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Published on Web 04/28/2007

Magnetic Tweezers Measurement of the Bond Lifetime−Force Behavior of the IgG−Protein A Specific Molecular Interaction

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Abstract: The bond lifetime−force behavior of the immunoglobulin G (IgG)−protein A interaction has been studied with magnetic tweezers to characterize the physical properties of the bond under nonequilibrium conditions. Super-paramagnetic microparticles were developed that have a high and uniform magnetization to simultaneously apply a piconewton-scale tensile force to many thousands of IgG−protein A bonds. A strong and a weak slip bond were detected with an effective bond length that is characteristic of short-range, stiff intermolecular interactions. These bonds are attributed to the interaction of protein A with the constant region (Fc) and heavy chain variable domain (V_H) of IgG, respectively. The IgG−V_H interaction appears to be one of the weakest specific molecular interactions that has been identified with a single molecule force measurement technique. This study demonstrates that magnetic tweezers can be used to rapidly characterize very weak biomolecular interactions as well as strong biomolecular interactions with a high degree of accuracy.

Introduction

The development of ultrasensitive force transducers has made it possible to directly measure inter- and intramolecular forces between and within individual macromolecules. Single molecule force measurements have been made in biomolecular systems with the atomic force microscope (AFM),[1] micropipette,[2,3] optical tweezers,[4] flow chamber,[5,6] and magnetic tweezers,[7] with each technique having an optimal force and displacement sensitivity range. The first four techniques have been used to characterize the physical behavior of specific molecular intermolecular interactions[1,6,8−11] and the mechanical properties of macromolecules under tensile load.[10,12,13] Torque can be applied with the magnetic tweezers technique, which has been used to control the supercoiling and extension of long strands of DNA,[14,15] characterize the mechanical properties of F-actin networks,[16,17] and stimulate specific receptors on living cells.[18] These studies have provided the basis for understanding the function of biomolecular systems under nonequilibrium conditions and the intramolecular forces responsible for macromolecule structure. This has resulted in new insight into the important role that force plays in cellular behavior.[18]

As single molecule force measurements have been applied to more biomolecular systems, it has become clear that it would be highly desirable to make these measurements with higher sensitivity and between larger numbers of ligand−receptor pairs. The magnetic tweezers technique can be used to transduce piconewton (pN) forces to many thousands of super-paramagnetic microparticles. This technique has been used to characterize specific molecular interactions to enhance the specificity of solid-phase immunoassays[19−22] and screen phage displayed libraries.[23] Wider application of the magnetic tweezers technique has been limited by at least two factors. First, it has been difficult

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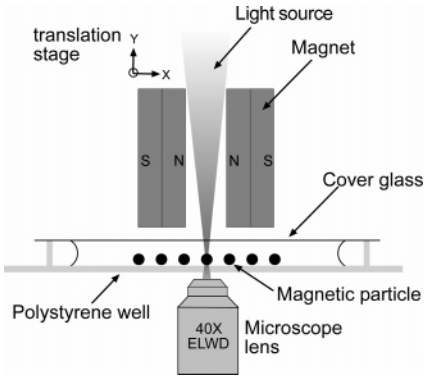

Figure 1. Schematic of the magnetic tweezers instrument that was built around an inverted optical microscope (see Supporting Information for details). The scale of the microreactor has been enlarged to illustrate the binding of the magnetic particles to the polystyrene surface.

to apply a uniform force to an array of magnetic microparticles because the super-paramagnetic particles that are commonly used have a nonuniform loading of magnetic nanoparticles.[21] This has limited the sensitivity and accuracy of array-based measurements. Second, the absolute magnitude of the force that can be accurately transduced to arrays of particles has been limited to less than 1 pN for micron diameter particles, which has limited the quality of the information that can be gained from these measurements. The usefulness of magnetic tweezers to the scientific and biotechnology communities would be greatly facilitated if one could overcome these limitations.

This report describes the magnetic tweezers measurement of the bond lifetime of the mouse IgG type 2a with protein A under a constant force. We chose to study the IgG−protein A specific molecular interaction as its structure and equilibrium binding properties have been well characterized.[24,25] Protein A is a 42 kDa protein containing five homologous ∼58 residue immunoglobulin-binding domains, designated A−E, that share 65−90% amino acid sequence identity.[26] Each of the protein A domains have been shown to bind to the Fc domain of immunoglobulins, with an affinity that is highly dependent on the species and isotypes of immunoglobulin. More recently it has been demonstrated that the protein A domains also bind to the V_H domain of IgG although the association constants are typically much smaller.[27] These strong and weak binding sites are incommensurate.

