Antigen Binding Forces of Single Antilysozyme Fv Fragments Explored by Atomic Force Microscopy

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We used atomic force microscopy (AFM) to explore the antigen binding forces of individual Fv fragments of antilysozyme antibodies (Fv). To detect single molecular recognition events, genetically engineered histidine-tagged Fv fragments were coupled onto AFM tips modified with mixed self-assembled monolayers (SAMs) of nitrotriacetic acid- and tri(ethylene glycol)-terminated alkanethiols while lysozyme (Lyso) was covalently immobilized onto mixed SAMs of carboxyl- and hydroxyl-terminated alkanethiols. The quality of the functionalization procedure was validated using X-ray photoelectron spectroscopy (surface chemical composition), AFM imaging (surface morphology in aqueous solution), and surface plasmon resonance (SPR, specific binding in aqueous solution). AFM force—distance curves recorded at a loading rate of 5000 pN/s between Fv- and Lyso-modified surfaces yielded a distribution of unbinding forces composed of integer multiples of an elementary force quantum of ~50 pN that we attribute to the rupture of a single antibody—antigen pair. Injection of a solution containing free Lyso caused a dramatic reduction of adhesion probability, indicating that the measured 50 pN unbinding forces are due to the specific antibody—antigen interaction. To investigate the dynamics of the interaction, force—distance curves were recorded at various loading rates. Plots of unbinding force vs log/loading rate revealed two distinct linear regimes with ascending slopes, indicating multiple barriers were present in the energy landscape. The kinetic off-rate constant of dissociation ($k_{\text{off}} \approx 1 \times 10^{-3} \text{ s}^{-1}$) obtained by extrapolating the data of the low-strength regime to zero force was in the range of the $k_{\text{off}}$ estimated by SPR.

Introduction

Molecular recognition, which involves multiple noncovalent bonds, plays a central role in cellular behavior and in immunology. Understanding the specific molecular forces between receptor and ligand molecules is therefore of great relevance for basic research (cell biology, microbiology, medicine). Furthermore, detecting individual molecular recognition events is an important step toward developing new analytical and biomedical devices (e.g., biosensors).

In the past decade, atomic force microscopy (AFM) has been increasingly used to measure the specific binding forces of individual ligand—receptor complexes.1–14 These studies imply attaching specific biomolecules on the AFM tip and on solid substrates and recording force—distance curves between the modified surfaces.12 Several important issues must be considered when functionalizing the tip and substrate with biomolecules. First, the forces which immobilize the molecules should be stronger than the intermolecular force being studied. Second, the attached biomolecules should have enough mobility so that they can freely interact with complementary molecules. Third, the contribution of nonspecific adhesion to the measured forces should be minimized. To fulfill these requirements, several flexible spacer molecules have been introduced, including poly(ethylene glycol)1 and carboxymethyl-amylose.8,13 The biomolecules are covalently bound and able to move and orient freely, while nonspecific adsorption is inhibited.

Here, we use AFM to probe the dynamics of the antigen binding strength of single antilysozyme antibodies. We chose the Fv fragment of a humanized version of the mouse antilysozyme antibody. About one-sixth the size of IgG, the Fv fragment is the two-chain heterodimer of the immunoglobulin variable light and heavy domains and is regarded as the smallest fragment of an antibody that...
still retains the specific binding of the whole antibody. The benefits of this selection include (i) this Fv fragment is a small protein (MW \(\approx 26\, \text{kDa}\)) which has been well characterized,\(^{(15,16)}\) (ii) it retains the lysozyme (Lyso) binding affinity of the whole antibody, \((K_d = 3.7 \times 10^{-9}\, \text{M})\) to hen egg white lysozyme;\(^{(11)}\) (iii) it was genetically modified with a His-tag for site-directed immobilization onto a NTA/EG-terminated tip. Lysozyme molecules were covalently bound onto a flat surface terminated with COOH/OH groups using the NHS/EDC chemistry. Using this immobilization strategy, Fv and Lyso are attached at a low surface density, thereby allowing detection of unbinding events at the single molecule level.

Figure 1. Scheme of the surface chemistry used to functionalize AFM tips and substrates with Fv antibody (Fv) and antigen (Lyso) molecules. Fv fragments were engineered with a His-tag for site-directed immobilization onto a NTA/EG-terminated tip. Lysozyme molecules were covalently bound onto a flat surface terminated with COOH/OH groups using the NHS/EDC chemistry. Using this immobilization strategy, Fv and Lyso are attached at a low surface density, thereby allowing detection of unbinding events at the single molecule level.

