anti-biotin antibodies (ABIO) and rabbit anti-mouse IgG control antibodies (AMOUSE) at 10 µg/ml. Most samples were immediately used, although some others were used after one day's storage at 4 °C. A series of experiments with many different tips and substrates and with many hundreds of approach-retract cycles was carried out, taking into account the statistical nature of a single interaction event. The functionalized sensor tips and substrates were always kept under buffer. Blocking was done with a solution of streptavidin (1 mg/ml) or biotin (1 mg/ml, FLUKA, Buchs, Switzerland). All

measurements were taken on a multimode NanoScope (Digital Instruments). Approach/retract cycles were performed as gently as possible (at 0.5 Hz). Here, the cantilevers were calibrated by SEM and resonance frequency measurements ( $k=0.014$  N/m, 5 % relative error, ~40 % absolute error).

## RESULTS

The coupling reaction of the proteins to the activated gold surface is illustrated in figure 1A, a model of the set-up configuration shown in figure 1B. The bioreactive succinimide head group of the self-assembled monolayer is capable of binding primary amines and the coupling reactions can be performed under physiological conditions. The reverse configuration, with the antibodies on the tip and the BBSA on the substrate, was also studied. Typical approach/retract cycles are shown in figure 2. Figure 2 A and B show typical curves taken between a BBSA covered sensor tip and an ABIO coated substrate, C shows a control where BBSA is replaced by BSA. To show that ABIO/BBSA curves originate from specific antibody/antigen interactions, a variety of control experiments was performed. In figure 3 A the whole series of experiments is summarized and the percentage reduction in the mean adhesion force of the last jump is shown. As a measure of the interaction strength, the size of the final jump off from the surface peak in each curve was automatically taken and averaged over a large number (total > 3000) of different curves ("mean adhesion force") (figure 3 B total > 1500). The mean adhesion force of the specific ABIO-BBSA is 111.5 pN. All measurements were included in the averaging process in order to avoid misinterpretation due to arbitrary choice of „good“ or "bad“ curves. It turned out that there is a clear difference between the (specific) ABIO-BBSA interaction and the (non-specific) ABIO-BSA interaction (figure 3A, nos. 1 and 2). Blocking by either biotin or streptavidin is possible (figure 3A nos. 3 and 4). If we use an antibody that is not specific for biotin (rabbit anti-mouse) we obtain mean adhesion forces similar to the nonspecific background, again indicating the specificity of the BBSA-ABIO reaction (Figure 3A, no. 7). The results for the reverse set-up with the antibodies on the sensor tip were similar to those from the set-up sketched in Fig 1B (data not shown). However, the reverse set-up often shows lower specific binding, indicating that the antibodies at the tip are more vulnerable to misorientation. The specific interaction can be suppressed by low pH (10 mM citrate, pH 1.8) (figure 3A, no. 5) or high pH (100 mM trimethylamine-HCl, pH 11.5) (figure 3A, no. 6) (Goldblatt, 1993; Harlow *et al.*, 1988). In all cases, the suppression is reversible, i.e., the full

adhesion force is reconstituted when standard buffer is used again.

Figure 3B shows a histogram of the analysis of the final jump off from the surface during the specific BBSA-ABIO interaction. We found maximum peaks of the measured adhesion forces composed of integer multiples of an elementary force of  $60 \pm 10$  pN. Although we are dealing with polyclonal antibodies which have been affinity purified, a quantization could be observed.

## DISCUSSION

The control experiments have confirmed that the force measurements represent specific interactions between biotin and anti-biotin antibodies. Due to the covalent anchoring of the antibodies and the (artificial) antigens it was possible to use the same tip for 100 measurements and more. Some major points have still to be discussed. Can an estimate for the binding force of an individual biotin and antibiotin pair be given? What are the possible applications of this approach in view of the experimental results, and how should future force experiments be designed?

The size of the peak of the final jump off from the surface in the approach/retract cycles that we used as a measure for the interaction force is certainly a direct determination of the strength of the bond formed between one or multiple individual biotin and antibiotin pair. Such a value will also depend on external parameters such as temperature, pH or buffer conditions, and on intrinsically statistical parameters such as the exact relative orientation and state of the complex and is the scope of future experiments. Thus, meaningful statements can only be made about force distributions. Whether statements about individual bonds can be made or not will depend on the sharpness of this distribution and on the force sensitivity of the instrument used. One solution is to look at the detailed shape of the approach/retract curves and focus on discrete steps. This will work well if the width of the force distribution is relatively small, for then multiples of a force quantum can be identified as such (Moy *et al.*, 1994). In our experiments we analyzed the size of the final „jump-offs“ (figure 3B) and a force quantization of  $60 \pm 10$  pN was observed. About 6 % of the curves show no adhesion, the others form a broad distribution ranging up to 500 pN with a concentration between 40 and 260 pN. In the future we plan to study systems with monoclonal antibodies well oriented on the surface, allowing a more pronounced observation of force quantization, and thus a correlation with data obtained from thermodynamic experiments. When discussing the force of separation of individual