Figure 1 presents a schematic of the magnetic tweezers instrument used in this study, in which a uniform magnetic field and field gradient were applied to the magnetic particles over a 25 mm^2 area. Several technical advances were made to improve the accuracy and sensitivity of the technique. First, highly uniform super-paramagnetic particles were created that have a magnetization of 50.6 emu/g, which is five times higher than the magnetization of the particles used in previous studies. This allows uniform forces as large as 12 pN to be simultaneously transduced to many thousands of particles. Second, a poly(ethylene glycol) (PEG) grafting chemistry was used to covalently immobilize IgG and protein A in a manner that minimizes nonspecific adhesive forces between the particles and surface. These technical advances have made it possible to measure the dissociation rate of the specific molecular interaction between IgG and protein A at forces between 1.5 and 12 pN. A strong bond was detected and was identified as characteristic of the interaction between protein A and the Fc region of IgG. However, a much weaker binding interaction was also detected that was consistent with the protein A interaction with the V_H region of IgG. The IgG−V_H interaction appears to be one of the weakest intermolecular interactions that has been detected with a single molecule force measurement technique. We believe the developments described in this report represent an important advance for the field of single molecule studies, as many important biomolecular interactions are of the same magnitude of energy as the IgG−V_H interaction.

Results

Magnetic Tweezers Technique. The magnetic tweezers technique uses an external magnetic field to apply force to one or more super-paramagnetic microparticles. The force $\vec{F}$ applied to a super-paramagnetic microparticle in a high magnetic field is

$$\vec{F} = mv\nabla\vec{H} \quad (1)$$

where m is the saturation magnetization of the microparticle per unit volume, v is the volume of the magnetic particle, and $\vec{H}$ is the external magnetic field. The commercial super-paramagnetic microparticles that have been used in these tweezers measurements are composed of Fe_2O_3 nanoparticles distributed in a polymer matrix. The bulk magnetization of these particles ranges between 2 and 20 emu/g, and the magnetization can vary by as much as a factor of 3 from particle-to-particle.[21] Force has been determined in the magnetic tweezers experiments using microfabricated cantilevers[20,22] or through the motion of a microparticle tethered to a surface through a molecule of known mechanical properties[14,15,28] as the magnetic moment of the super-paramagnetic microparticles has not been known.

In this study uniform tweezing forces have been simultaneously applied to arrays of thousands of magnetic particles with a uniform magnetization in a well-defined magnetic field. These particles are produced using an emulsion based self-assembly technique that produces microparticles composed of 90% by weight Fe_3O_4 nanoparticles.[29] Figure 2A presents a TEM micrograph of these microparticles illustrating that they are composed of densely packed nanoparticles, spherical in shape, and uniform in size. The average diameter of the particle used in this study was determined from TEM to be 1.07 μm with the coefficient of variance less than 10%. The magnetic properties of these particles were characterized with a MPMS SQUID magnetometer (Quantum Design, San Diego, CA) operated at room temperature. Figure 2B presents the results of the magnetization measurements. These particles have a saturation magnetization of 50.6 emu/g at 10 kOe with no measurable hysteresis or coercivity. The saturation magnetization of these super-paramagnetic microparticles is the highest

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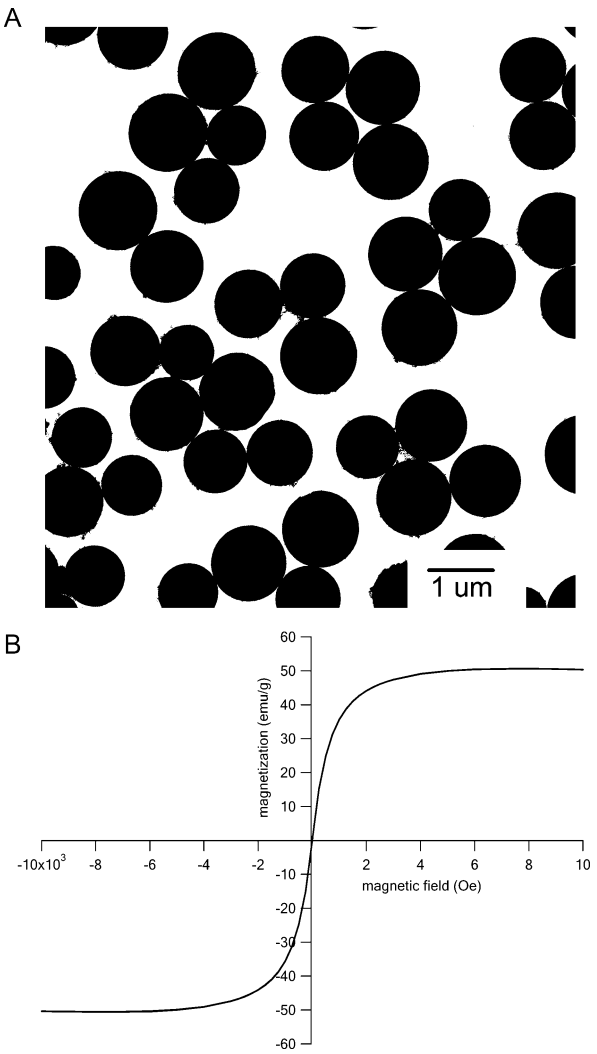

