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Feasibility of measuring antigen–antibody interaction forces using a scanning force microscope

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Abstract

The molecular affinity scanning force microscopy (MASFM) described in this study was developed in an effort to test the possibility of antigen–antibody binding measurement using force-separation distance profiles. The MASFM configuration was comprised of a spherical glass bead as an MASFM probe, to which the fluorescein antigen has been covalently attached, and a silicon dioxide-based substrate, to which the anti fluorescein IgG antibody was covalently bound. The bead was glued to the tip of a commercial SFM cantilever. Adhesion forces have been measured between two different specific antigen–antibody pairs and between nonspecific surfaces bearing only glycidoxypyrilsilane immobilization chemistry. In force-separation (F-s) measurements, nonspecific forces displayed relatively few force discontinuities and mean adhesion forces lower than those found for specific antigen–antibody measurements. Force-separation profiles measured between specific antigen–antibody pairs showed many discontinuities and had higher mean forces. Positive controls revealed that the mean forces were slightly reduced by the addition of free ligand. The magnitude of mean forces did not correlate with the respective activation enthalpies of the proteins, as would be expected. At lower force values the force histograms for the specific pairs and for positive controls were indistinguishable. None of the force-separation data sets could fit a Poisson discrete-force model. This statistical analysis showed a large relative contribution from nonspecific interactions. It is concluded that the use of the large sphere as an SFM probe is counterproductive: while the large sphere does sample a larger number of specific interactions during each measurement, it also samples at the same time a large proportion of nonspecific forces. The presence of the nonspecific force contributions is likely due to the deformation of the polymerized GPS spacer layer which is thought to be delaminated from the surface upon the application of tension across the specific antigen–antibody bonds.

Keywords

Antigen–antibody recognition forces; Protein immobilization; Nonspecific adhesion forces

1. Introduction

Molecular recognition is an important event in biology. Many recognition interactions occur between immobilized partners, such as in the case of a blood cell receptor binding to a ligand on the vessel endothelium, or that of cell–cell adhesion in tissue formation. In the presence of external forces such as shear due to fluid flow, or internal forces such as cellular contraction, the binding partners are nonetheless required to remain bound and maintain a certain tension. The unique combination of salt bridges, hydrogen bonds, hydrophobic associations, and other interactions, coupled with complementary size and shape (the so-called lock-and-key mechanism [1,2]) assure that the ligand will be bound to the receptor for some required duration. Hence, a molecular recognition bond, although thermally labile, is able to withstand some tensile force. Several authors have developed models for calculating the strength of biological bonds [1–5]. The force required to break a single bond, F_s , equals G/d , where d is the length of the bond, and G is the binding free energy. The instantaneous tensile strength of an average bond calculated using this method is from 400 to 120 pN (which is approximately the same force as is required to uproot an entire protein from a lipid membrane) [5].

The formation of affinity bonds from bulk solution is dependent not only on the surmounting of the activation energy barrier, but also on rotational and translational diffusion of the binding partners. In the case where the reacting partners are restricted to two dimensions, as in a membrane, diffusion will be reduced. Receptors bound to a membrane can generally translate throughout the membrane as needed, although not as freely as in the bulk medium, and cannot rotate. The probability of receptor–ligand association is decreased when one or both members are bound to a membrane; several degrees of freedom are removed when this is the case [3]. In the case where the receptors and ligands are covalently linked to the substrate, they will not be able to diffuse laterally. Rotational diffusion is also limited to the length of the spacer linking the molecule to the substrate.

Because of its amphipathic nature, a protein is intrinsically surface-active [6]. Once adsorbed, proteins have been shown to change their structure (i.e. ‘denature’) in order to maximize favorable interactions. This loss of conformation will potentially render the protein nonfunctional. It has also been noted that protein adsorption is seldom reversible, so if a protein does desorb from the surface, it may not immediately regain its structure or function [7]. Several cell adhesion models also consider the contribution of nonspecific background interactions to the overall adhesive force [8,9]. It is recognized that once long range attraction or repulsion forces are overcome and contact is established, various background surface forces are inevitable to some extent and must be distinguished from specific forces.

Since its development in 1986 [10], the use of scanning force microscopy (SFM) for measuring interaction forces on a near-molecular scale has become common. A logical extension of this technique, which allows the measurement of forces between surfaces with a contact area of a few hundred nm², is to measure and characterize direct forces between specific biological molecules which are immobilized on opposing surfaces. This extension of SFM is termed here molecular affinity scanning force microscopy (MASFM). The

concept of MASFM has gained attention only in the past few years. Initially, several groups were able to detect specific binding by measuring changes in thickness or morphology at a sample surface bearing ligands [11,12]. Subsequently, several groups including our own have reported using chemically and biologically modified SFM systems to measure molecular binding [13–20]. All of these systems utilize differing types of chemistries and interacting pairs, but most see relatively similar trends. A common protein–ligand pair is the streptavidin–biotin system, used in many studies due to its unusually strong affinity. Several groups have immobilized biotin to the SFM tip and sample by physically adsorbing biotinylated bovine serum albumin (bBSA) which carries 7–12 biotin groups per molecule [13,14,16]. Avidin is then attached to the bBSA on one surface in a sandwich-type geometry. Another group measured the interactions between a bBSA-functionalized tip and a surface bearing anti-biotin antibodies [18]. The antibody surface was fabricated using a self-assembled monolayer (SAM) bearing amine-reactive groups to which the antibodies were covalently bound. SAM techniques have been used by other groups as well to immobilize thiol-containing molecules, such as DNA [19] or functionalized amphiphiles [21], to a gold-coated surface. There is, however, a concern with using noncovalently bound molecules due to their ability to desorb during the experiment. To the best of our knowledge there has been only one report on the measurements of molecular forces between completely covalent anchored ligands and receptors using SFM [20].