Materials and Methods

Production of Gold Surfaces. For AFM force measurements, cantilevers were coated using electron beam thermal evaporation with a 5-nm-thick Cr layer followed by a 30-nm-thick Au layer.

Preparation of Fv- and Lyso-Terminated Surfaces. The E. coli strain 25F2 was transfected with the Fv-encoding vector plasmid pAK19 which was generously provided by the Patrick Stayton Lab (Department of Bioengineering, University of Washington, Seattle, WA). They obtained the original gene from the Jefferson Foote Lab (Fred Hutchinson Cancer Research Center, Seattle, WA) and introduced a hexahistidine fusion peptide to the C-terminal end of the variable heavy (VH) domain.\(^{(18)}\) The protein expression was carried out following published procedures.\(^{(18)}\)

For the AFM and XPS experiments, the Fv fragments were immobilized onto gold-coated substrates and AFM tips using the specific binding between histidine-tagged proteins and the tri- diacetic acid (NTA)-terminated self-assembled monolayers (SAMs).\(^{(20)}\) To this end, the gold surfaces were first cleaned for 5 min by UV/ozone treatment (Jelight Co., Irvine, CA), rinsed with ethanol, and dried with a gentle nitrogen flow. They were immersed overnight in ethanol solutions containing 0.05 mM NTA-terminated (5%) and tri(ethylene glycol)(EG)-terminated (95%) alkanethiols (for details on the synthesis of these molecules, see ref \(20\)) and rinsed with ethanol. Sonication was briefly applied to remove alkanethiol aggregates that may be adsorbed. Then, the SAM-coated surfaces were immersed in a 40 mM aqueous solution of NiSO4 (pH 7.2) for 1 h and rinsed with phosphate buffer saline (PBS; pH 7.0; 150 mM NaCl, 3 mM KCl, 4.3 mM Na2HPO4, 7H2O, 1.4 mM KH2PO4). Finally, the samples were incubated in PBS containing 2.5 mg/mL His-tagged Fv fragments for 2 h and further rinsed several times with PBS.

Lysozyme was covalently immobilized onto mixed SAMs of carboxyl (COOH)- and hydroxyl (OH)-terminated alkanethiols.\(^{(21,22)}\) Cleaned gold surfaces were immersed for 3 h in ethanol solutions containing a 1 mM mixture (20:80, mol/mol) of H5S(CH2)15-COOH (Aldrich, used as received) and H5S(CH2)15-OH (Aldrich, used as received) and then rinsed with ethanol. Sonication was briefly applied to remove alkanethiol aggregates that may be adsorbed. The SAMs were immersed for 30 min into a PBS solution 170 mM N-hydroxysuccinimide (NHS) (Aldrich) and 260 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) (Sigma), briefly sonicated, and rinsed with PBS. The activated surfaces were then incubated with 10 \(\mu\)g/mL chicken egg white lysozyme (Sigma) in PBS for 1 h, rinsed, and kept in PBS solution.

Surface Characterization. For XPS analysis, samples were rinsed with water and dried by flushing with a gentle nitrogen flow and then immediately introduced in the XPS vacuum chamber. The analyses were performed on a Kratos Axis Ultra spectrometer (Kratos Analytical, Manchester, UK) equipped with a monochromatized aluminum X-ray source. The samples were fixed on a stainless steel multispecimen holder by using double-sided conductive tape. The angle between the normal to the sample surface and the electrostatic lens axis was \(90^\circ\). The constant pass energy of the hemispherical analyzer was set at 40 eV. The following sequence of spectra was recorded: survey spectrum, C 1s, N 1s, O 1s, Au 4f, S 2p, and C 1s again to check the stability of charge compensation in function of time and the absence of degradation of the sample during the analyses. The binding energies were

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(20) Luk, Y.-Y.; Tingley, M. L.; Hall, D. J.; Israel, B. A.; Murphy, C. J.; Bertics, P. J.; Abbott, N. L. Langmuir 2003, 19, 1671.
calculated with respect to the C(−(C,H)) component of the C1s peak of adventitious carbon fixed at 284.8 eV. Following subtraction of a linear baseline, molar fractions were calculated (CasaXPS program, Casa Software Ltd., UK) using peak areas normalized on the basis of acquisition parameters, sensitivity factors, and the transmission function provided by the manufacturer.

Water contact angles were measured under ambient atmosphere at 20 °C using the sessile drop method and an image analysis of the drop profile. The water droplet volume was 0.3 μL.