Figure 2. A. TEM micrograph of the super-paramagnetic microparticles produced by emulsion-based self-assembly. The image was taken on a Philips CM-10 TEM (Hillsboro, OR) operating at 80 kV. B. The magnetization curve of a microparticle sample measured with a MPMS SQUID magnetometer (Quantum Design, San Diego, CA).

of that we are aware.[30] The magnitude and uniformity of magnetization of these particles make them well suited for magnetic tweezer experiments.

A permanent magnet assembly was designed to produce a large and uniform magnetic field and field gradient normal to the microreactor surface using finite element analysis. Figure 3A presents the result of a simulation of the magnetic field generated by the set of permanent magnets in which the poles face each other across a 1 mm gap. This magnet assembly produces a strong magnetic field gradient just below the air gap between the poles of the magnet (area delineated by the red arrows) and is symmetrical at the center of this gap. Figure 3B presents the calculated magnetic fields and field gradients in the air gap 1 mm from the surface of the magnets. The magnitude of the magnetic field is in excess of 7500 G, which is sufficient to saturate the super-paramagnetic microparticles. The magnetic field gradient at the center of the gap was 15600 G/cm, which is approximately 2 orders of magnitude higher than the field gradient generated at the surface of one of these

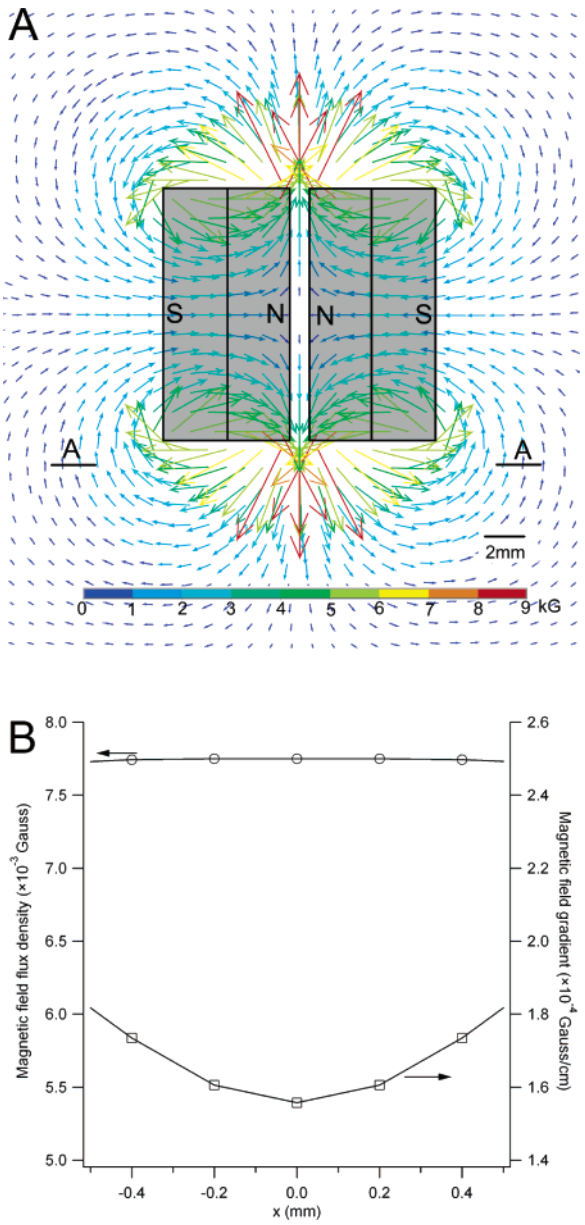

Figure 3. A. Cross-section of the magnetic field distribution calculated for a pair of permanent magnets with a 1 mm air gap using finite elements analysis (ANSYS, Canonsburg, PA). The magnetic field is presented in vector form with the magnitude of the field proportional to the length and color of the arrows. The microreactors were placed in the plane with the maximum field and field gradient, which is illustrated by the A−A markers. B. The calculated magnitude of the magnetic field and field gradient 1 mm below the gap of the magnet assembly.

magnets. The magnetic field and gradient vary by 0.1% and 1%, respectively, in the 220 × 165 μm^2 analysis area 1 mm below the magnets.