Several other techniques have also been used to measure forces between specific biological molecules. The surface forces apparatus (SFA), a benchmark technique for accurately measuring force-distance laws on a very small scale [22], has also been used to measure specific ligand-receptor binding forces. Helm et al. [23] and Leckband et al. [24,25] have immobilized both streptavidin/biotin and anti-fluorescein IgG/fluorescein to mica SFA surfaces using Langmuir–Blodgett lipid monolayer deposition. It was found, by normalizing over the relatively large contact area, that the rupture force between a streptavidin–biotin bond was 80 pN [24]. However, in studying forces between streptavidin and several biotin analogs with lower affinity, it was found that the mechanism for failure occurred within the membrane interior at nearly all bond affinities considered.

In another type of assay, adhesion forces between IgG-coated spheres and protein A-coated glass surfaces were studied in a radial fluid flow chamber [26]. Evans et al. [27–30] have developed an elegant micromechanical method which measures the rupture strength of molecular bonds between two blood cell membrane capsules. The rupture force measured between different types of molecules using this technique was determined to be in the range of 10 to 20 pN, and the failure appeared to be a function of the magnitude and duration of the detachment force. This detachment value is rather low, and the technique was shown to be capable of measuring forces two orders of magnitude lower than those measured using the SFM.

The work presented in this study is an attempt at using the MASFM in a sphere-flat surface geometry to measure the forces between IgG and its ligand. The choice of the geometry was based on a desire to sample a larger number of biological pairs on the mesoscopic level at the same time, rather than measure a single receptor–ligand pair force. In addition, the geometry allowed the formulation of the model described below, which was used to evaluate

the statistical significance of the MASFM measurements. All measurements in this work were done with the biological molecules immobilized on surfaces using covalent chemistry, such that the point of weakest tensile strength was the affinity bond itself.

2. A simple model of multiple receptor – ligand rupture events

Because biological bonds dissociate thermally after some time, the force required to break a bond decreases with a decrease in receptor–ligand separation rate, where the thermal energy can begin to contribute. However, for instantaneously developed tension across a receptor–ligand bond, the statistical nature of bond breakage and re-formation is not important, and the force required then to break multiple bonds, F_m , is simply equal to $F_s N_b$, where N_b is the number of instantaneously disrupted bonds [3]. The experimental implementation of MASFM allows a relatively fast separation between the liganded MASFM probe and the flat surface with the receptors; on the order of 1000 nm s^{-1} . At this rate the separation between ligand and receptor is practically instantaneous. In the present study the MASFM probe was chosen to be a silica sphere ($R = 10 \text{ }\mu\text{m}$) glued to the SFM cantilever (Fig. 1(a)). The silica sphere is rendered molecularly specific by immobilizing ligand molecules to it using covalent chemistry. Because the contact area of such a probe with the flat surface can encompass many molecular receptor radii, it is thought that instantaneous bond ruptures occur as discrete groups of events, as depicted in Fig. 1(b). In this scenario, molecules located on the outer perimeter of the probe will be at the furthest distance from the corresponding molecules on the flat surface. As the surface is pulled away from the probe, the cantilever deflects and its increase in restoring force imposes tension on these outer pairs first. Once the first set of molecular bonds is ruptured, the cantilever moves away from the surface until the next set of bonds is challenged, and so on.

In using SFM, the force information is typically displayed in the form of a force versus separation (F-s) plot. For the sequential bond rupture discussed here, one of these force-separation plots could appear as shown in Fig. 1(c), where the first jump from contact has the largest force since the largest number of bonds is breaking. The upward deflection of the cantilever upon bond rupture will only progress until the next set of bonds is challenged, at which time the cantilever will again be pulled in conjunction with the sample over the next bond length. A statistical model was generated to mimic this scenario by creating ‘reactive sites’ in a lattice. In order to make this simplistic model somewhat more realistic, a random assignment of the percentage of reactive receptor and ligand sites in each of several circumference sets is used while keeping the total fraction of the reactive receptor sites between 0 and 0.5 (50%) and the ligand sites between 0 and 1 (100%). Thus, for example, by changing the receptor fraction incrementally from 0.5 to 0 one can simulate a competitive ‘titration’ with free ligands in solution that will block the receptor sites.

The circumferential sets were used to create a lattice with one central site and $n+6$ sites in each concentric set. A lattice with a total number of 1261 sites was used in the simulation employing 21 circumference sets. The sites in one lattice, e.g. ‘ligands’, make a pair with the sites in a second identical superimposed lattice, e.g. ‘receptors’. If both opposing sites are filled with reactive moieties, a bond is created that will rupture. All bonds in each circumference set will ‘break’ at the same time, producing a discrete force equal to the sum

of the number of bonds. The sequential breakage of the set of bonds over the total interaction area is dependent on the vertical separation distance between each set because the curvature of the probe introduces an increase in separation distance with increasing radial distance from the center, such that the outermost bonds will be challenged first. The forces from every circumference set will also vary in magnitude for every simulation event due to the random assignment of the reactive sites. Fig. 1(d) shows an example of three different areas in which the variation of site population will result in a different magnitude of the force.