antibody/antigen pairs, it is important to compare numbers obtained from the energetics of the reaction. If the free enthalpy  $\Delta H$  is known and the effective range of the potential  $d$  can be estimated, then the unbinding force is given by  $F = \Delta H/d$ . Typical antibody/antigen complexes with  $K_{\text{ass}}$  from  $10^2$  to  $10^{10}$  L/mol have free enthalpies of about 30 to  $100 \cdot 10^{-21}$  J/ pair (van Oss., 1994). With a binding pocket of 0.6 nm from Davis (1990, obtained for vitamin K1 and antibody) or 0.93 nm for biotin/streptavidin (Moy *et al.*, 1995), this gives a force of about 35 to 165 pN (20 to 100 pN, respectively). In round numbers, this corresponds with steps ( $n \cdot 60 \pm 10$  pN) that we commonly see in the final jump off from the surface in our approach/retract curves, see figure 2 and figures 3B, C. It is not entirely clear if  $\Delta H$  or rather the free energy  $\Delta G$  describes the antibody/antigen complex formation better (Kelly *et al.*, 1993; Webster *et al.*, 1994; Moy *et al.* 1994). In this study, however, we are dealing with polyclonal antibodies and cannot distinguish between the two. However, from the above estimate and from geometrical considerations (the tip radius of our tips with the gold coating is about 60 nm, the diameter of an antibody about 10 nm, BBSA is slightly smaller), it is clear that only very few, say less than ten but often only one, antibody/antigen complexes are formed and subsequently broken in an approach experiment.

There is one important point to be taken into account when looking at the detailed shape of approach/retract curves. It seems surprising that the adhesion peaks reach about 100 nm into the retracting regime of the force curves. This appears to be contradictory to the fact that the molecules have a diameter of less than 15 nm. However, the numbers on the distance axis do not represent the relative tip/surface distance but the bending of the cantilever. This distance is quasi zero in that attractive regime where the spring tension is built up necessary to overcome the antibody/antigen binding. True tip movement is on the other hand represented by the steps along the force axis. Here, 200 pN correspond to a cantilever bending of 20 nm and this distance is compatible with the size of the molecules. We interpret the fine structure as the breaking of multiple contact point that are likely to be antibody/antigen pairs but nonlinear convolution of multiple unbinding processes. Additionally, the effect of lateral movement of the sensor tip during the process of approach or withdrawal has to be considered. For geometrical reasons, there will be a force on the tip that is parallel to the surface reflecting the finite angle between the cantilever and the surface (typically  $10^\circ$ - $20^\circ$ ). To a first approximation (no bending) the lateral movement  $\Delta x$  is proportional to the vertical movement  $\Delta z$  with  $\Delta x = \tan \vartheta \Delta z$ , where  $\vartheta$

is the mounting angle of the cantilever. In future experiments, this effect should be software corrected by moving the sample laterally while taking approach/retract cycles. This effect is rather small (a 10 nm vertical movement in contact might cause a 2 nm lateral drift) but makes it more difficult to interpret the detailed shape of the fine structure.

Another important question to be addressed in the light of these experiments concerns the future applications of the force method. Direct measurements with a force measuring device such as the AFM are complementary to the existing immunotechnology based on biosensors or labeled antibodies and can give additional information about the spatial distribution of the receptors or the antigens. New immunosensors of high throughput rate, combined with minute sample quantities, could be used in the screening of novel active substances. Such applications might be in reach if some requirements can be fulfilled. The main design principle for future experiments will be that the reaction between the bioactive molecules takes place under well controlled conditions as close as possible to the physiological. Therefore, the force measuring device has to be extremely gentle, to avoid conformational changes or even destruction of the molecules. Furthermore, lateral drift needs to be corrected. Taking the rapid progress of electronics into account, these conditions might be realized soon. In addition, the preparation of the sensor tips is essential, including well-oriented antibodies (Brada and Roth., 1984; Ill *et al* 1995) or  $F_{ab}$  fragments, dense packing without steric hindrance, and the suppression of unspecific background.