Protein Immobilization Chemistries. The chemistries used to immobilize IgG and protein A to the microparticle and microreactor surfaces were designed to minimize nonspecific adhesive interactions and control the density of protein A. Bare microparticles were found to strongly adhere to bare polystyrene surfaces in the presence of proteins. This behavior is attributed to protein−surface bridging interactions that have been reported to produce adhesive forces as large as 1000 pN.[31] The surface forces between IgG−poly(ethylene glycol) (PEG)-coated micro-

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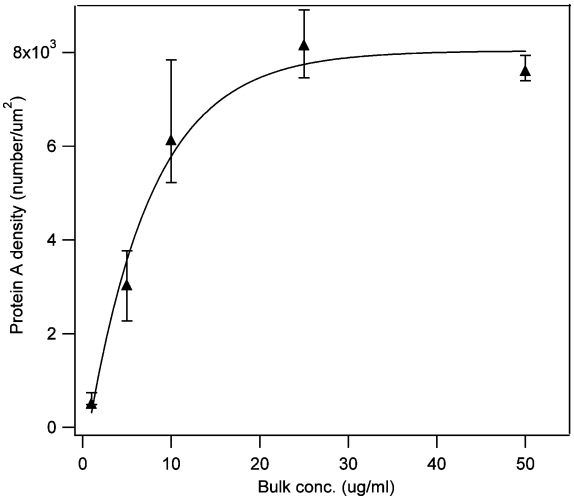

Figure 4. Protein A coverage as a function of the concentration of protein A used in the grafting solution.

particles and a methoxy-terminated PEG microreactor surface were characterized with the magnetic tweezers. It was found that less than 1% of the microparticles remained bound to the surface after a 1 pN force was applied for 0.5 s. The remaining fraction of particles was observed to adhere to the surface regardless of the duration of an applied 10 pN tweezing force. Thus, it appears that the nonspecific interactions of IgG on the microparticles with the microreactor surface were largely suppressed by coating both the particles and surface with dense PEG monolayers.[19] This is consistent with the fact that the PEG monolayers are known to minimize nonspecific adsorption of proteins on surfaces through a combination of steric and osmotic forces.[32]

The microreactor surface was functionalized with a mixed monolayer of amine and methoxy-terminated PEG to allow the density of protein A to be controlled. Figure 4 presents the protein A density in the microreactors that have been created by the reaction of a specified concentration of protein A with the PEG monolayers. The protein A coverage in the microreactor wells was measured using a colorimetric assay based on an IgG−horse radish peroxidase (HRP) conjugate, which has been described in the Supporting Information. This assay was carefully calibrated with immobilizing HRP to account for the mass transport effects imposed by the diffusion of the colorimetric substrates to the surface.[33] The immobilization of protein A appears to follow Langmuir adsorption behavior that is commonly observed for protein−surface reactions. That is, at low concentrations of protein A the rate of reaction appears to be controlled by diffusion of protein A to the surface, and at concentrations higher than 25 μg/mL the surface was saturated with protein A (the rate of reaction at the surface was limited by the availability of surface sites).