The result of the bond rupture simulations is shown in Fig. 2 as the number of ‘bonds’ ruptured at each circumference set for a low (0.05, Fig. 2(a)) and a high (0.5, Fig. 2(b)) average fraction of reactive sites. Note that the bonds ruptured in the outer-most circumference set is marked in Fig. 2 with ‘separation 1’ and the last central site is at ‘separation 21’. The results correspond to the sequential and discrete breakage of bonds, and can be seen to display distinct force jumps as the probe is separating from the surface. Summing all bonds ruptured in each simulation event allows one to perform a titration simulation by varying the average fraction of one type of reactive sites while keeping the other average fraction constant. Such titration results are shown in Fig. 3 in which histograms were compiled for the cases of a fixed receptor coverage of either 0.5 (Fig. 3(a)) or 0.05 (Fig. 3(b)). The coverage of the opposing surface (i.e. ligands) was simulated to be 0.5, 0.25 and 0.05. A total of 40 simulations for each combination were compiled and the sum of ruptured ‘bonds’ plotted in the histograms. It can be seen that for a higher fixed coverage on one surface (Fig. 3(a)), the variation between different surface coverages on the opposing surface is clearly distinguishable. On the contrary, for a lower constant surface coverage (Fig. 3(b)), the distinction is more difficult to make and the number of bonds is barely discernable as data sets begin to overlap. Thus, in order to more easily distinguish different numbers of individual bonds or perform a competitive titration, it is important to obtain adequate surface coverage of the interacting molecules.

The MASFM described in this study was developed in an effort to test the possibility of quantitative measurement of receptor–ligand unbinding events. The configuration of the instrument comprises a spherical glass bead as an MASFM probe, to which the specific ligand has been covalently attached, and a silicon dioxide-based substrate, to which the receptor of interest was also covalently bound. The bead was glued to the tip of a commercial SFM cantilever. Binding interactions were studied with and without excess of free ligand solution and while varying other experimental conditions such as loads, separation rates and pH. Control experiments were performed to determine contributions from background (nonspecific interactions). The forces measured are compiled into histograms and statistically analyzed for discrete behavior, which would correlate with the rupture of specific receptor–ligand bonds.

3. Materials and methods

3.1. Materials

Quartz microscope slides were from ESCO Products (Oak Ridge, NJ) and were cut to 1×2.5 cm for TIRF experiments using a diamond circular wafering blade. Glass coverslips were

obtained from VWR Scientific (Boston, MA). Glass beads (5–50 μm diameter) were from Duke Scientific (Palo Alto, CA). The silane reagent, 3-glycidypropyltrimethoxysilane (GPS) was obtained from Hüls/United Chemical Technologies (Bristol, PA). 1,1-carbonyldiimidazole (CDI) was from Aldrich (Milwaukee, WI). 5-(((2(carbohydrazino)methyl)thio)acetyl) aminofluorescein was from Molecular Probes (Eugene, OR). Glycine-*p*-nitroanilide (g-pNA) was purchased from Sigma (St. Louis, MO). Two anti-fluorescein IgG isoforms, 4-4-20 and 9-40, were a kind gift from J.N. Herron (University of Utah). Purified protein was refrigerated in a concentrated form in phosphate buffer (PB) pH 8 and 0.01% sodium azide, and was used within 3 months. All deionized (DI) water used was subject to reverse osmosis filtering and UV treatment. All buffers and reagents used were fresh and of a high quality commercial grade. Toluene from EM Science (Gibbstown, NJ) was assured to contain less than 0.01% residual water. All glassware was machine-washed and dried at 160°F before use. The protocols below are described assuming flat surfaces are used. They were also used in the same way for derivatizing the glass beads, the only difference being that the derivatized beads were washed after each step using a Millipore (Bedford, MA) vacuum filtration system with 0.65 μm pore size filtration disks. These disks are compatible with all chemicals used except acetone, which was not used to wash the beads in this way.

3.2. Surface modification steps

Fig. 4 outlines the chemical steps involved in covalently binding the protein and ligand to sample surfaces using GPS. These steps were used for all samples, although the sample material was slightly different (but always silicon dioxide) depending on the experiment. Glass spheres were used as the probe in all cases, to which only the fluorescein was bound. Antifluorescein IgG was immobilized to flat surfaces. Surface fluorescence experiments required transparent samples, preferably with a refractive index equal to the quartz prism used, and so quartz plates were used for these measurements. The SFM experiments were done simultaneously with optical microscopy measurements; the microscope objective was configured for a 0.17 mm glass coverslip (#1-1/2), and therefore this substrate was derivatized with proteins for use with these experiments.

3.2.1. Cleaning of surfaces—Silicon dioxide (quartz or glass) surfaces were submerged in piranha solution (70% H_2SO_4 :30% H_2O_2) for 15 min and subsequently rinsed thoroughly with DI water. After drying in a vacuum desiccator, the surfaces were cleaned further with oxygen plasma (200 mTorr, 50 W) (PLAS-MOD, Tegal) for 2 min.

3.2.2. GPS derivatization—Immediately after cleaning, the surfaces were placed in an Erlenmeyer wide-mouthed reaction vessel to which was added 1% (v/v) GPS and 0.2% (v/v) triethylamine in dry toluene. The vessel was equipped with a CaCl_2 drying tube and was suspended in a heated water bath, where it was heated to 70°C for 8 h, stirred overnight at room temperature, and heated another 8 h before washing with toluene and acetone. The GPS surfaces were then dried in vacuum overnight for curing.

3.2.3. Preparation for reacting GPS surfaces with amines—The epoxide groups of GPS were first hydrolyzed and then derivatized with a diimidazole crosslinker (CDI). The

hydrolysis was achieved by immersing the surfaces in a 100 mM NaCl solution adjusted to pH 4 with 10 mM HCl, and heating to 70°C for 30 min, with a subsequent rinse with DI water and drying in vacuum. The resulting glycol surfaces were then immersed in a solution of 20 mg CDI per 10 ml *p*-dioxane, which was stirred at room temperature for 1 h [31]. They were then rinsed with dioxane and acetone and dried. These GPS-CDI surfaces were stored in a dry atmosphere until further use to avoid hydrolysis of the imidazole groups.