In conclusion, the specific interaction between polyclonal biotin-directed antibodies and biotinylated bovine serum albumin has been measured directly by force microscopy. Specificity has been demonstrated by a number of control experiments. It has been estimated that only a very few antigen/antibody complexes contribute to the measured binding force. Provided that certain limitations can be overcome, the correlation to data from thermodynamic experiments of individual antigen/antibody interactions should be possible. In future research, this technique has obvious applications in many different fields.

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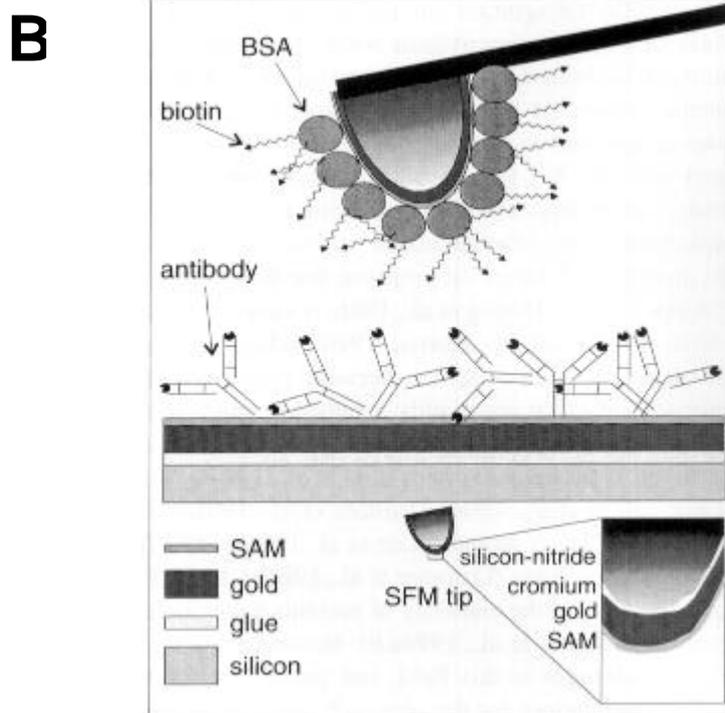
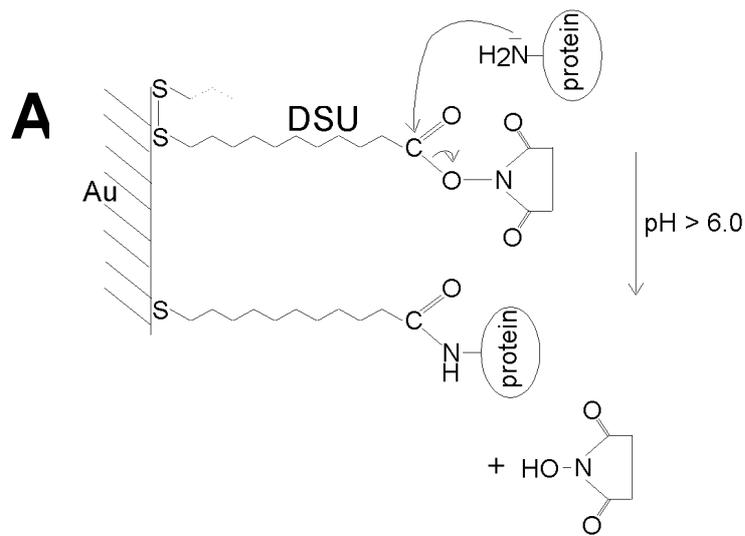


FIGURE 1: (A) Immobilization procedure: Both the antibodies and the antigen contain free amine groups which can be coupled to gold surfaces via DSU crosslinker (see text, drawing not to scale, the thickness of the crosslinker monolayer is 1.7-1.9 nm) (B) Sketch of the experimental set-up: biotinylated bovine serum albumin (BBSA) was covalently immobilized on a sensor tip. The anti-biotin antibodies (ABIO) are coupled to a flat gold substrate. In control experiments the BBSA is replaced by normal BSA or the ABIO by non-specific antibodies. The interaction can be inhibited by adding biotin or streptavidin or suppressed by low or high pH buffer. The set-up can be reversed, i.e. the antibodies can be located on the sensor tip.