Table 1 summarizes the calculated reaction efficiency of protein A and the density of active protein A on the surface that can react with IgG on the microparticles as a function of added protein A concentration. The grafting efficiency has been defined as the percentage of total protein added to each microreactor well that was immobilized on the surface. A

Table 1. The Grafting Efficiency and Density of Protein A Molecules on the Polystyrene Surface as a Function of Protein A Concentration[a]

protein A-SATA conc. (μg/mL)	grafting efficiency (%)	protein A molecules available
1	10	45
5	9.3	270
10	9.3	545
25	5.1	726
50	2.3	677

[a] The average number of protein A molecules for each particle was calculated by the area of the sphere that is within a distance of 15.7 nm of the surface and the grafting densities of protein A.

maximum grafting efficiency of 10% was achieved at the 1 μg/mL protein A concentration, but the efficiency decreased to 2% at 50 μg/mL protein A as the functional PEG groups became saturated. The number of protein A molecules on the microreactor surface that are theoretically capable of reacting with the IgG on the particle was estimated from the grafting density of protein A and the total area of protein A−IgG reaction[23,34] (the interaction area was determined from the geometry of a sphere-on-flat contact and the 25.7 nm length of the IgG−protein A conjugate). These calculations indicate that there are 45 to 677 protein A molecules available to react with the IgG on the sphere (this was significantly less than the number of IgG molecules on the sphere).[23] The large number of protein A molecules that are available to react with IgG suggest that the magnetic particles will attach to the surface through multiple IgG−protein A interactions. However, it was found that only 10% of the total particles added to the surface remained on the surface after the application of a 10 pN force for 0.5 s at 1 μg/mL of protein A. This behavior indicates that the majority of particles were not bound to the surface through a specific molecular interaction. We conclude that the immobilization of IgG and protein A results in a significant decrease in their availability to react with groups on opposing surfaces.

Characterization of the Microparticle−Surface Interactions. The bond lifetime−force behavior of the IgG−protein A interaction was characterized by measuring the number of microparticles bound to the microreactor surface as a function of the protein A grafting density, time, and force. Figure 5A presents the particle dissociation behavior as a function of time and protein A concentration under an applied load of 12 pN. The bound fraction of microparticles has been calculated in terms of the total number of microparticles on the surface after a 0.5 s interval, rather than the total number of microparticles added to the surface, to differentiate microparticles that are specifically bound to the surface from those that are not specifically bound to the surface. Several trends can be observed in the dissociation behavior of the microparticles in Figure 5A. First, the fraction of bound microparticles at any given time is dependent on the density of protein A on the surface. Higher protein A densities result in a higher bound fraction of microparticles as can be clearly observed in comparing the dissociation profiles at 1 and 50 μg/mL protein A. Second, the microparticles appear to dissociate from the surface at two rates. This behavior can be clearly observed in the 5 μg/mL protein A measurement where 70% of the microparticles leave the surface in less than 5 s while the remaining microparticles appear to escape with a time constant greater than 30 s.

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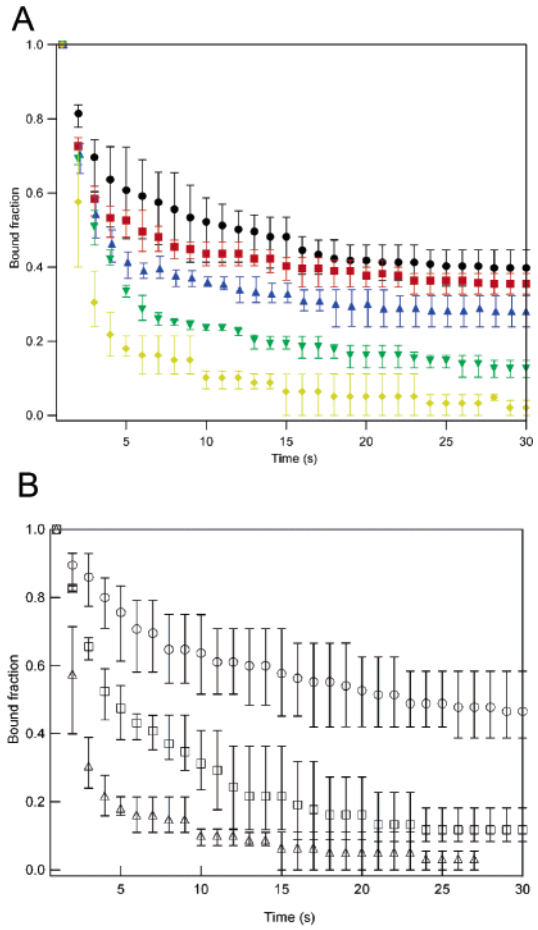

Figure 5. A. Dissociation of super-paramagnetic microparticles bound to surfaces as a function of protein A concentration of 1 (♦), 5 (▼), 10 (▲), 25 (■), 50 (●) μg/mL. Each measurement has been made in triplicate on approximately 150 microparticles at an applied force of 12 pN. B. Bound fraction of super-paramagnetic microparticles bound to the surface at 1 μg/mL protein A as a function of the applied force of 1.5 (○), 8 (□), and 12 (△) pN. The data points are experimental measurements that have been made in triplicate on approximately 150 microparticles.