3.2.4. Protein immobilization to CDI-activated surfaces—Protein amines were reacted directly with GPS-CDI surfaces by covering the surfaces with protein solution. A large (ap;125 μ l) drop of 1 mg ml⁻¹ protein solution in carbonate buffer at pH 9.0 was placed on the flat GPS-CDI surface and incubated at room temperature in an enclosed container for 1.5 h. The surface was then washed with 50 mM PB pH 7.4. The protein coated surfaces were either used immediately or stored refrigerated in phosphate buffer for no more than 2 days.

3.2.5. Fluoresceination of CDI-activated surfaces—CDI-activated surfaces were placed in a 1 mM solution of 5-(((2-(carbohydrazino)methyl)thio)acetyl)aminofluorescein in 10 mM PB pH 7.4, and stirred for 1 h at room temperature. They were rinsed with buffer, DMF, and DI water and dessicated.

3.2.6. Contact angle measurements—Contact angles were measured using the sessile drop method. Contact angles were measured after each chemical step, i.e. for clean glass and surfaces derivatized with GPS, hydrolyzed GPS and GPS-CDI-protein. At least three samples of each type of surface were measured, on two different areas for each sample. A 10 μ l drop of triple-distilled DI water (stored in an acid-cleaned container) was placed on the surface and the contact angle was recorded using the protractor portion of a goniometer.

3.2.7. X-ray photoelectron spectroscopy (XPS)—XPS spectra were measured using a Hewlett-Packard 5950B ESCA instrument. XPS spectra were obtained for clean glass, GPS, and GPS-CDI-protein derivatized surfaces. The atomic percentages of surface elements were calculated by correcting the area under each elemental peak with its Scofield cross section. Due to the absence of conductivity of the sample material, the flood gun was always operated at 10 eV to minimize charging effects, which results in a downward shift of the binding energy peaks from their expected values by this amount.

3.2.8. Chemical determination of epoxide groups—A 10 μ M solution (stoichiometric excess) of g-pNA in acetone was reacted with 2 g of GPS epoxide-coated beads in a closed vial at 80°C for 4 h. The beads were rinsed well with acetone and vacuum dried, then weighed for surface area determination. To cleave the *p*-nitroanilide (pNA), 10 ml of 4 N NaOH was added to the beads and stirred for 2 h. The absorbance at 410 nm of the pNA/NaOH solution was then read using a Perkin-Elmer Lambda 6 UV/VIS Spectrophotometer. The concentration of pNA, *C*, was calculated using $A_{410} = \epsilon C d$, where $\epsilon = 8.8 \times 10^3$ (M cm)⁻¹. The number of molecules per unit surface area was then calculated from *C* and assumed to be equal to the epoxide group surface density.

3.2.9. Radiolabelling—A solution containing 500 μg of 9–40 IgG in 300 μl of phosphate buffer was radiolabelled with 0.3 mCi Na^{125}I according to reference [32], being oxidized with chloramine-T and reduced with sodium metabisulfite. Once eluted from a PD-10 (Pharmacia) size-exclusion column, the concentration of radiolabelled protein was measured in a UV spectrophotometer for calibration with the counts measured using a γ -irradiation counter. The solution was mixed in equal parts with unlabelled protein solution. A 125 μl aliquot was placed onto a GPS-CDI silica plate of 1×1 cm and allowed to incubate at room temperature for 1.5 h. The plate was washed with buffer and placed on the γ -counter, which measured the radiation from the immobilized proteins. The counts (in kCi min^{-1}) from the sample were calibrated to concentration using the data from the protein in solution.

3.2.10. Fluorometry—An ISS PC1 photon counting spectrofluorometer was used to measure solution fluorescence. The binding affinity of 9–40 IgG for fluorescein in solution was measured by adding 10 μl aliquots of 4×10^{-6} M fluorescein stepwise to a 3×10^{-7} M solution of antibody in phosphate buffer, and also to a buffer reference. The solutions were excited at 488 nm and the emission was measured at 515 nm. Binding of fluorescein by anti fluorescein IgG quenches its fluorescence; therefore the degree of binding could be determined by comparing the sample fluorescence to that of the reference. A Scatchard plot was constructed from the data to determine binding constant and purity of the protein. The binding constant for 4-4-20 IgG has been well characterized and literature values were assumed.

3.2.11. Total internal reflection fluorescence (TIRF) spectroscopy—TIRF was employed to measure both the binding of protein to ligand-bound flat surfaces and the binding of ligand to protein-bound flat surfaces. A schematic of the TIRF setup is shown in Fig. 5. The laser used was an argon ion (Ar^+) laser, Model 5400 (100 mW) manufactured by Ion Laser Technology (Salt Lake City, UT). The shutter (Model 846), polarizer and $\lambda/4$ plate, mirrors and mounting components were from Newport. The monochromator (1681C 0.22 m Spectrometer) was from Spex (Edison, NJ), and the Series 200 CCD camera with cooling unit was from Photometrics (Tucson, AZ).