Surface Plasmon Resonance Measurements. To prepare the NTA-functionalized surface for SPR measurements, clean glass chips were coated by e-beam evaporation, first with 10 Å of Pt and then with 500 Å of gold. The freshly prepared chips were immersed overnight in 0.1-mM C11-oligo(ethylene glycol)-terminated (EG) thiol (absolutely ethanol as solvent). After the chips were removed from the solution, they were rinsed sequentially by ethanol and dioxane then immersed in 50-mM 1,1-carbonyldiimidazole (CDI) in dioxane at 50–60 °C for 3 h. At the end of the reaction, the chips were rinsed with dioxane, blown dry with nitrogen, and transferred immediately to an aqueous solution of N,N′-bis(carboxymethyl)-L-lysine hydrate (100 mM; the pH of the solution was adjusted to 10–11 with NaOH). The NTA functionalization was continued for 4 h at 50–60 °C and ended by rinsing the chips with DI water. The chips were then immersed in water at room temperature for 30 h to quench any remaining imidazolyl carbamate groups and finally blown dry with nitrogen.

A PLASMON–II SPR liquid sensing system was used for the SPR measurements. Basically, it uses the planar prism (Kretschmann) configuration. A white light source was passed through the prism and projected on the backside of the gold-coated glass chip at a predetermined angle. Interactions at or above the gold sensor surface are observed by monitoring the wavelength shift of the SPR minimum. A Teflon flow cell containing a flow channel with small chamber was used to guide the flow of liquid samples during the experiment. A peristaltic pump was utilized to deliver liquid samples to the chamber of the flow cell. During SPR measurements, buffers and solutions were sequentially delivered to the substrate surface at a flow rate of 50 μL/min. All the buffers and protein solutions used in this study contained 0.05% Tween 20, except as noted.

AFM Measurements. AFM images and force–distance curves were obtained at room temperature, using a commercial microscope (Nanoscope III, Digital Instruments, Santa Barbara, CA). The bottom side of hydrated substrates was quickly dried using precision wipes (Kimwipes, Kimberly-Clark), and the substrates were then immobilized on a steel sample puck using a small piece of adhesive tape. The mounted samples were immediately transferred into the AFM liquid cell, while avoiding dewetting. Imaging was performed in TSC solutions with silicon nitride triangular-shaped cantilevers (Microlevers, ThermoMicroscopes, Sunnyvale, CA). For the force measurements, cantilevers were functionalized as described above. Blocking control experiments were performed by injecting 10-μM lysozyme solutions into the liquid cell.

The spring constants of the cantilevers were estimated as follows. The geometrical dimensions of each functionalized cantilever were accurately measured by scanning electron microscopy, and the free resonance frequency was measured. Then, their mechanical properties were adjusted in order to match the calculated frequencies to the measured ones. The determined mechanical properties and the measured geometrical

### Table 1. Surface Chemical Composition and Water Contact Angles of Solid Substrates after the Various Steps of the Immobilization Procedures

<table>
<thead>
<tr>
<th>sample</th>
<th>mole fraction (%)</th>
<th>water contact angle (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Au</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
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<tr>
<td>O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NTA/EG</td>
<td>38.2</td>
<td>47.9</td>
</tr>
<tr>
<td>NTA/EG + Fv</td>
<td>36.4</td>
<td>45.3</td>
</tr>
<tr>
<td>COOH/OH</td>
<td>36.2</td>
<td>54.0</td>
</tr>
<tr>
<td>COOH/OH + Lyso</td>
<td>25.9</td>
<td>59.6</td>
</tr>
</tbody>
</table>

* Mole fraction of elements excluding hydrogen. Duplicate XPS measurements yielded the same trend. * Mean of three independent experiments.

Results and Discussion

Surface Chemistry for Preparing AFM Tips and Substrates. Functionalization of AFM tips and substrates using appropriate procedures is a key prerequisite for successful, reliable receptor–ligand force measurements. To explore the antigen binding forces of single antilysozyme Fv antibodies, hexahistidine peptide tags (His-tag) were genetically linked to the C-terminus of VH, which is located opposite to the lysozyme binding domain, and the His-tagged Fv fragments were coupled on NTA-terminated AFM tips (Figure 1). This approach offers several advantages: first, binding of the Fv fragments is stronger than the intermolecular force being studied; second, the attached molecules have enough mobility for antigen binding; third, the site-directed NTA–His system should allow all fragments to be uniformly oriented on the surface with their binding domains exposed; fourth, the use of mixed NTA/EG-terminated SAMs allows the density of biomolecules on the tip to be sufficiently low to permit the detection of single interactions; fifth, the EG-terminated SAM inhibits nonspecific protein adsorption which would otherwise affect the measured forces.