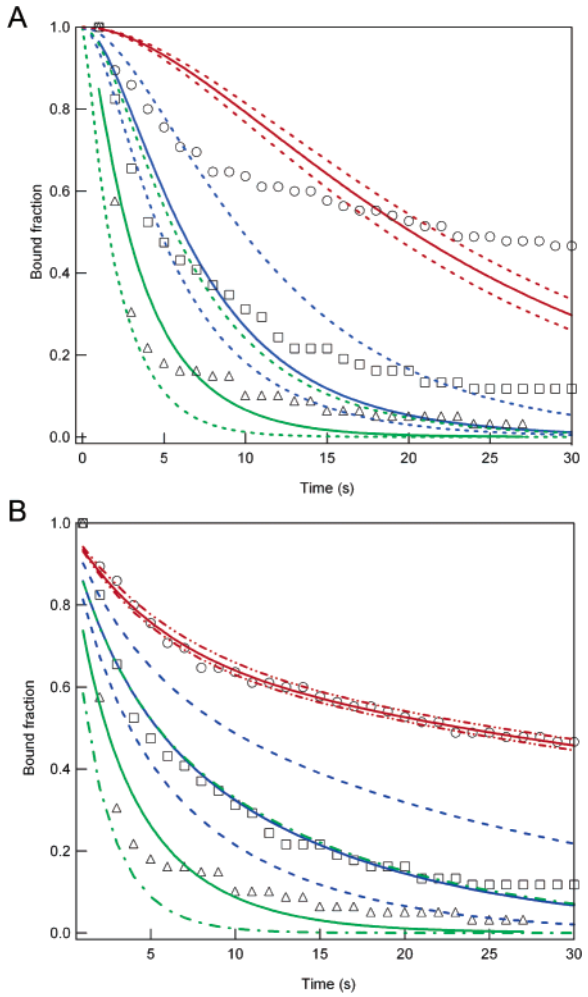

Figure 6. The bound fraction of super-paramagnetic microparticles bound to the surface as a function of the applied force at 1 μg/mL protein A concentration with the force of 1.2 (○), 8 (□), and 12 pN (△). A. The solid lines are the fitted curves using eq 4, and the dashed lines are the predicted uncertainty due to the distribution in particle size. B. The solid lines are the fitted curves using eq 5, and the dashed lines are the predicted uncertainty due to the distribution in particle size.

Figure 5B presents the microparticle dissociation behavior for surfaces functionalized with 1 μg/mL of protein A at loads of 1.5, 8, and 12 pN. The rate at which the bound microparticles leave the surface is highly dependent on the applied force. That is, a 12 pN force produces a rapid dissociation of the microparticles while a 1.5 pN force results in a much slower rate of dissociation. There also appears to be more than one rate of dissociation of the microparticles at any given force. This behavior can be clearly observed in the 1.5 pN results where the microparticles leave the surface rapidly in the first 10 s.

Discussion

Characterization of the Particle Dissociation Behavior. The form of the particle surface interaction can be determined through quantitative analysis of particle dissociation behavior. The dissociation constant (k_d) for a stiff, single, specific intermolecular interaction under a constant applied external force (F) is

$$k_d = k_o \exp(F/f) \qquad (2)$$

where k_o is the dissociation constant of the interaction in the absence of a force and f is a scaling force that is representative of the form of the bond (it is sometimes easier to think of the scaling force in terms of an effective bond length $x_o = kT/f$, where k is Boltzmann's constant and T is absolute temperature).[35] The fraction of bound particles (N) is exponentially related to the dissociation rate of the molecules at constant force

$$N = \exp(-tk_o \exp(F/f)) \qquad (3)$$

where t is the period of time that the force has been applied to the bond. Attempts to fit the dissociation profiles in Figure 5 with a single intermolecular interaction expression produced poor results. This leads us to conclude that the observed microparticle dissociation behavior did not result from a simple, single, specific intermolecular interaction. Rather, it appeared that the particles were either bound to the surface through two similar bonds or a single bond of two types.