For the IgG pair, in one experiment, hydrazino-fluorescein was bound to the GPS-CDI surface, increasing concentrations of 9–40 anti fluorescein IgG were titrated into the TIRF cell and fluorescence quenching was monitored. In the other experiment, 9–40 IgG was immobilized to the GPS-CDI surface through its primary amine groups, and fluorescein-ethylenetetramethylrhodamine (FeT, a gift from A.P. Wei, University of Utah) was titrated. The experiments were performed by injecting a 1 ml aliquot of the fluoresceinated solution, allowing it to incubate for 1 min, and measuring fluorescence intensity. Then a 1 ml aliquot of buffer was injected into the flow cell to wash away any loosely bound protein, and the fluorescence was again recorded. This sequence was repeated with a dilution series of the corresponding binding partners. Control surfaces, prepared by immobilizing only half of the quartz surface with the specific molecule while leaving the other half to carry background chemistry, were also studied within the same experimental system. In these cases, the TIRF flow cell was moved vertically to position each type of surface without altering the optics in any way. Subsequent to each experiment, the data were compiled by using custom-written

software to sum each intensity reading over its emission range (≈ 100 nm). These values were then plotted versus concentration to determine binding affinities.

3.2.12. SFM probe attachment—Prior to each experiment, the SFM cantilevers were rendered chemically specific by attaching a bead with immobilized fluorescein to the tip area. The cantilevers used were Microlevers (Park Scientific, Sunnyvale, CA) with integral tips, having nominal spring constants, k , between 0.02 and 0.1 N m^{-1} . A $15 \mu\text{m}$ diameter tungsten wire was secured to micromanipulator arm and was dipped into a droplet of adhesive activator (Locquic Activator 707, Loctite, Newington, CT), then placed on the cantilever of choice to apply the activator. While the activator was drying, another set of tungsten wires were attached to the micromanipulator. One of these was used to apply a small amount of glue (Loctite Speedbonder 325) to the activated cantilever, and the other was used to pick up a bead electrostatically and place it onto the cantilever. The cantilevers were then stored in a petri dish and allowed to dry overnight. Prior to use, they were normally cleaned by flushing them with buffer in the fluid cell.

3.2.13. SFM force-separation measurements—The stand-alone SFM head of the Topometrix Explorer allows for convenient integration with an inverted microscope by simply placing it onto the microscope stage, where the cantilever and the bead can be imaged from below. A custom-made open fluid cell was machined from Teflon for this purpose. It was designed to hold a standard 1 inch diameter round glass coverslip on which the IgG was immobilized. The fluid cell was pressed into a custom-made plate and fitted onto the stage of a Nikon Diaphot 200 inverted microscope. A $100 \times 1.25 \text{ N.A.}$ oil immersion objective (Leitz) was used to observe the SFM experiments optically. SFM force measurements were recorded using commercial Point Spectroscopy software (Topometrix). The sample surfaces were subject to force-displacement analysis at each stage of chemical derivatization in order to determine the effect of background molecular interactions. All measurements were made in solution in order to eliminate the strongly adhesive effects which can arise due to capillarity. They were all performed in phosphate buffer of low (10 mM) ionic strength, in order to maintain constant conditions without overly shielding electrostatic interactions. For all surfaces, adhesion forces were measured from six to 40 times at one location on the surface, and this was performed at two or three locations per surface. The fluid cell required the cantilever probe to be removed each time the solution needed to be changed. For this reason, it could not be assured that force measurements were performed on exactly the same location after each change.

The immobilization chemistry was always the same on both probe and sample in order to create a symmetrical system. The background forces were also studied between the symmetrical systems of clean glass sample and clean glass sphere and GPS-CDI-derivatized sample and sphere. In addition, a series of measurements was performed on all of these surfaces after the adsorption of a non-specific protein layer, which was formed by incubating the sample in a 1 mg/ml solution of avidin for 30 min. As a positive control, the specific free ligand (fluorescein) was added in excess (1 mM) into the sample solution in order to determine whether competitive displacement of the bound ligands occurs. Saturation of specific binding sites by the free ligand was expected to result in a reduction or

elimination of specific forces. All raw force-displacement measurements were converted into force-separation (F-s) plots as described [15]. The magnitude of each of the force jumps in all force curves was measured and included in the respective histograms.

4. Results

4.1. Contact angle

Water contact angle measurements were performed on derivatized surfaces after each chemical step. In general, the contact angle values followed expected trends. The clean glass had a very low contact angle of $6 \pm 3^\circ$ and would ideally be expected to be close to 0° . GPS surfaces had contact angles of $47 \pm 5^\circ$, whereas hydrolyzed GPS which carries two hydroxyl groups had lower contact angles of $35 \pm 4^\circ$. Protein-coated surfaces had a lower contact angle, as would be expected due to the proteins amphipathic nature; GPS-protein surfaces had contact angles of $25 \pm 2^\circ$.

4.2. XPS

The atomic percentages of elements determined using XPS are tabulated in Table 1. The appearance of some carbon on a clean glass surface, 6 at.% in this case, is typical for slight contamination and is difficult to avoid.

4.3. Chemical methods

The glycine-*p*-nitroanilide assay used to determine surface coverage of epoxide groups on GPS surfaces showed an average of 0.35 epoxides nm^{-2} from three experiments. This is $\approx 10\%$ of the capacity of silicon dioxide and is a reasonable amount for glass which contains other less reactive oxides. Immobilization of ^{125}I -labeled antfluorescyl IgG to a GPS surface showed a protein surface density of $0.5 \mu\text{g cm}^{-2}$, or one molecule per 40 nm^2 . This corresponds to slightly less than a monolayer, which has been determined to be between 0.6 and $2.1 \mu\text{g cm}^{-2}$ for IgG, depending on the molecular orientation [33].

4.4. Fluorometry

Analysis of the Scatchard plot generated from solution binding data gives a binding affinity constant for 9–40 IgG of $K_a = 1.3 \times 10^7 \text{ M}^{-1}$. This is comparable to the reported value of $3.7 \times 10^7 \text{ M}^{-1}$ [34].