The lysozyme-coated substrates were prepared by attaching Lyso covalently onto flat gold substrates modified with alkanethiol SAMs. To visualize the small Lyso molecules, gold surfaces exhibiting uniform flat morphology over large areas were prepared using the TSG method. Lyso molecules were reacted, via NHS/EDC, with long-chain COOH alkanethiols diluted in a matrix of shorter OH alkanethiols to provide mobility to the attached molecules and to reduce the protein surface density.

Validation of the Modified Surfaces Using XPS, AFM, and SPR. Model substrates functionalized with Fv and Lyso molecules were thoroughly characterized using XPS, AFM imaging, and SPR. Table 1 presents the surface chemical composition determined by XPS for the substrates after the various steps of the immobilization procedure. Following treatment with NTA/EG-terminated alkanethiols, the gold sample showed significant oxygen, sulfur, and nitrogen concentrations, consistent with the presence of the NTA/EG-terminated SAM. This is also illustrated by the carbon (C1s) spectra, Figure 2, which showed three main components, one at 284.8 eV due to the aromatic backbone (C=C), one at 285.6 eV due to the aromatic backbone (C=N), and a third small one localized between 288.1 eV (C=O) and 289.3 eV (O=C=O)
corresponding to carbon making two and three bonds with oxygen, respectively. These results, together with the intermediate water contact angle values, are consistent with the presence of a mixed NTA/EG-terminated SAM. After incubation with Ni$^{2+}$ and His-tagged Fv fragments, a large increase of the nitrogen concentration was noted, together with an increase of the 287.8 eV component ($C\equiv O$) of the C 1s peak, indicating the presence of a significant amount of proteins at the surface. This was also reflected by the lower contact angle value.

As expected, the gold surface treated with OH- and COOH-terminated alkanethiols showed significant oxygen and sulfur concentrations and a small contact angle (Table 1); the small amount of nitrogen may be attributed to contamination. The carbon spectra (Figure 2) showed three components at 284.8 eV ($C-(C,H)$), 286.3 eV ($C-O$), and 289.3 eV ($O-C=O$), the latter two components being attributed to alcohol and carboxyl functions, respectively. We also note that the carbon atoms linked to carboxyl functions ($CH_2-COOH$) should be chemically shifted about 0.75 eV by the carboxyl group, thus leading to the broadening of the main peak. Treatment with NHS-EDC and Lyso lead to an increase of the nitrogen concentration and to the appearance of a 287.8 eV component ($C=O$), reflecting essentially the amide bonds in the proteins.

Figure 3A shows an AFM topographic image in aqueous solution of a gold substrate prepared using the TSG method. As can be seen, the surface was remarkably smooth, with an rms roughness in 500 nm × 500 nm areas of 0.14 ± 0.02 nm and randomly distributed small holes. Modifying the gold substrates with a mixed COOH/OH alkanethiol SAM, Figure 3B, did not dramatically change the surface roughness (rms roughness = 0.19 ± 0.04 nm) and gave images that were devoid of aggregates, supporting the idea that smooth SAMs are formed. Figure 3C shows that functionalization of the SAM-coated substrates with a 10-µg/mL solution of Lyso gave rise to a significant increase of surface roughness (rms roughness = 0.34 ± 0.09 nm) and to the appearance of dots of 2 ± 0.6 nm height and 15–20 nm in diameter uniformly distributed across the surface. The dot height is consistent with previous AFM studies and in the range of the Lyso dimensions, i.e., 3 nm × 3 nm × 4.5 nm, suggesting each dot reflects an individual protein. The large diameter can be attributed to tip broadening and compression of the molecule by the tip. Interestingly, scanning the surface repeatedly at low forces did not cause reorganization of the molecules, supporting the notion that they were firmly anchored and not simply adsorbed. Finally, we note that the 10-µg/mL concentration was found to be optimal for our AFM measurements (data not shown): a smaller concentration (1 µg/mL) yielded a very small surface coverage of the surface by individual Lyso molecules while a larger concentration (100 µg/mL) produced loosely bound, large aggregates (>20 nm in height).

To further characterize the lysozyme layer, a small area was first recorded at large forces (10 nN) for short period of times, followed by imaging a larger image of the same area under normal load. Figure 3C shows that imaging at high forces resulted in pushing the grafted material aside, thereby revealing the underlying substrate. The
thickness of the removed layer was found to be 3 ± 0.5 nm, which is consistent with the Lyso dimensions, thus pointing to the presence of a protein monolayer at the surface. Note that performing such experiment on the SAM-coated substrates did not cause any morphological change, indicating they resisted the high imaging forces.