Let us consider the dissociation behavior that would result from microparticles that are bound to the surface through multiple specific molecular interactions of the same type. In the special case where only two bonds form, force is initially

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distributed evenly across both bonds, and the rate of bond reformation is slow, the fraction of bound particles mediated with two bonds ($N_{2\rightarrow0}$) is

$$N_{2\rightarrow0} = 1 - P_{2\rightarrow1}P_{1\rightarrow0} = 1 - (1 - \exp(-tk_0 \exp(F/2f))) (1 - \exp(-tk_0 \exp(F/f))) \quad (4)$$

where $P_{2\rightarrow1}$ is the fraction bimolecular interactions in which one bond dissociates and $P_{1\rightarrow0}$ is the fraction of the single molecular interactions that dissociate. The microparticle dissociation behavior was analyzed using the double bond model identified in eq 4, and the results are presented in Figure 6A. This model was found to fit the 8 and 12 pN results for a spontaneous dissociation rate of 0.07 ± 0.04 s^{-1} and scaling force of 2.94 ± 1.5 pN, respectively. However, the microparticle dissociation results measured at the 1.5 pN tweezing force, which was the most accurate measurement, cannot be fit with the parameters derived at the higher forces. The spontaneous dissociation rate derived from this model is also 5 times smaller than the rate that has been previously been measured for the protein A−Fc interaction using the micropipette technique.[25] Multiple bond lifetimes associate with differences in reaction pathways have been used to describe changes in rupture forces measured as a function of loading rate.[6] However, we believe that it is unlikely that the IgG−protein A interaction could have multiple reaction pathways that are relevant over the long periods at which these measurements have been made. These observations lead us conclude that it is highly unlikely that the microparticles are bound to the surface through two or more identical bonds, which is consistent with our qualitative observations in Results.

The second possibility is that the microparticles are bound to the surface through a single molecule interaction of more than one type. In the special case where two irreversible bonds form between the microparticle and surface, the fraction of bound particles is

$$N_{1\rightarrow0} = A \exp(-tk_{o,1} \exp(F/f_1)) + B \exp(-tk_o \exp(F/f_2)) \quad (5)$$

where A is the initial fraction of the first type of bonds, $k_{o,1}$ is the dissociation rate of the first species, f_1 is the scaling force of the first species, B is the initial fraction of the second type of bonds, $k_{o,2}$ is the dissociation rate of the second species, and f_2 is the scaling force of the second species. Figure 6B presents the best fit of eq 5 to the experimental measurements at all three tweezing forces for strong and weak bonds that make up 67 and 33% of the particle interactions, respectively, and have a constant dissociation rate and effective bond length. The relatively good fit of the measured particle dissociation profiles supports the two bond model.

Single Molecule Bond Behavior. The improved magnetic tweezers technique described in this study has been used to characterize the form of the microparticle−surface interaction as well as the physical properties of the specific IgG−protein A interaction at a constant force in a range between 1.5 and 12 pN. The advantage of using a range of forces is that both weak and strong intermolecular interactions can be characterized by tuning the magnitude of the tweezing force. The spontaneous dissociation rate and effective bond length of the strong bond are 0.011 ± 0.001s^{-1} and 0.88 ± 0.21 nm, respectively. These bound properties are in close agreement with results from the micropipette aspiration technique for the IgG−protein A interaction where $k_d = 0.014$ s^{-1} and $x = 0.96$ nm,[25,36] but different from the much stronger IgG−protein G interaction.[37] The physical properties of the weaker bond identified with magnetic tweezers were $k_{o,2} = 0.14 \pm 0.01$ s^{-1} and $x_2 = 1.04 \pm 0.19$ nm. The dissociation rate of the weak bond is 1 order of magnitude faster than that of the strong bond, but the scaling forces are statistically indistinguishable.

Isothermal titration calorimetric analysis of the protein A interaction with the V_H domain of IgG has indicated that the enthalpy of the interaction is -7.1 ± 0.4 kcal/mol.[27] This enthalpy is approximately three times smaller than that measured for the interaction of protein A with the Fc region of IgG, which was -24.6 ± 0.6 kcal/mol. The relative magnitude of the enthalpy of binding of protein A for two domains of IgG suggests that the dissociation rate of the V_H domain should be 1 order of magnitude lower than that of the Fc region. These results lead us to conclude that the strong and weak bonds are associated with the interaction of protein A with the Fc region and V_H domain of IgG, respectively.