4.5. Total internal reflection fluorescence

Results of TIRF measurements are shown in Fig. 6. Binding of solution 9–40 antfluorescyl IgG to surface-immobilized fluorescein resulted in a quenching of fluorescence indicating that the IgG did indeed bind to the fluorescein. The normalized isotherm shown in Fig. 6(a) displays a typical S-shaped binding curve when plotted against the log of antibody concentration. The concentration at which 50% of antibody is bound is $\approx 5 \times 10^{-9} \text{ M}$. Titration of solution fluoresceinrhodamine to the surface immobilized 9–40 IgG (Fig. 6(b)) showed that most of the fluorescein remained bound to the surface IgG between the bulk measurement, where free ligand was present in the solution, and the wash step, where any loosely associated ligand was removed by washing the cell with buffer. On the contrary, the control with no bound protein showed very little nonspecific binding.

4.6. SFM force-separation measurements

Typical adhesion force-separation measurements obtained for background surfaces at each intermediate chemical step displayed relatively straightforward behavior as shown in Fig. 7. While clean glass showed little or no adhesion (Fig. 7(a)), hydrolyzed GPS surfaces showed adhesion forces between 0 and 1.5 nN (Fig. 7(b)). Adhesion force-separation measurements were also obtained for the case of background surfaces, both clean and GPS-derivatized, which had been incubated with nonspecific protein solution. These measurements began to show discrete force ‘jumps’ upon separation. Fig. 7(c) shows an example of nonspecific forces between avidin-coated GPS surfaces. The respective histogram is shown in Fig. 7(d). The overall mean force, μ , was ~ 1 nN found from 28 trials. The mean force for protein coated glass surfaces was 0.70 nN (data not shown). Thus, nonspecifically adsorbed protein appears to contribute significantly to adhesion force.

The histograms of forces measured for the GPS-CDI surfaces bearing specific fluorescein-antifluorescyl IgG pairs are shown in Figs. 8 and 9 together with typical examples of force-separation profiles. F-s experiments between surfaces bearing specific pairs consistently displayed discrete force ‘jumps’ and discontinuities which occurred over a larger separation distance than did the background forces. This behavior is partially attributed to the rupture of distinct bonds or groups of bonds which generate tension on the cantilever at different vertical or lateral distances. However, as shown by the positive control experiments with an excess of free ligand in solution (Fig. 8(c) and (d), Fig. 9(c) and (d)), these discontinuities may also be due in large part to nonspecific interactions as well. Although the shape of the force-separation profiles for the specific interactions were distinctly different from those of the background surfaces, the average maximum magnitude of adhesion of any one jump was only slightly higher than that for most of the control surfaces. The mean adhesion force for GPS-CDI-IgG 9-40 versus GPS-CDI-fluorescein interactions was 2.19 nN and in the presence of 1 mM solution fluorescein the mean force decreased to 0.95 nN. Similarly, the mean adhesion force for GPS-CDI-IgG 4-4-20 versus GPS-CDI-fluorescein interactions was 1.83 nN. Upon the addition of free fluorescein, this mean force decreased only to 1.48 nN.

5. Discussion

Characterization of GPS-derivatized surfaces showed that the various chemical derivatization steps produced the desired surface properties. XPS analysis showed that the expected elements were present on surfaces, although it was difficult to directly quantify their respective surface coverage (Table 1). Similar conclusions were derived from the water contact angle results, which showed the expected changes after each chemical surface modification step. Information about the surface heterogeneity on a microscopic level was not available from these analyses. Adhesion mapping experiments performed on surfaces with similar chemistries have shown that the hydrolysis of the epoxide group of GPS results in an increase of surface heterogeneity on a μm -sized scale due to the creation of two populations of hydroxyl groups [35]. Solid state [^{13}C]NMR spectra have shown that one type of hydroxyl group produced by the hydrolysis of GPS is the C(5) primary hydroxyl with which CDI mainly reacts (H.P. Jennissen, unpublished). Based on this difference in

reactivity, one expects an uneven distribution of immobilized IgG and fluorescein molecules on surfaces. Force-separation measurements obtained for surfaces with only GPS background chemistry showed different characteristics than those for clean silica: a single jump out of contact, a different characteristic than that seen for clean silica (Fig. 7(b)). On the contrary, forces recorded on a surface bearing nonspecific protein layer (Fig. 7(c)), showed the first jump out of contact at 20 nm separation and complete separation at 230 nm. This force-separation profile was similar to the stress-strain curve of a yield process often found in polymers. The yield process may be due to the adsorbed protein layer which bridged the probe and the flat surface and was being stretched upon separation.

The force-separation profiles for specific pairs, such as in Figs. 8 and 9, display an even greater number of discontinuities or discrete force ‘jumps’, which occur over a larger separation distance. This type of behavior has been observed by other groups studying molecular recognition interactions and is generally attributed to the breaking of specific bonds or groups of bonds [16,17]. In other words, as the ligand-receptor bonds are challenged due to the separation of probe and sample, those on the periphery or with the shortest spacing will generate tension on the cantilever, causing it to deflect, until the energy stored in the cantilever spring overcomes the bond energy and the first set of bonds ruptures with a characteristic force. The cantilever will then begin to return to its resting position as the sample continues to separate, pulling all remaining bonded molecules along until the next set is challenged, causing another deflection or jump, and so on, until all bonds are broken. This scenario was an initial hypothesis for the current measurements (Fig. 1(b) and (c)). However, it failed to explain why are the separation distances at which these jumps occur as large as several hundred nanometers and often greater? By combining the SFM experiments with reflection interference contrast microscopy (RICM) it was verified that the observed discontinuities have indeed occurred at such large probe-flat surface separations [36]. Given that an IgG molecule is on the order of 10 nm, the large separation distances at which the force jumps have been observed would require that the protein layer is stretched to many times its original thickness. Alternatively, this stretching could be due to the deformation of the polymerized network formed by the GPS molecules.