We also imaged the surface of flat gold substrates after modification by NTA/EG alkanethiols and subsequent binding of Fv fragments. As shown in Figure 3D, the surface was devoid of dotlike structures or aggregates and showed a fuzzy contrast that was clearly different from that of the native NTA/EG surface. Figure 3D also reveals that imaging the surface at high forces caused the removal of a 0.6-nm-thick layer from the surface that we attribute to Fv since this behavior was not observed on native NTA/EG surfaces (not shown). The measured thickness was surprisingly small in view of the size of the Fv fragment (MW 26 kDa). However, because Fv molecules were immobilized on flexible, bulky NTA groups at low surface density (5% NTA), they are expected to have significant mobility and therefore are easily compressed or pushed aside by the AFM tip.

Next, we used SPR to measure the specific binding of Fv and Lyso on solid substrates. Figure 4 shows the SPR sensorgram obtained from exposing the NTA/EG surface sequentially to NiSO₄, Fv, and Lyso. Between each step, the surface was rinsed with pure buffer. SPR wavelength shifts of 3.0 and 1.7 nm were observed for Fv and Lyso exposure, respectively. In a control experiment, no detectable SPR wavelength shift was observed upon injecting the NiSO₄- and Fv-exposed surface to the nonspecific protein BSA (data not shown). This confirmed that Lyso was specifically bound to the immobilized Fv. After accounting for the different molecular weights of Fv (≈26 kDa) and Lyso (~14 kDa), the molar ratio of adsorbed Lyso to Fv was determined to be ~1 on the Ni−NTA/EG surface, as expected if all the Fv molecules were uniformly oriented on the surface with their binding domains exposed. The dissociation rate constant (kd) was derived using eq 1:

\[
R(t) = (R_0 - R_b)e^{-kd(t-t_0)} + R_b
\]

where \( R(t) \) is the SPR response at time \( t \), \( R_0 \) is the SPR response at \( t_0 \), corresponding to the beginning of the dissociation process, and \( R_b \) is the baseline SPR response prior to the injection of lysozyme. It was estimated that \( kd \) was ~0.003 s⁻¹, in agreement with the literature.\(^{17}\)

Taken together, the above data validate the quality of the mixed COOH/OH-terminated and NTA/EG-terminated SAMs and demonstrate their suitability for grafting stable, homogeneous antigen and antibody monolayers.

**AFM Force Spectroscopy of the Fv/Lyso Interaction.** Force−distance curves were recorded between the Fv-modified tip and Lyso-modified substrate at a loading rate of 5000 pN/s. As shown in Figure 5, 26% of a total of 900 curves displayed single or multiple unbinding events, the remaining curves exhibiting no adhesion. This relatively low unbinding force frequency, which is not uncommon for single-molecule AFM force measurements, may reflect the low density of biomolecules attached on tip and substrate, as well as the random orientation of the Lyso molecules. The corresponding histogram of unbinding forces showed several maxima centered at 54 ± 11, 105 ± 9, and 154 ± 19 pN (Figure 5B). Several observations lead us to believe that the 54-pN unbinding force reflects the rupture of

![Figure 4](image-url)  
**Figure 4.** SPR sensorgram showing the wavelength shifts detected after exposing the NTA surface to NiSO₄ (200 mM), Fv (50 µg/mL), and Lyso (1 mg/mL). The inset figure shows the curve fitting result for the Lyso dissociation process. Both the x(time) and y(SPR response) axes were offset to fit the standard dissociation equation, \( y = y_0 e^{-kd(t-t_0)} \).

![Figure 5](image-url)  
**Figure 5.** Unbinding forces between individual Fv and Lyso molecules. (A) Typical retraction force−distance curves between the Fv-terminated tip and the Lyso-terminated substrate. (B) Histogram of the unbinding forces of the last rupture events from 900 curves. The curves were obtained using a loading rate of 5000 pN/s. An adhesion probability of 26% is observed, with the distribution of unbinding forces showing several maxima.
Kinetic parameters on the Fv/Lyso unbinding process from SPR measurements. The support of the Région wallonne, of the National Foundation for Scientific Research (F.N.R.S.), of the Université catholique de Louvain (Fonds Spéciaux de Recherche), of the Federal Office for Scientific, Technical and Cultural Affairs (Interuniversity Poles of Attraction Program), and of the Research Department of Communauté Française de
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