A large number of specific molecular interactions have now been studied with single molecule force techniques. Analysis of these interactions has revealed that at least two types of nonequilibrium behavior may result, depending on the relative stiffness of the transition and the bound states.[38] If the bound state is stiffer than the transition state, bond dissociation will be accelerated by the application of stress and, hence, the bond will slip. If the bound state is more flexible than its transition state, stressing the bond will actually cause the bond to catch and thus decrease the dissociation rate. The effective bond length of the protein A interaction with the Fc and V_H fractions of IgG was not dependent on tweezing force, which leads us to conclude that both bonds are 'stiff' and that there is a single transition state. This is consistent with structural studies of the Fc and V_H binding sites, which indicate that protein A binds to the surface of IgG in both of these interactions.[27]

Accuracy of Measurements. The accuracy of the tweezing force applied to an array of specific molecular interactions is determined by the size distribution of the microparticles, the accuracy with which we can determine the position of the magnet assembly, and the relative position of the microparticles in the air gap. A 10% variation in size of microparticles will result in a 33% change in this force. The absolute position of the magnet was determined within 0.1 mm and this will result in a 5% error in the magnitude of the magnetic field gradient applied to the microparticles. Thus, the size variation of the magnetic particles is the variable that produces the highest uncertainty in the magnitude of the magnetic force. The influence of the variation in the magnitude of the force has been shown in Figure 6 with dashed lines that bound the dissociation behavior expected from the particles. The impact of this level of uncertainty appears to be minimized in the analysis of the physical properties of the bonds by the large number of simultaneous microparticle measurements.

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Comparison of Magnetic Tweezers to Other Techniques. Single molecule force measurements are the primary experimental means for characterizing the role of force in molecular interactions as techniques such as surface plasmon resonance do not have a direct means for modulating force. It is useful to compare the magnetic tweezers technique described in the paper with other single molecule techniques. There are three limitations to using magnetic tweezers. First, the magnetic tweezers technique we describe does not currently provide a highly sensitive measurement of the microparticle displacement. This information is not required for the measurement of dissociation rates but can provide direct access to the mechanical properties of molecules. Second, the temporal resolution of the technique is currently limited to approximately 0.5 s by the manner in which the magnetic field is applied and the displacement of the particles is determined. Third, the effective magnetic tweezers force is dependent on the quality of the superparamagnetic microparticles and surface chemistries used to coat the microparticles and surfaces.

There are at least three advantages of using magnetic tweezers to measure specific molecular interactions. First, weak specific molecular interactions can be characterized. The protein A interaction with the heavy chain variable domain of IgG is the weakest bond of which we are aware that has been studied with a single molecule technique. There also appears to be the resolution to study even weaker bonds, as magnetic tweezers can apply forces as low as 0.01 pN. Second, the magnetic tweezers measurements appear to provide the means to characterize hundreds of single molecule interactions in a matter of hours. That is, we have focused on measuring several hundred microparticle interactions, but in each measurement, uniform forces were simultaneously applied to approximately hundreds of thousands of microparticles. This suggests that a large number of specific molecular interactions could be simultaneously studied. Finally, it appears that the use of freely diffusing microparticles rather than a fixed probe makes it possible to identify the full ranges of specific molecular interactions that take place. Previous measurements of the specific molecular interaction between IgG and protein A have failed to detect the protein−heavy chain interaction, which may be due to the fact that the weak bond was slow to form and difficult to identify.

Conclusions

We have implemented several technical improvements to the magnetic tweezers technique that have made it possible to measure the dissociation rate and effective bond length of protein A with IgG. Analysis of the microparticle dissociation behavior revealed that two molecular bonds were formed that were consistent with the interactions of protein A with the Fc fragment and V_H fragment of IgG, respectively. The effective bond length of these interactions indicates that these bonds result from stiff, short-range interactions. Further advances in magnetic tweezers technology promise to provide high accuracy measurements at loads as small as 0.01 pN. This promises to allow some of the weakest biological intermolecular interactions to be characterized and provide the means to probe the full range of specific biomolecular interactions in living cells. We also believe that this technique could be used to improve the specificity of sensing and high throughput screening assays if more sophisticated particle detection schemes were implemented, such as microfabricated cantilevers or giant magnetoresistance sensors.[21]

Acknowledgment. This work has been supported by the NASA Institute for Nanoelectronics and Computing at Purdue University (NASA NCC 2-1363). We would also like to thank G. Sagvoldend, S. Metzger, and G. Cassell for their valuable comments.

Supporting Information Available: Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

JA071215C