When either IgG or fluorescein were immobilized and the complementary molecule was allowed to bind from solution the binding proceeded in a specific manner as compared to the respective controls (Fig. 6). Similarly, in the SFM specific force measurements the mean adhesion forces in general were greater than the mean forces in either positive or negative controls. However, the magnitude of smaller force jumps recorded in the experiments employing the specific pairs was often indistinguishable from those found on control surfaces. The histograms for interactions between surfaces immobilized with 9–40 IgG and fluorescein (Fig. 8(a) and (c)) showed that both specific and control experiments display a force ‘peak’ at ≈ 0.7 nN, and another small grouping at 1.2 nN. Therefore it is assumed that these smaller forces are nonspecific in their origin. These force values could indicate discrete quanta corresponding to dissociation events, although they did not occur at regular intervals. The histograms of the interactions for 4–4–20 IgG samples also indicated that the specific measurements (Fig. 9(a)) showed very little difference from positive control measurements (Fig. 9(c)) at the lower range of forces. One could distinguish a greater frequency of forces at certain higher values in the case of specific pairs, marked by asterisks

(Fig. 8(a) and (c), Fig. 9(a) and (c)). The mean forces measured for two IgG isomers did not follow the same trends that their respective binding affinities would imply. It has been suggested that dissipative and entropy driven processes occur beyond the rupture point during forced unbinding; further, since equilibrium conditions do not apply, the measured force is likely that of the activation or transition state enthalpy [14]. One can modify the equation: $F_s = G/d$, by substituting the activation enthalpy, H_a , instead of G , in order to calculate the single bond force, F_s . The activation enthalpy for 4-4-20 antiferrofluorescyl IgG [37] has been determined to be 20 kcal mol⁻¹ (1.39×10^{-19} Nm molecule⁻¹). With the effective bond length of 1.5 nm for IgG [38] one finds that the instantaneous force per bond is 93 pN for 4-4-20 IgG. Values for 9-40 IgG activation enthalpy are not known but are assumed to be smaller in proportion to the smaller affinity of 9-40 IgG. However, the experiments showed that the mean force was greater for 9-40 IgG than for 4-4-20 IgG.

The statistical analysis of force-separation measurements using histograms requires large amounts of data in order to generate meaningful results [39], which can be subject to error due to sample damage induced by such a large number of measurements. The attempt throughout this work was to minimize damage to the proteins which could occur by repeated pressure and forced unbinding by the probe. An alternative method for analyzing and distinguishing single chemical bond forces from a smaller number of SFM force curve measurements has recently been presented by Beebe et al. [21,40]. This method applies Poisson statistics to compare mean forces with force variances for several data sets. The model calculates the probability of occurrences of an event (in this case, an event is a bond or group of bonds breaking simultaneously) in each of several intervals (intervals being sampling locations), with the assumption that an occurrence in one interval does not affect that in another interval; i.e. the occurrences are independent [41]. This situation holds true for MASFM measurements, in which binding and dissociation of a certain number of bonds at one location does not affect whether or to what extent this occurs at another location. Assuming that the total contact force between probe and sample is an event which is the sum of many discrete bond forces, F , then the total force for each measurement should vary as a Poisson distribution with mean $\mu = mF$ and variance $\sigma^2 = mF^2$, where m is the mean number of discrete bonds. A plot of variance against mean force should then be linear with a slope of $F = \sigma^2/\mu$. Convergence calculations showed that, for similar types of measurements, stable values of F could be obtained from as little as ten to 45 repetitions per data set [21].

This analysis was extended to include the relative contribution of nonspecific interactions to observed force values [40]. In the absence of non-specific interactions, the intercept of the $\mu(\sigma^2)$ plot is zero. The presence of nonspecific forces, F_o , is accounted by additional parameter, nF_o , such that $\mu = mF + nF_o$ and $\sigma^2 = mF^2 + \mu F_o$. The slope of the $\mu(\sigma^2)$ plot is again F , but the intercept is now nFF_o . The parameter, n , is introduced here to reflect any variation in nonspecific forces at each measurement location. The analysis of the data presented in this work showed that the mean force of each set of measurements taken at different surface region was different from its variance. In other words, none of the observed events followed the Poisson model. Furthermore, the contribution from the nonspecific forces deduced from the intercept, nFF_o , of the $\mu(\sigma^2)$ plot amounted to $\approx 1/5$ (IgG 9-40) and $1/2$ (IgG 4-4-20) of the respective discrete forces quantum, F . Such a large interference of

nonspecific forces indicated that the use of the large sphere as an SFM probe is counterproductive: while the large sphere does sample a larger number of specific interactions during each measurement, it also samples at the same time a larger proportion of nonspecific forces.

The current hypothesis for the observed forces is that the GPS-CDI-protein layer is being delaminated from its surface. The trimethoxyl groups of GPS are able to crosslink with each other through condensation in the plane of the surface, resulting in a film of GPS which, although continuous over some area, might have only sparse siloxane bonds to the glass surface. In this scenario the immobilized ligands bind specifically to the immobilized IgG molecules and, when tension is imposed across these bonds, the GPS film is pulled away from the surface. At some point the specific bonds will rupture; however, the specific forces are now convoluted with the forces required to stretch the polymerized spacer layers. In other words, although a difference between the mean forces in specific experiments and positive controls has been observed, the discrete unbinding which occurs upon dissociation of a receptor-ligand pair was not distinguishable using this MASFM configuration.