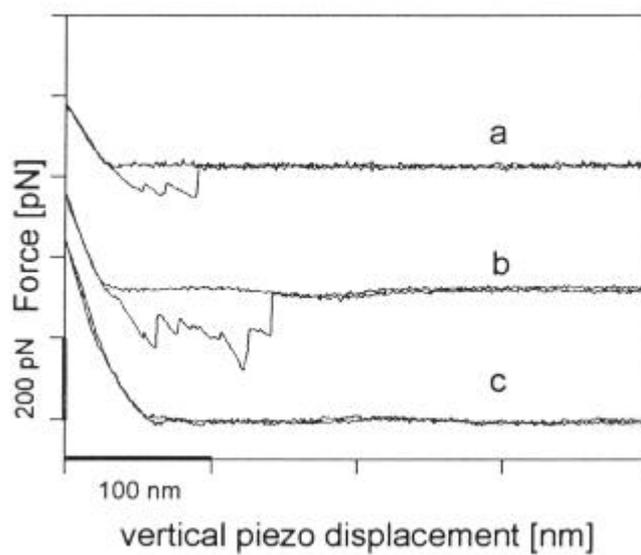


FIGURE 2: Typical approach/retract cycles taken between a BBSA coated sensor and a ABIO coated surface. The adhesion peaks are in the piconewton range and often multiple (curves a and b) (discussion in text), whereas the BSA control (curve c) shows hardly any adhesion.

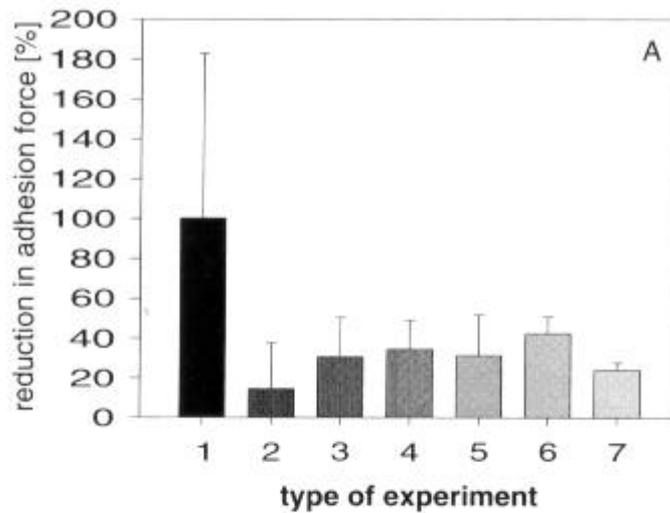


Table 1: Summary of the control experiments of the antibody-antigen interaction of Figure 3A (see text).

	surface/tip	surface/tip	medium	force [pN]	force [%]
1	ABIO	BBSA		111.5±98.6	100.0±88.4
2	ABIO	BSA		17.5±26.5	15.7±23.8
3	ABIO	BBSA	biotin	33.8±22.5	30.3±20.2
4	ABIO	BBSA	streptavidin	39.6±15.1	35.5±13.5
5	ABIO	BBSA	high pH	34.7±23.8	31.1±21.3
6	ABIO	BBSA	low pH	48.5±7.9	43.5±7.1
7	AMOUSE	BBSA		26.8±4.7	24.0±4.2

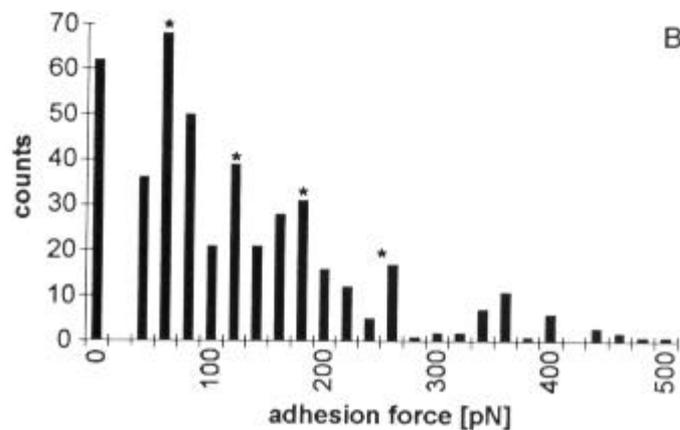


FIGURE 3: (A) Summary of the control experiments (>3000 curves): bar no 1 shows the mean value of 111.5 pN  $\pm$  for the specific ABIO-BBSA interaction (last jump off the surface). The other bars show the percentage reduction of the interaction force corresponding to the 100% force of the specific interaction. In no. 2 BBSA was replaced by BSA; nos. 3 and 4 show blocking by biotin and streptavidin, respectively. Nos. 5 and 6 represent the (reversible) change of the buffer from PBS to low pH and high pH respectively, whereas in no. 7 ABIO was replaced by a non-specific antibody AMOUSE; errors are standard deviations. (B) Histogram of the adhesion force between biotinylized bovine serum albumine and antibiotin IgG. Analysis of the peak of the final jump off from the surface during approach/retract cycles.