6. Conclusions

Interaction forces have been measured between two different GPS-CDI-immobilized fluorescein and GPS-CDI-immobilized IgG pairs and between nonspecific surfaces bearing glycidoxypopylsilane (GPS) layers. In MASFM experiments, nonspecific forces displayed relatively few characteristic force discontinuities and had mean adhesion forces lower than those found in the specific antigen-antibody measurements. Force-separation profiles measured between specific antigen-antibody pairs showed many discontinuities and had higher mean forces. Positive controls revealed that the mean forces were slightly reduced by the addition of free ligand. The magnitude of mean adhesion forces did not correlate with the assumed relative activation enthalpies of the two IgGs, as would be expected.

Force histograms for the specific pairs and for positive controls were indistinguishable at lower force values. None of the data sets for specific pairs could be fitted to the Poisson model, and the statistical analysis showed a large relative contribution from nonspecific interactions. It is concluded that while the use of a large probe does allow a larger number of specific interactions to be sampled during each measurement, it also samples at the same time a larger proportion of nonspecific forces. The presence of nonspecific force contributions is likely due to the deformation of the polymerized GPS spacer layer which is thought to be delaminated from the surface upon the application of tension across the specific antigen-antibody bonds. An alternative probe configuration or immobilization chemistry may facilitate further quantification of receptor-ligand tensile forces and energetic parameters.

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Fig. 1.

Concept of molecular affinity scanning force microscopy. (a) Functional receptors are covalently immobilized to a flat surface, and the corresponding ligands are immobilized to the spherical SFM probe. (b) A simple model for molecular affinity force measurements. An array of receptor–ligand bonds is formed, each at a vertical distance from the surfaces determined by the curvature of the probe. The outer molecules begin to be stressed first as the surfaces are pulled apart. (c) Sequential rupture of fewer and fewer bonds upon separation results in a periodic force discontinuities of decreasing magnitude. (d) The spherical probe samples a different number of bonds in each circumferential set depending on the actual distribution of functional receptors and ligands.

Fig. 2.

Simulated force-separation plots generated using the simple model. The 'separation' is in arbitrary units and reflects relative radial position, with '1' being the outermost circumferential set of bonds and '21' being the central contact site. The number of bonds is proportional to force and the negative sign signifies the adhesion. (a) Simulated force-separation plot for low surface coverages of 0.05 (5% reactive sites). (b) Simulated force-separation plot for higher surface coverages of 0.5 (50% reactive sites).

Fig. 3.

Results of force-separation simulation showing the effects of surface coverage of reactive binding sites. (a) One surface has a fixed coverage of 0.5, and the opposing surface coverage is varied from 0.5 to 0.05, with 40 simulation events in each group. (b) One surface has a fixed coverage of 0.05, and the opposing surface coverage is varied from 0.5 to 0.05, with 40 simulation events in each group. At this lower surface coverage, the number of bonds in each group is barely distinguishable as data sets begin to overlap.

Fig. 4.
Schematic of GPS-CDI immobilization reactions.

Fig. 5.
Schematic of total internal reflection fluorescence (TIRF) experimental configuration.

Fig. 6.

Total Internal Reflection Fluorescence specific antigen–antibody binding measurements. (a) Titration of 9–40 antifluorescyl IgG solutions onto a GPS-fluorescein surface. (b) Titration of fluorescein-tetramethylrhodamine (FeT) onto a GPS-CDI-surface with immobilized 9–40 IgG. The ‘bulk’ and ‘wash’ titration data correspond to the fluorescence signal prior to and after washing the FeT reagent out of the TIRF cell, respectively. The ‘control’ data show titration of the same FeT solution onto a GPS-CDI surface with no protein.

Fig. 7.

Force-separation results for nonspecific surfaces. Characteristic adhesion force-separation profiles are shown for (a) clean glass flat surface vs. clean glass probe; (b) hydrolyzed GPS flat surface vs. hydrolyzed GPS probe; and (c) nonspecifically adsorbed avidin on hydrolyzed GPS flat surface vs. avidin on the hydrolyzed GPS probe. (d) Histogram of adhesion forces for the nonspecifically adsorbed avidin-coated hydrolyzed GPS surfaces. The mean force, μ , and number of trials, N , are indicated.

Fig. 8.

Force-separation results for the specific pair consisting of GPS-CDI-IgG 9-40 (flat surface) and GPS-CDI-fluorescein (probe). The mean adhesion force, μ , and number of trials, N , are indicated in each histogram. (a) Histogram of measured forces for the specific pair. (b) Typical adhesion force-separation profile for the specific pair. (c) Histogram of forces for the positive control experiment in which free ligand (0.001 M fluorescein) was present in solution. (d) Typical adhesion force-separation profile for the specific pair in the presence of free ligand in solution.

Fig. 9.

Force-separation results for the specific pair consisting of GPS-CDI-IgG 4-4-20 (flat surface) and GPS-CDI-fluorescein (probe). The mean adhesion force, μ , and number of trials, N , are indicated in each histogram. (a) Histogram of measured forces for the specific pair. (b) Typical adhesion force-separation profile for the specific pair. (c) Histogram of forces for the positive control experiment in which free ligand (0.001 M fluorescein) was present in solution. (d) Typical adhesion force-separation profile for the specific pair in the presence of free ligand in solution.

Table 1

Atomic percentages of elements found on the modified glass surfaces

	C (at.%)	O (at.%)	Si (at.%)	N (at.%)
Glass surface	6.0	63.8	30.2	0
GPS surface	46.3	36.3	17.3	0
GPS-CDI surface	30.9	45.5	21.1	2.5
GPS-CDI-IgG 9–40	54.4	26.6	7.0	12.0